UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOMEDICINA COMPARATA ED ALIMENTAZIONE

SCUOLA DI DOTTORATO IN SCIENZE VETERINARIE

Curriculum Unico

Ciclo XXVIII

PhD Thesis

INTO THE BLUE:

Spoilage phenotypes of *Pseudomonas fluorescens* in food matrices

Director of the School: **Illustrious Professor Gianfranco Gabai**
Department of Comparative Biomedicine and Food Science

Supervisor: **Dr Barbara Cardazzo**
Department of Comparative Biomedicine and Food Science

PhD Student: **Andreani Nadia Andrea**
1061930

Academic year 2015

To my family of origin and my family that is to be

To my beloved uncle Piero

Science needs freedom,

and freedom presupposes responsibility…

(Professor Gerhard Gottschalk, Göttingen,

30th September 2015, ProkaGENOMICS Conference)

Table of Contents

Table of Contents VII

List of Tables XI

List of Illustrations XIII

ABSTRACT XV

ESPOSIZIONE RIASSUNTIVA XVII

ACKNOWLEDGEMENTS XIX

ABBREVIATIONS XXIII

PREFACE XXVII

BACKGROUND 1

References 3

AIM 5

CHAPTER 1: The Microbiological Quality of Food: *Pseudomonas* and Related Genera 7

1.1. Abstract 9

1.2. Introduction to *Pseudomonas*: historical cues and significance of the genus 9

1.3. Identification and tracking methods of foodborne pseudomonads: from classical microbiology towards -omics 10

1.4. Genus *Pseudomonas*: characteristics and ecology 13

1.4.1. *Pseudomonas fluorescens* group 14

1.5. Spoilage potential of the *Pseudomonas* strains: a focus on the *P. fluorescens* group ... 17

1.5.1. Pigment production 20

1.5.2. Enzyme production 21

1.5.3. Other types of spoilage 25

1.6. Related genera: *Xanthomonas* and *Shewanella* 26

1.6.1. *Shewanella* spp. 27

1.6.2. Spoilage potential of *Shewanella* spp. 28

1.6.3. *Xanthomonas* spp. 31

1.6.4. Spoilage potential of *Xanthomonas* spp. 34

1.7. Conclusion and future perspectives: biofilm formation and control of spoilage defects 35

1.8. References........ 36

CHAPTER 2: Tracking the blue: A MLST approach to characterise the *Pseudomonas fluorescens* group........55

2.1. Abstract........57

2.2. Introduction........57

2.3. Materials and methods........60

2.3.1. Bacterial strains........60

2.3.2. Phenotypic characterisation........61

2.3.3. Primer design........67

2.3.4. DNA extraction and PCR amplification........67

2.3.5. MLST data treatment and phylogenetic analyses........69

2.3.6. Clonal and population analyses........70

2.3.7. Pigment production on mozzarella cheese........70

2.4. Results........71

2.4.1. Phenotypic characterisation........71

2.4.2. MLST scheme, allelic diversity and the nucleotide substitution analysis of housekeeping genes........73

2.4.3. Phylogeny of *P. fluorescens*........75

2.4.4. Recombination analysis of the population structure........77

2.4.5. Pigment production on mozzarella cheese........78

2.5. Discussion........79

2.6. Acknowledgements........84

2.7. References........84

2.8. Supplementary data........90

2.9. Appendix to Chapter 2........98

CHAPTER 3: A genomic and transcriptomic approach to investigate the blue pigment phenotype in *Pseudomonas fluorescens*........101

3.1. Abstract 103

3.2. Introduction 103

3.3. Materials and methods 106

3.3.1. Bacterial strains 106

3.3.2. Genome sequencing and analysis 106

3.3.3. Assessment of pigment production in MBM broth 109

3.3.4. Transcriptome sequencing and analysis 109

3.3.5. Assessment of indole-derivative production test 111

3.3.6. Biochemical characterisation of the blue pigment 111

3.3.7. Mass spectrometry 111

3.4. Results and discussion 112

3.4.1. Genome sequencing and analysis 112

3.4.2. Growth curves and pigment production 118

3.4.3. Transcriptome sequencing and analysis 118

3.4.4. Analysis of *trp* cluster as putatively involved in the blue pigment biosynthesis .. 122

3.4.5. Biochemical characterisation of the blue pigment 124

3.5. Conclusions 128

3.6. Acknowledgments 129

3.7. References 129

3.8. Supplementary data 137

3.9. Appendix to Chapter 3 142

3.9.1. Screening of the strains for the presence of *trp* genes 142

3.9.2. Is the second copy of *trp* genes the consequence of a duplication or of Horizontal Gene Transfer? 144

3.9.3. References 148

CHAPTER 4: Transposon-induced pigment deficient mutants of a blue *Pseudomonas fluorescens* strain 149

4.1. Abstract 151

4.2. Introduction 151

4.3. Materials and methods 154
4.3.1. Bacterial strains 154
4.3.2. Transposon mutagenesis and screening of the mutants 155
4.3.3. Tn5-flanking sequences of selected mutants 155
4.3.4. Genetic analyses and evaluation of gene expression in *Pseudomonas fluorescens* strains 157
4.3.5. Phenotypic characterisation of the mutants 158
4.4. Results and discussion 161
4.4.1. Transposon mutagenesis of ps_77 161
4.4.2. Mapping of genes disrupted by transposon-insertion 161
4.4.3. Investigation of presumptive gene function in blue pigment production 164
4.4.4. Evaluation of gene expression 175
4.4.5. Phenotypic characterisation of the mutants 176
4.5. Conclusion 184
4.6. Acknowledgements 185
4.7. References 185
GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES 195
APPENDIX 197
LIST OF ORIGINAL PUBLICATIONS 231
Book chapters 231
Journal papers 231
Abstracts 232
CURRICULUM VITAE 235
RINGRAZIAMENTI 237

List of Tables

Table 1.1 - Discolouration caused by *Pseudomonas* in food products. 20

Table 1.2 - Enzymatic spoilage caused by *Pseudomonas*. 24

Table 1.3 - *Shewanella* in food products. 32

Table 2.1 - Origins and typing data of *Pseudomonas fluorescens* strains analysed in this study. 62

Table 2.2 - Primers used for amplification and sequencing..68

Table 2.3 - Phenotypic traits of the *Pseudomonas fluorescens* strains. 71

Table 2.4 - Nucleotide diversity observed within the *Pseudomonas fluorescens* strains characterised in this study...74

Table 2.5 - Origins and typing data of *Pseudomonas fluorescens* strains analysed in this study. 99

Table 3.1 - Complete and draft genomes analysed in the phylogenomic investigation using Gegenees...108

Table 3.2 - Genes involved in indigoidine biosynthesis used as query for the Blastp investigation of ps_20, ps_22, ps_40 and ps_77 draft genomes. 108

Table 3.3 - Summary of genomic data obtained through the analysis of the draft genomes of ps_20, ps_22, ps_40 and ps_77. 113

Table 3.4 - Relative abundance of COG..115

Table 3.5 - Summary of transcriptomic data..119

Table 3.6 - Read count of core genome and accessory *trp* genes in blue-pigmenting strains. 124

Table 3.7 - Up- and down- regulated genes obtained through Differential Gene Expression Analysis.. 137

Table 3.8 - Strains investigated for the presence of *trp* genes. 142

Table 3.9 - Primers used for amplification and sequencing.. 143

Table 4.1 - Primers used for the mapping procedures. 156

Table 4.2 - Transposon-induced mutants..162

Table 4.3 - COGs of disrupted loci in the investigated mutants. 165

Table 4.4 - Number of transcriptome reads mapping to ps_22 and ps_77 genomes. 176

Table 4.5 - Growth rates calculated through Combase.. 177

List of Illustrations

Figure 0.1 - Blue discolouration caused by *P. fluorescens* strains. 2

Figure 1.1 - The genus *Pseudomonas*. 16

Figure 2.1 - Maximum Likelihood (ML) tree of the dataset. 76

Figure 2.2 - Structure analysis. 78

Figure 2.3 - Pigment production on mozzarella cheese. 79

Figure 2.4 - ML tree based on *glnS* sequencing. 90

Figure 2.5 - ML tree based on *gyrB* sequencing. 91

Figure 2.6 - ML tree based on *ileS* sequencing. 92

Figure 2.7 -ML tree based on *nuoD* sequencing. 93

Figure 2.8 - ML tree based on *recA* sequencing. 94

Figure 2.9 - ML tree based on *rpoB* sequencing. 95

Figure 2.10 - ML tree based on *rpoD* sequencing. 96

Figure 2.11 - ML tree of the field strains of the dataset. 97

Figure 2.12 - ML tree of the expanded dataset. 100

Figure 3.1 - Phylogenomic analysis. 113

Figure 3.2 - Pangenome analysis. 114

Figure 3.3 - Graphical representation of the 84,616 bp gene clusters in contig_0004 of ps_77 draft genome. 116

Figure 3.4 - Differential gene expression analysis. 120

Figure 3.5 - Comparative representation of *trp* gene cluster. 123

Figure 3.6 - Indigo biosynthetic pathway. 126

Figure 3.7 - Indole production test. 126

Figure 3.8 - ML phylogenetic tree of TrpA proteins. 145

Figure 3.9 - ML phylogenetic tree of TrpB proteins. 146

Figure 3.10 - ML phylogenetic tree of TrpC proteins. 146

Figure 3.11 - ML phylogenetic tree of TrpD proteins. 147

Figure 3.12 - ML phylogenetic tree of TrpF proteins. 147

Figure 4.1 - Growth curves in KB and in iron-free KB. 177

Figure 4.2 - 96-well plate for the evaluation of growth and pigment production of the strains. 178

Figure 4.3 - 96-well plate for the execution of the CAS assay. 179

Figure 4.4 - Biolog GN2 test. 181

Figure 4.5 - Ratio of tested strains during the competition experiment in normal KB and iron-limited KB. 182

Figure 4.6 - Competition experiment in food matrices. 183

Figure 4.7 - Ratio of the strains during the competition experiment on food matrices. 184

ABSTRACT

Spoilage induced by *Pseudomonas* strains is commonly found in a wide range of food products as a result of the ubiquitous presence of these strains and their ability to induce alteration through different mechanisms. Particular attention has been recently paid on those *P. fluorescens* strains able to induce a blue discolouration on several food matrices (e.g. dairy or meat products). Actually, poor data are available about this curious event that draw the attention of European consumer from 2010.

In the present manuscript a step-by-step investigation of the spoilage potential of *Pseudomonas fluorescens* species complex strains is reported, focusing in particular on the ability to produce an unpleasant blue pigment in food.

Firstly, some general information is given to the reader to understand the *P. fluorescens* group as food spoiler. Then, the application of a polyphasic approach is described with the aim to investigate 136 *Pseudomonas fluorescens* group strains. Additionally, the achievement and the analyses of draft genomes and transcriptomes for 4 *P. fluorescens* strains are described to investigate the biosynthetic pathways involved in the blue pigment production. The attempt to chemically characterise the blue molecule using MALDI-TOF mass spectrometry is also reported. Finally, the execution of a transposon-mediated mutagenesis is described to confirm previously obtained genomic data and to highlight further genes involved in the blue-pigment production.

The phenotypic and genotypic characterisation, based on the combination of classical microbiological tests and a MLST scheme, allowed the reconstruction of phylogenetic relationships among the isolates and the identification of a monophyletic group (named "the blue branch") grouping all the blue-pigmenting and few uncoloured strains. The real involvement of these strains in the blue mozzarella event was confirmed by their ability to induce a blue discolouration on mozzarella cheese during a challenge test.

The genomic investigation confirmed the strict phylogenetic relationship between the strains belonging to the "blue branch". Additionally, comparative genomic tools revealed the

presence of a genetic cluster unique to the blue pigmenting strains containing a second copy of five *trp* genes, clearly involved in the blue pigment production. The biochemical characterisation of the pigment, hampered by strong issues of solubility, led to the conclusion that the molecule is an indigo-derivative.

Transposon-induced mutants confirmed the involvement of the previously identified unique cluster and the association of several genes affecting directly or indirectly the blue molecule production. Furthermore, the phenotypic characterisation of the mutants revealed a key role of iron in the production of the pigment, such as absence of any advantage of the wild-type strain in co-culture with a non-pigmenting mutant.

To conclude, the present work represents an exhaustive investigation of the spoilage potential of the blue-pigmenting *P. fluorescens* strains, giving to food industry reliable approaches to identify, track and prevent spoilage related to the growth of these interesting bacteria.

ESPOSIZIONE RIASSUNTIVA

Le alterazioni causate da ceppi di *Pseudomonas* sono solitamente riscontrate in una grande varietà di alimenti a causa del loro essere ubiquitari e dalla loro capacità di indurre modificazioni organolettiche negli alimenti mediante diversi meccanismi. Particolare attenzione è stata posta su alcuni ceppi di *P. fluorescens* in grado di indurre una colorazione blu in diverse matrici alimentari (quali prodotti lattiero-caseari o carne). In realtà, poche informazioni sono ad oggi disponibili riguardo al curioso caso che ha attirato l'attenzione pubblica a partire dal 2010.

In questo lavoro è riportata un'analisi a più livelli del potenziale alternate dei ceppi appartenenti allo *Pseudomonas fluorescens species complex*, ponendo particolare attenzione alla capacità di produrre un indesiderato pigmento blu negli alimenti.

In primo luogo, ai lettori sono date delle informazioni generali per una migliore comprensione di *P. fluorescens* come alterante alimentare. In seguito, è descritta la messa a punto e applicazione di un approccio polifasico con l'obbiettivo di indagare 136 ceppi appartenenti al gruppo *P. fluorescens*. Inoltre, sono descritti l'ottenimento e le analisi dei genomi *draft* e dei trascrittomi di 4 ceppi di *P. fluorescens* con la finalità di comprendere il *pathway* biosintetico coinvolto nella produzione del pigmento blu. In aggiunta, è riportato il tentativo di caratterizzare chimicamente il pigmento mediante la metodica della spettrometria di massa MALDI-TOF. Infine, è riportata l'esecuzione della mutagenesi random con la finalità di confermare i risultati genomici precedentemente ottenuti e di individuare ulteriori geni coinvolti nella produzione del pigmento blu.

La caratterizzazione fenotipica e genotipica, basata sulla combinazione di metodiche di microbiologia classica e di uno schema MLST, ha permesso la ricostruzione delle relazioni filogenetiche tra gli isolati e l'identificazione di un gruppo monofiletico (chiamato "ramo blu") che raggruppa tutti i ceppi pigmentanti e pochi ceppi non-pigmentanti. Il reale coinvolgimento dei ceppi blu nei casi di mozzarella blu è stato confermato dalla possibilità degli stessi di indurre un'anomala colorazione blu su mozzarella durante un *challenge* test.

Le analisi genomiche hanno confermato la stretta vicinanza filogenetica tra i ceppi del “ramo blu”. Inoltre, analisi di genomica comparativa hanno rivelato la presenza di un *cluster* genico unicamente presente nei ceppi blu, contenente una seconda copia di cinque dei sette geni per la biosintesi del triptofano, chiaramente coinvolto nella produzione del pigmento blu. La caratterizzazione biochimica del pigmento, resa difficoltosa da problemi di solubilità, ha portato alla conclusione che la molecola blu sia un derivato dell’indigo. I mutanti ottenuti mediante l’applicazione di trasposoni hanno confermato il coinvolgimento del *cluster* genico precedentemente identificato nella produzione del pigmento e l’associazione di ulteriori geni che influenzano direttamente o indirettamente la produzione della molecola blu. Inoltre, la caratterizzazione dei mutanti ha rivelato il ruolo importante del ferro nella produzione del pigmento e l’assenza di un effettivo vantaggio del ceppo *wild-type* posto in co-cultura con un mutante non pigmentante.

In conclusione, questo studio rappresenta un’indagine esaustiva del potenziale alterante dei ceppi blu, dando inoltre all’industria alimentare sistemi efficaci per identificare, tracciare e prevenire l’alterazione indotta da questi interessanti ceppi.

ACKNOWLEDGEMENTS

Recently, a friend's mum, reading his acknowledgements, said: "You forgot to say THANKS to the dog", joking about the endless list of people. I will not do it... I will not forget the dog!

These 3 years of PhD have been one of the most amazing experiences of my life.
I am grateful to the **PhD School of Veterinary Science** of the University of Padova that supported my education: thanks for this great opportunity.

I have to say thanks to my supervisor **Barbara Cardazzo**: thank you for giving me the opportunity to undertake the amazing journey of the PhD course. I never felt alone, having, on the other hand, my intellectual independence.
Not only Barbara, but also the whole research group of "Ispezione degli alimenti" has been fundamental for the development of my critical eye and my intellectual background. Special thanks to my teacher and friend **Maria Elena**: you never stopped to motivate me, also when far from each other. Thanks to **Luca Fasolato**, called "Il Maestro": your teachings, your frankness, your irony and your way to be yourself made our collaboration a bitter-sweet and precious friendship. Thanks to my mate **Lisa Carraro**: working with you is every time amazing! Thank you for your advice, your kindness and your shyness. It was just as working with a sister!
I really want to thank the entire Pat-Barg group: **Serena, Massimo, Rafaella, Roberta, Francesco, Massimiliano, Giulia**, but also other friendly faces of my Department as **Matteo, Giulia and Eleonora.** An extra thanks goes to **Marianna** for the hospitality and for having feed me during the drawing up of the thesis.
I have to say thanks also to **Prof. Gianfranco Gabai** for his advice along the PhD route: your helpfulness in several situations made me free to ask for advice when I was looking for self-confidence and help.

Finally, I have to recognize that these 3 years would not have been the same without my students. All of you gave me a little piece of the complex puzzle of my PhD project. I have been very lucky to find you on my way **Alberto, Andrea** (the *Pseudomonas* guys)**,** but also **Alessia, Valentina, Luca, Silvia, Angelica, Raissa…**

An important step of my growth was my internship to Cornwall from January to July 2015. First, I have to say thanks to **Ing. Aldo Gini Foundation** that supported it economically. During this experience, I met some extraordinary people, starting from **Michiel Vos**, my Dutch-English supervisor: thank you for accepting me in your research group and letting me work on my beloved *Pseudomonas fluorescens*. At the end of this six-month experience I was enriched, both professionally and personally.

I would like to thank also the whole Michiel's group: **Prof**. **William Gaze**, **Lihong** and **Andy,** and the crazy female counterpart (**Abigail**, **Aimee**, **Anne** and **Amy**). It was a great pleasure and, above all, fun to work with you.

Cornwall gave me also a second family, composed by **Jo, Simon, Ruby** and **Isaac**: I felt at home for all my stay there with you! Thank you for having made me feel a Cain.

Friendship was a great disclosure, as I could not imagine what great relationships I could have created in this too short period abroad. I need to thank **Leo** for having been my safe haven: thank you for your hug when nostalgia was around the corner and thank you for having taught me to smile. On the other hand, **Tomasa** has been my unsafe haven: the sisterhood that ties us goes behind the incompatibility of our nature. It was a great pleasure that our ways crossed, Tom: thank you for the person you made me. A special "Thank you" to the amazing "Launchers" group constituted by **Sidan**, **Sam**, **Dave** and **Atta,** for the great time spent together. I would like to thank also **Francesca**, **Giulia**, **Alice** and **Iris** (my left and right arms), **Claudio**, **Daniela** and **Pawel**, **Eduardo, Prabhu, Shivangi, Adeline, Faryal…**

By the way, in Italy is my heart, as here are the best friends I could desire.

A first thanks to **Elena** for being “my everything”, a friend, a sister, a mum and a supporter. I never feel alone. Thanks to her daughter **Gloria**, my foster sister, for her special smile and for having taught me that everything depends on the way you look at the world.

Special thanks to **Jessica** for being herself, namely my crazy little sister, and for letting me taking care of her. Thanks because you never gave up and for the friendship with **Elena** that binds three so different minds in an indissoluble relationship.

I really need to thank **Vale** e **Nico** for remembering me how much loved I am, for our holidays together and because looking at them I always remember how much lucky I am to have them and to have Simo.

Thanks to **Paolo**, **Daniela**, **Francesco**, **Enrica** and **Padre Giovanni** for saying always yes at our meeting.

I cannot forget to thank **Letizia**, **Erika**, **Fabio** and **Monica, Mario** for being in every occasion by my side.

A special thanks to all my relatives, but in particular to my **Aunt Elena** for the time spent together reading the newspaper on her knees.

I have a wonderful family that gave me a complete support in this long way: I really hope that **Paolo** and **Giulia** will be proud of me and that their smile, their smartness and their enthusiasm won’t never be weakened. You are two special guys and I love you more than myself. **Luisa** gave me the example that distances don’t matter, because nothing can divide two sisters.

Mum and **Dad** are great: they are the centre of my life. They supported me in all the aspects of this endless academic career, never claiming me to be more than I am, but always convincing me that I can do everything with my smartness, my smile and the way I tend to be. This thesis is, above all, for you. Thank you for having taught me that working always pays the effort. My only desire at the moment is that you are proud of me.

This thesis is dedicated to you, my beloved parents and brother and sisters, because I love you.

Last but not least, this Manuscript takes its origin in my family that has to be… in who incited me to do the PhD, even knowing that this choice would have taken me far from home for three years more… the patience you showed in these eight years spent together is more than I expected (and, probably, than I deserve). Thanks for the opportunity you gave me to leave and undertake the Cornish internship. Thank you for having made me your “child prodigy”: in these two words I could always find the pride that sometimes you cannot find the way to show. Thank you for having always waited for me, for the support in every step of this amazing route, for the love that I can read in your mute eyes, for the dream of a life together. Thank you, **Simone**, for the person I am from when we are together. You made me complete and I am really looking forward to being your family, forever.

A special thanks to **Claudio** and **Luciana** for having given to me the best man I could desire and for treating me as a daughter.

Thanks to everybody!

Nadia

ABBREVIATIONS

6PGDH	6-Phosphogluconolactonase
Acetyl-CoA	Acetyl-Coenzyme A
Arg	Arginine
ATP	Adenosine triphosphate
A_w	Water Activity
BSSF	Bacterial Spot of Stone Fruit
CAS	Chrome Azurol S
CDS	Coding DNA Sequence
CFC PAB	Cetrimide Fucidin Cephalosporin Pseudomonas Agar Base
CFU	Colony Forming Unit
CL	Cardiolipin
COG	Cluster of Orthologous
DGE	Differential Gene Expression
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethylsulfoxide
dN	Non-synonymous substitution
dNTP	Deoxynucleotide triphosphate
dS	Synonymous substitution
EYA	Egg Yolk Agar
G6PDH	Glucose-6-phosphate 1-dehydrogenase
gDNA	Genomic DNA
Gly	Glycine
HDTMA	Hexadecyltrimethyl ammonium
HGT	Horizontal Gene Transfer
His	Histidine
HPLC	High Performance Liquid Chromatography
HT-NGS	High-Throughput Next-Generation Sequencing
Hx	Hypoxanthine
IA	Iron Agar
IMP	Inosine MonoPhosphate
ISO	International Standardizations Organization
ITS	Intergenic Spacer
Kan^R	Kanamycin Resistant
KB	King’s B broth

KIA	Kligler Iron Agar
KOALA	KEGG Orthology And Links Annotation
LB	Luria Bertani broth
LC	Liquid Chromatography
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MAPs	Modified Atmosphere Packages
MBM	Minimal Bacterial Medium
MCMC	Markov Chain Monte Carlo
ML	Maximum Likelihood
MLST	Multilocus Sequence Typing
MS	Mass Spectrometry
MPV	Minimally Processed Vegetables
NA	Nutrient Agar
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NGS	New Generation Sequencing
nt	Nucleotides
O.D.	Optical Density
OF	Oxidative-Fermentative
ORF	Open Reading Frame
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PFGE	Pulsed-Field Gel Electrophoresis
PHA	Polyhydroxyalkanoates
Phe	Phenylalanine
Phl	3,4-diacetylphloroglucinol
Pro	Proline
PRPP	Phosphoribosyl pyrophosphate
Q-TOF	Quadrupole Time of Flight
RAPD	Random Amplification of Polymorphic DNA
RASFF	Rapid Alert System for Food and Feed
REP-PCR	Repetitive Extragenic Palindromic Sequence Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
r/m	Recombination to mutation
RNAseq	RNA sequencing
rpm	Revolutions per minute

rRNA	Ribosomal RNA
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
Ser	Serine
SIM	Local Similarity Program
SIM agar	Sulphur-Indole-Motility agar
spp.	Species
SRA	Sequence Read Archive
SSO	Specific Spoilage Organism
ST	Sequence Type
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic Acid
TFA	Trifluoroacetic Acid
Thr	Threonine
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
tRNA	Transfer RNA
Trp	Tryptophan
Tyr	Tyrosine
TSB	Tryptic Soy Broth
UHT	Ultra-High Temperature
UV	Ultra-Violet
VBNC	Viable But Non Culturable bacteria
WGS	Whole Genome Sequencing
WT	Wild-Type

Symbols

π	Nucleotide diversity per site
θ	Average number of nucleotide differences per site
λ	Wavelength
K	Populations
ω	dN/dS

PREFACE

When I started the drawing up of this thesis, I was not completely sure whether to write it from the beginning or in the format of collection of my papers. The writing process took me a long time during these three years of PhD course. As you will see in following chapters, I had the opportunity to write a chapter of a book and three original papers, all about my PhD topic. For this reason, I finally decided to reap the benefits of my work and to assemble the thesis adapting already published, submitted or in preparation papers, in order to have time to finish the writing process of my last manuscripts.

Moreover, two publicity materials have been divulgated: a piece for a children scientific magazine and an early-independent reader book. These two publications, even if cannot be considered proper scientific material, have been an important step of my PhD course. For this reason, after much thought, I decided to include them in the Appendix of the present work.

Only in this way, I can say this thesis is really mine and it reflects to the best my dedication and passion toward this topic.

I really want this thesis to be a story and I am happy to be the storyteller of this amazing project that, personally, I love.

"A scientist in his laboratory is not a mere technician:

he is also a child confronting natural phenomena that impress him

as though they were fairy tales."

(Marie Curie)

BACKGROUND

Microbiological spoilage is the complex batch of modification caused by bacteria, yeasts and moulds that can affect the quality of the aliment in terms of nutritional value, texture, flavour and, sometimes, consumer compliance (Gram *et al.,* 2002). This activity can be fulfilled by microorganisms through different actions such as the breakdown of food components, production of extracellular molecules (such as CO_2, pigmented molecules or polysaccharide material) or through the growth of bacteria themselves (Gram and Huss, 1996). Spoilage caused by bacteria can induce changes in organoleptic properties through visible growth (formation of colonies or slimes on food surfaces), changes in the texture of food matrices or through the production of off-odours and off-flavours that might reduce consumer acceptance of food.

Usually, spoilage activity is evident when bacteria reach 10^7-10^9 CFU/g of food and can be the consequence of interactions among different species of spoilers (Gram *et al.,* 2002).

Food environment plays an important role in the development of spoilers. Indeed, food characteristics, such as pH value, composition of nutrients, water activity (A_w), oxido-reduction potential and presence of molecules that can inhibit the growth of spoilage microorganisms, directly influence bacterial growth and their spoilage activity. Moreover, temperature of storage influences the predominance of particular species in the food environment.

Pseudomonas strains, and specifically *Pseudomonas fluorescens* are considered among the most important food spoilers, thanks to the ubiquitous nature, their recovery in a wide range of food products, their wide growth temperature range and their ability to induce the spoilage effect through different mechanisms (Dogan and Boor, 2003; de Oliveira *et al.,* 2015).

The most outstanding event related to contamination of strains belonging to the *Pseudomonas fluorescens* group was the identification, in 2010, of unpleasant blue discolourations on mozzarella cheese.

In that occasion, some European consumers noticed the formation of an undesirable blue pigmentation on the surface of mozzarella cheese. Analyses conducted by health authorities identified *P. fluorescens* as responsible of the outbreak (Marro *et al.,* 2011). Specifically, in 2010 the Annual Report of Rapid Alert System for Food and Feed (RASFF) reported the involvement of two main species belonging to the *Pseudomonas fluorescens* group: *P. tolaasii* and *P. libanensis*. The microbiological analyses of the contaminated blue cheese revealed high concentrations of these strains (up to 10^6 CFU/g of cheese; Bogdanova *et al.,* 2010). No information was given about the chemical nature of the blue pigment.

Actually, not only mozzarella cheese can be involved in such event, as visible in Figure 0.1.

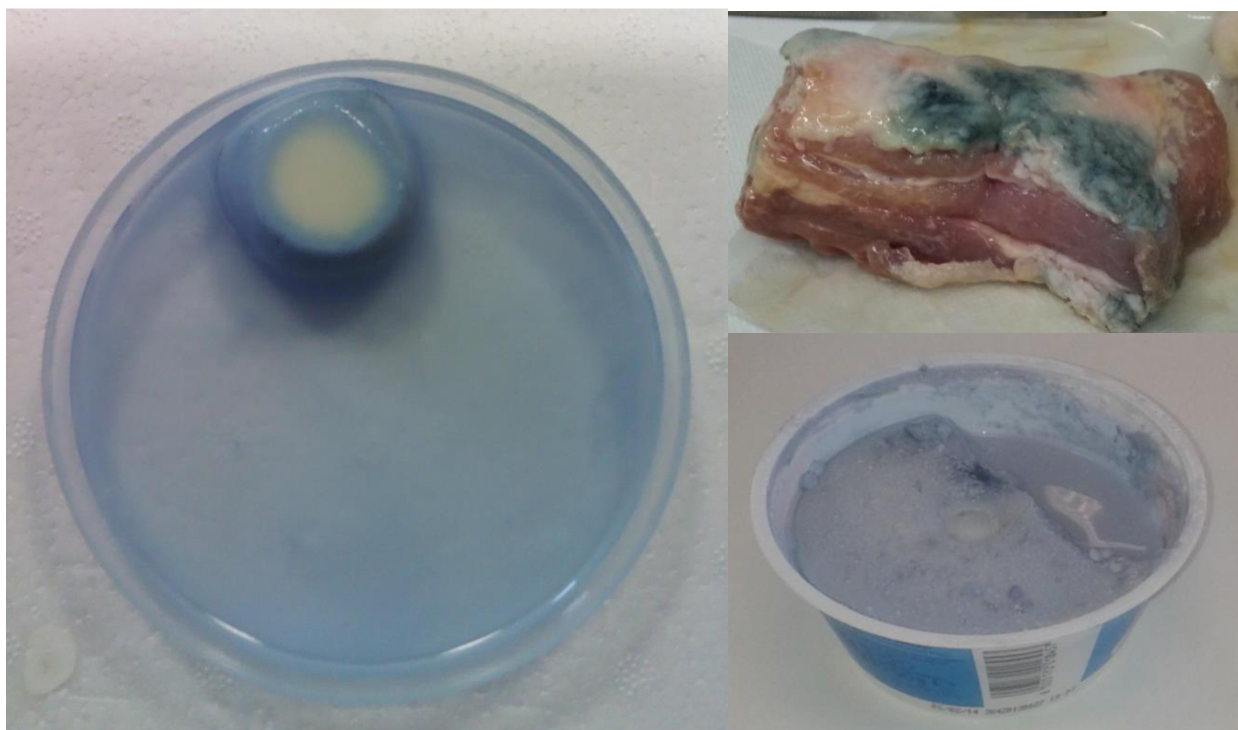


Figure 0.1 - Blue discolouration caused by P. fluorescens strains.
Spoiled food products due to the production of the blue pigment on mozzarella cheese, pork and ricotta cheese. Mozzarella cheese was experimentally contaminated during a challenge test. Pork and ricotta cheese were seized after the sale.

Despite the fact that strains of the *P. fluorescens* group were blamed of the blue discolouration, an imprecise background was given at the time of the blue mozzarella event. This was due to the fact that characterisation of the responsible strains was mainly conducted using quite dated approaches that often cannot distinguish among different strains. As a matter of fact, *P. tolaasii* and *P. libanensis* were identified as causing the blue discolouration even if the Type strains are usually defined as unpigmented.

Among pigment-producer *Pseudomonas fluorescens*, *P. lemonnieri* (now *P. fluorescens* biovar IV) was reported to be responsible of discolouration in a traditional Turkish fresh cheese, because of the production of an intracellular light-blue pigment (Starr *et al.,* 1976; Martin *et al.,* 2011).

To clarify the responsibility of specific strains and species, to identify the molecular structure of the pigment and to understand the function of the blue pigment a deep investigation has to be performed.

Classical microbiological approaches have been for several years the reference methods for the investigation of food contaminants, so that most official standards are based on quite old techniques. Actually, nowadays microbiology can trust the innovative support of biomolecular methods and New Generation Sequencing (NGS) approaches.

In particular, an efficient coupling of microbial tests to genetic investigation can be resolved with the aim to correlate specific phenotypes to particular genetic information. Moreover, the investigation of the genomic information, as well as gene expression data might help in shedding light on obscure mechanisms at the bottom of interesting phenotypes (Diaz-Sanchez *et al.,* 2012).

References

- Bogdanova, T., Flores Rodas, E.M., Greco, S., Tolli, R. and Bilei, S., 2010. Indagine microbiologica di mozzarella in occasione dell'allerta <<Mozzarella blu>>". XII Congresso Nazionale S.I.Di.L.V., Volume Atti, 117-118.
- de Oliveira, G.B., Favarin, L., Luchese, R.H., McIntosh, D., 2015. Psychrotrophic bacteria in milk: How much do we really know?. Brazilian Journal of Microbiology, 46, 313-321.
- Diaz-Sanchez, S., Hanning, I., Pendleton, S., D'Souza, D., 2012. Next-generation sequencing: the future of molecular genetics in poultry production and food safety. Poultry Science, 92, 562-572.

- Dogan, B., Boor, K.J., 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Applied and Environmental Microbiology, 69, 130-138.
- Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology, 33, 121-137.
- Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002. Food spoilage--interactions between food spoilage bacteria. International Journal of Food Microbiology, 78, 79-97.
- Marro, S., Griglio, B., Testa, A., Piovesan, F., Civera, T., 2001. Alterazioni organolettiche negli alimenti causate da Pseudomonadaceae e possibili ricadute per la sanità pubblica. Rapporto tecnico scientifico 1/2011 Ce.I.R.S.A.
- Martin, N. H., Murphy, S.C., Ralyea, R.D., Wiedmann, M., Boor, K.J., 2011. When cheese gets the blues: *Pseudomonas fluorescens* as the causative agent of cheese spoilage. Journal of Dairy Science, 94, 3176-3183.
- Starr, M. P., Knackmuss, H., Cosens, G., 1967. The intracellular blue pigment of *Pseudomonas lemonnieri*. Archiv für Mikrobiologie, 59, 287-294.

AIM

As reported in the background, microbial food spoilage is a complex process that can be controlled through a complete understanding of the causes and the spoilage mechanisms, as well as the environmental conditions in which bacteria can induce alterations.

Poor information is available about the ability of some *P. fluorescens* strains to induce blue discolouration in food products, the responsible strains, the chemical nature of the secreted pigment, the conditions enhancing the production, as well as the biosynthetic pathway of the blue molecule itself.

To solve these issues, the major aim of the present work was a complete investigation of the spoilage potential of *P. fluorescens* in food, focusing in particular on the production of the blue pigment.

Initially, general information about *P. fluorescens* spoilage potential was reported, including all the characteristics that make *Pseudomonas* one of the most important food spoilers.

Then, this study aimed to the investigation of the strains causing the blue discolourations, by developing and applying a polyphasic approach based on microbiological tests, a MLST approach and a challenge test to distinguish blue-pigmenting strains.

Reached this goal, the attention was focused on the understanding of the genetic determinants at the bottom of the blue pigment production, through the application of comparative genomic and transcriptomic approaches.

The chemical nature of the blue pigment and the environmental conditions of its production were further subject matters of the present research.

Finally, a deep understanding of the biosynthetic pathway was aimed, by the application of a random mutagenesis process and a further study of the so-obtained non-pigmenting mutants.

In addition to the goals of advancing research in the understanding of the spoilage topic, this study aimed to give to food industry relevant methods to identify, track and prevent the contamination of blue-pigmenting *Pseudomonas*.

CHAPTER 1: The Microbiological Quality of Food: *Pseudomonas* and Related Genera

---

Nadia Andrea Andreani and Luca Fasolato

Submitted

Keywords:

Pseudomonas, *Xanthomonas*, *Shewanella*, pigment production, enzyme production, volatile compound production, SSO.

1.1. Abstract

Spoilage induced by *Pseudomonas*, *Shewanella* and *Xanthomonas* is commonly found in a wide range of food products as a result of the ubiquitous presence of these strains and their ability to induce alteration through different mechanisms.

In the present chapter the investigation of the spoilage potential of the strains of these three genera is reported. Historical cues, taxonomic issues, identification and isolation methods are reported, as well as the different spoilage mechanisms through which the strains are able to induce alteration of several food matrices. Finally, recent control techniques proposed in bibliography are reported.

1.2. Introduction to *Pseudomonas*: historical cues and significance of the genus

The name *Pseudomonas* was proposed initially by Professor W.E.F.A. Migula of the Karlsruhe Institute of Germany at the end of the 19th century (Migula, 1894, 1900; Palleroni, 2010) and it was reported for the first time in the Bergey's Manual of Determinative Bacteriology in 1923. The choice of name seems to be due to its similarity in size and motility to the nanoflagellate Monas (from the Greek: "*pseude*"= false).

The first description of *Pseudomonas* was inaccurate: Professor Migula described *Pseudomonas* as "… cells with polar organs of motility. Formation of spores occurs in some species, but it is rare… ". *P. pyocyanea* (now *P. aeruginosa*) was proposed as the type species. In 1926, the extreme versatility of *Pseudomonas* was highlighted by L.E. den Dooren de Jong in his thesis (den Dooren de Jong, 1926; Palleroni, 2010) focused on bacteria of soil.

By the middle of 1900, more than 800 species had been ascribed to *Pseudomonas*, creating a confusing background for researchers interested in the genus. The major cause of this erroneous classification was the trend to categorise any Gram-negative, strictly aerobic, nonsporulating, motile bacillus as a representative of the genus *Pseudomonas* (Scales *et al.*, 2014). The turning point in this view was the development of first biomolecular approaches

that, alongside classical microbiology, unravelled the difficult classification of the strains of the genus. Around the beginning of the third quarter of the 20th century, DNA/DNA hybridization revealed deep differences among phenotypically similar strains (Pecknold and Grogan, 1973). Subsequently, RNA/DNA hybridization showed the presence of five different rRNA groups (rRNA group I, II, III, IV and V; Palleroni *et al.,* 1972). *Pseudomonas* rRNA group I (called *Pseudomonas sensu stricto*) comprised *P. aeruginosa*, all the fluorescent *Pseudomonas* and some non-fluorescent *Pseudomonas* (such as *P. stutzeri*, *P. alcaligenes*, *P. pseudoalcaligenes* and *P. mendocina*). A more in depth analysis of genetic differences among *Pseudomonas* species was conducted with the study of 16S sequence homologies: despite the low discriminatory power of rRNA, the study allowed the identification of distinct phylogenetic groups (Laguerre *et al.,* 1994; Anzai *et al.,* 2000).

From 2000, a great advance in *Pseudomonas* study was represented by siderophores study by Meyer and colleagues that allowed an excellent characterisation of several species (Meyer, 2000; Meyer *et al.,* 2002).

At the present time (December 2015) the genus comprises 244 species, as reported at http://www.bacterio.net/, having different characteristics.

In this chapter, the importance of the genus *Pseudomonas* and related genera as food spoilers is described. Taxonomic organization, identification methods, spoilage mechanisms and control plans are reported, with the goal of highlighting the extreme complexity of the spoilage potential of the genera *Pseudomonas, Xanthomonas* and *Shewanella*.

1.3. Identification and tracking methods of foodborne pseudomonads: from classical microbiology towards -omics

Several traditional methods have been applied to isolate and characterise *Pseudomonas* spp. strains from foodstuffs; however, recently these approaches have been replaced by more exhaustive and effective nucleic acid-based investigation methods (de Jonghe *et al.,* 2011).

Three ISO standards are available for the enumeration of *Pseudomonas* species. ISO 16266:2006 reports enumeration methods of *Pseudomonas aeruginosa* from bottled water and is based on the plating on cetrimide agar medium with nalidixic acid; a double incubation is suggested (4 and 42 °C). ISO/TS 11059:2009 and ISO 13720:2010 are official methods to isolate and count *Pseudomonas* spp. in milk and dairy products and in meat and meat products, respectively. ISO 13720:2010 reports the use of Pseudomonas Agar Base, a selective agar medium containing cetrimide, fucidin and cephalosporin (CFC supplement), three antibiotics selective for other non-*Pseudomonas* strains, and incubation at 25 °C for 44 hours (Tryfinopoulou *et al.,* 2001). On the other hand, ISO 11059:2009 suggests incubation in a medium containing penicillin and pimaticin at 25 °C for 48 hours.

Routinely applied tests for *Pseudomonas* are based on the identification of morphological, phenotypic and biochemical characteristics of the strains. Some commercial kits are available for strain identification, allowing a great saving of money and time. The most common kit is the API 20 NE system (No-Enterobacteriaceae; Biomerièux, Lyon, France). The API 20 NE system was proposed by Peladan and Monteil in 1988 as an effective method to characterise *Pseudomonas* and it was applied in further studies, for example the investigation of microbial diversity in milk and dairy plants conducted by Dogan and Boor (2003). As an alternative, BIOLOG GN microtiter plates can be used to characterise *Pseudomonas* strains (Johnsen *et al.,* 1996; Arnaut-Rollier *et al.,* 1999; Martin *et al.,* 2011). Other analyses are based on the growth of strains on differential agarised media, on Gram staining, on strain nutritional requirements, on motility tests and on the ability to grow at different temperatures.

The recent increasing interest in molecular approaches led to the development of several typing methods that avoid the lack of information inherent in classical methods. These new approaches allowed not only the identification of the species within the genus, but allowed also the description of phylogenetic relationships among the species. Examples are the application of REP-PCR (Repetitive Extragenic Palindromic PCR; Johnsen *et al.,* 1996), RFLP (Restriction Fragment Length Polymorphism*;* Franzetti and Scarpellini, 2007), DGGE (Denaturing Gradient Gel Electrophoresis*,* De Jonghe *et al.,* 2011), and PGFE (Pulsed Field Gel Electrophoresis; Nogarol *et al.,* 2013) to the identification of foodborne *Pseudomonas*

species. Sequencing of 16S rRNA genes has been widely used to identify and categorise several species of the genus isolated from food or other environments (Laguerre *et al.,* 1994; Moore *et al.,* 1996; Anzai *et al.,* 2000); however, because of the relatively high conservation of the 16S gene, not all species can be discriminated using this approach (Moore *et al.,* 1996; Anzai *et al.,* 2000; Yamamoto *et al.,* 2000). For this reason, the investigation by sequencing of 16S rRNA locus has been replaced by the combined analysis of several sequences of defined housekeeping genes for identification, characterisation and spoiler tracking purposes. Some studies have focused on the phylogeny of the whole genus (Yamamoto *et al.,* 2000; Hilario *et al.,* 2004; Mulet *et al.,* 2010), while others have concentrated on subgroups of the genus, such as the investigation of blue-pigmenting strains isolated from food products and belonging to the *P. fluorescens* group by Andreani and collaborators (2014). Both classical microbiological and biomolecular approaches require the isolation of the strains from their origin environments (culture-dependent methods). For this reason, these techniques have to take into account the problem of viable but non culturable bacteria (VBNC). VBNC are bacteria whose metabolic activity is too low to allow reproduction. Sometimes, the induction of VBNC forms is due to stresses to which vital cells are subjected within the food chain (e.g. refrigeration of fresh products, use of sanitisers or heating treatments).

Apart from culture-dependent biomolecular methods (based on the study of single cells isolated and grown as planktonic cultures), recently, the spread of Next-Generation Sequencing (NGS) approaches (e.g. 454 Pyrosequencing and Illumina sequencing) has allowed a more in depth knowledge about food and its spoilers (Andrews-Polymenis *et al.,* 2009; Holt *et al.,* 2008; Gilmour *et al.,* 2010). The ever-decreasing cost of NGS techniques has made it easier to obtain complete or draft genomes of bacterial species (Diaz-Sanchez *et al.,* 2012; Solieri *et al.,* 2012). Consider that, taking into account an average *Pseudomonas fluorescens* genome size of 6 Mbases, in a single run of MiSeq 300 pair-end (Illumina Technology) with an estimated output of 15 Gbases (data obtained from Illumina website in December 2015), up to 10 genomes could be multiplexed to obtain a coverage that allows robust genomic and comparative investigations (Kisand and Lettieri, 2013). Recently, several

draft genomes of strains belonging to the *P. fluorescens* species complex have been published, among which there are some food spoilers (Andreani *et al.,* 2015; Lo *et al.,* 2015). Investigation of such data might disclose the genetic information underlying the spoilage potential of *Pseudomonas* spp. strains. Similarly, NGS methods can be used to investigate the expression of genes involved in food spoilage, as in the application of microarray experiments by Mohareb and co-workers (2015), to identify potential biomarkers of meat alteration in *P. putida*. Bacterial communities can also now be investigated through NGS technologies: metagenomics studies (namely the sequencing of all genetic material extracted from different types of sample, such as food samples) are expected to be one of the future strategies for genome sequencing of interesting microbes. It is clear the innovation of NGS approaches has not been exploited only for the purposes of food microbiology.

1.4. Genus *Pseudomonas*: characteristics and ecology

The genus *Pseudomonas* is composed of a heterogeneous group of bacteria characterised by important ecological significance (de Oliveira *et al.,* 2015). The strains ascribed to the genus *Pseudomonas* are Gram-negative, straight or slightly curved rods, 0.5 to 1.0 µm by 1.5 to 5.0 µm in length, catalase-positive and are motile by means of one or several polar flagella. These bacteria are characterised by an obligate respiratory metabolism, a lack of gas formation from glucose and a positive result from the oxidase test. Even if they are aerobic strains, some *Pseudomonas* can grow under anoxic conditions in the presence of nitrate, fumarate or other electron acceptors (Palleroni, 1984).

As they are mesophilic bacteria, their optimal growth temperature is about 25-35 °C; however, most of them are psychrotrophic microorganisms that means they are able to growth at refrigeration temperatures, and they do not tolerate high temperatures. Cold adaptation of *Pseudomonas* spp. is linked to high levels of unsaturated lipid in the cell membrane and to a wide range of mechanisms that allow the tolerance of cold-induced stresses (Fonseca *et al.,* 2011; Samaržija *et al.,* 2012; Moreno and Rojo, 2014).

The optimum pH environment for *Pseudomonas* strains is above 5.8: lower pH values slow down bacterial growth.

Pseudomonas strains have very simple nutritional requirements and have the ability to use different organic compounds as carbon and energy sources. For these reasons, *Pseudomonas* can be defined as a ubiquitous bacterium. *Pseudomonas* is present in a wide range of different environments such as organic material under decomposition, atmospheric dust and vegetation and it has various animal and plant hosts (Anzai *et al.,* 2000: Frapolli *et al.,* 2007).

Some strains are important bacteria of soil and water and are responsible for the degradation of several molecules. For this reason, they are very important in the mineralization process in nature, in sewage treatment and in the degradation of xenobiotic substances, such as pesticides or chemicals. They are also important agents in bioremediation of the environment (Stanier *et al.,* 1966; Wasi *et al.,* 2013).

Some species are human pathogens, such as the well-known *P. aeruginosa*, but also *Pseudomonas paucimobilis*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas acidovorans* (Tümmler *et al.,* 2014). Other species are plant pathogens, for example *Pseudomonas pseudoalcaligenes*, *Pseudomonas savastanoi*, *Pseudomonas syringae* and others are animal pathogens, mainly against fishes and birds (*Pseudomonas anguilliseptica*, *Pseudomonas chlororaphis*, *Pseudomonas aeruginosa*; Peix *et al.,* 2009). Some *Pseudomonas* are well-known food spoilers, such as *Pseudomonas fluorescens* that is responsible for the spoilage of dairy products, raw fish and meat and eggs, because of their psychrotolerant character (Jay, 2003; Palleroni, 2010).

1.4.1. *Pseudomonas fluorescens* group

A recent investigation based on the sequencing of four loci (16S rRNA, *gyrB*, *rpoB* and *rpoD*) by Mulet and collaborators (2010) of 107 *Pseudomonas*, allowed the identification of two major lineages (*P. aeruginosa* and *P. fluorescens*). The *P. fluorescens* lineage has itself been divided into six groups (*P. syringae, P. lutea, P. putida, P. anguilliseptica, P. straminea* and *P. fluorescens*). In particular, the *P. fluorescens* group has been identified as a well-separated monophyletic group, comprising nine further subgroups and characterised by strains with

high spoilage potential. Since the recent classification by Mulet and colleagues, at least 52 species are now considered as part of the *Pseudomonas fluorescens* group (2010). The schematic representation of the lineages, groups and subgroups within the genus is reported in Figure 1.1.

In the history of microbiology, the *Pseudomonas fluorescens* group has not been intensively studied (Palleroni, 2010) and only recently this group has been seriously considered due to its high spoilage potential.

Pseudomonas fluorescens possesses a number of functional traits that allow to grow in several environments, such as the production of several secondary metabolites or siderophores (Cornelis, 2010), the presence of several secretion systems and the ability to form biofilms, together with the adaptability of its genome (Spiers *et al.,* 2000; Silby *et al.,* 2011).

The major issue concerning *Pseudomonas fluorescens* group strains (and specifically *P. fluorescens* subgroup and *P. fluorescens*) is food spoilage and their importance is related to public hygiene and the food trade. In fact, even though *P. fluorescens* group strains are easily destroyed by the heat treatments usually applied to food, they are able to produce a wide range of enzymes (mainly proteases, lipases and lecithinases), or pigments, that are in some cases thermostable. Most of the reported spoilage cases by *Pseudomonas fluorescens* are attributable to post-process contaminations.

An early investigation into the pathogenicity of *Pseudomonas fluorescens* identified it as a harmless bug (Baader and Garrer, 1887). In this study, *Pseudomonas fluorescens* was defined as non-pathogenic as no reaction took place when a culture was applied subcutaneously to animals or injected into the peritoneum. Additionally, ingestion of cultures resulted in no harm to the gastro enteric tract. However, a recent review by Scales and co-workers (2014) reported *Pseudomonas fluorescens* as a possible human pathogen as it has been identified as part of the microflora of several anatomical locations (i.e. mouth, stomach and lungs). Usually, bacteraemia cases involving *Pseudomonas* are milder than those involving its cognate *Pseudomonas aeruginosa* and they are usually related to nosocomial infections.

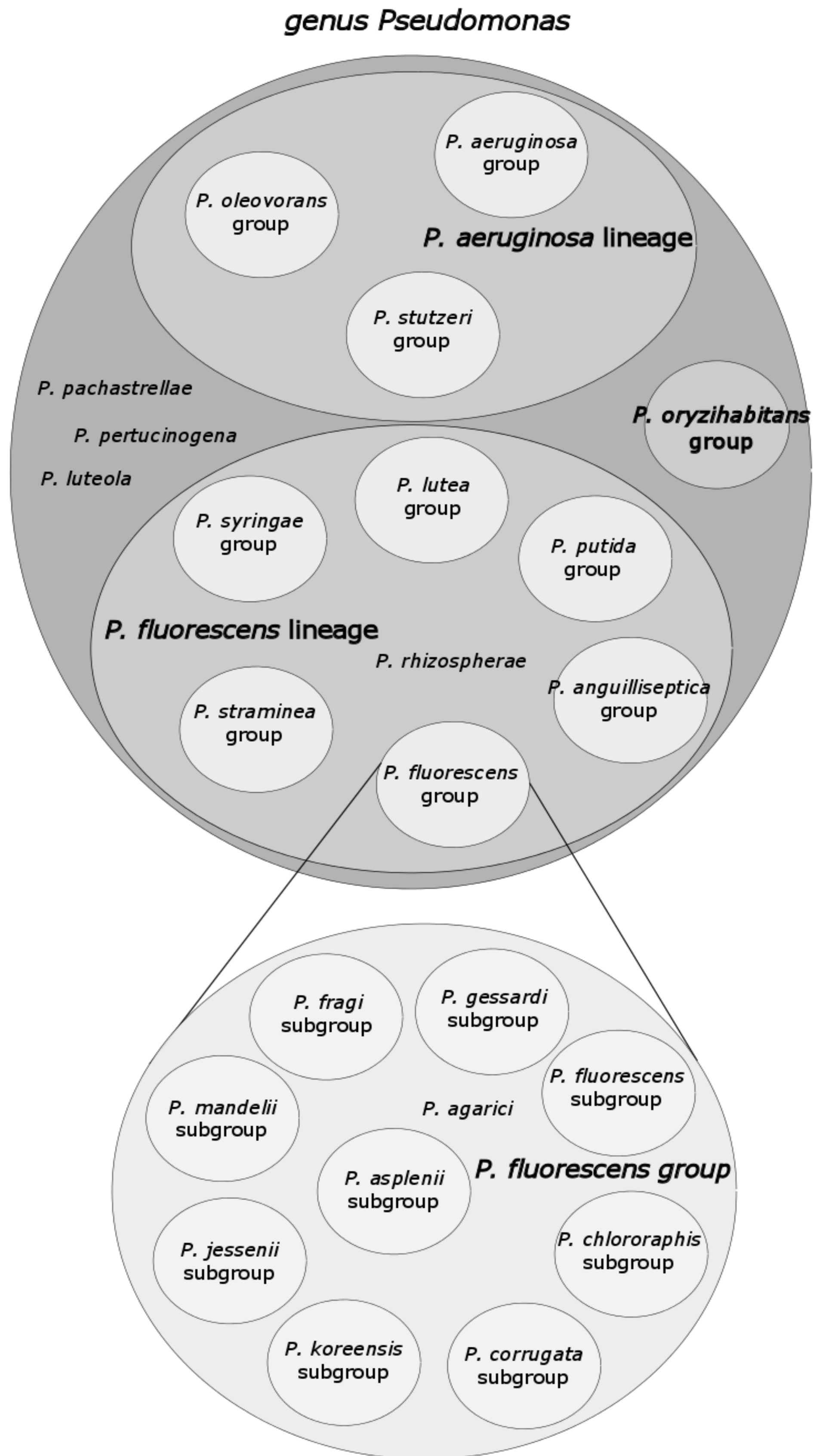


Figure 1.1 - The genus Pseudomonas.
Schematic representation of lineages, groups and subgroups of the genus Pseudomonas, as reported by Mulet et al., 2010.

1.5. Spoilage potential of the *Pseudomonas* strains: a focus on the *P. fluorescens* group

Pseudomonas spp. strains are reported to be some of the most common food spoilers (Annual Report of RASFF, 2010). Strains belonging to the *Pseudomonas fluorescens* species complex have been isolated from a wide range of foods, even when the spoilage was not visible in the food matrices.

Because of its mesophilic and psychrotolerant nature, *Pseudomonas* is often found in a wide range of aliments. Even though its optimum temperature is substantially higher, *Pseudomonas fluorescens* can replicate at the refrigeration temperatures at which fresh food is usually stored during its shelf life. This favours psychrotrophs, as competition with mesophilic bacteria that are effectively controlled by cooling, is reduced. Furthermore, storage in inappropriate thermal conditions could further enhance the growth of these spoilers. The combination of increasing shelf life, to meet consumer and marketing demand, and low temperatures has been demonstrated to enhance the growth of psychrotrophs (Munsch-Alatossava and Alatossava, 2005). Additionally, *Pseudomonas* is characterised by a brief generation time (less than four hours) that is the shortest between 0-7 °C, which further worsens food spoilage (Sørhaug and Stepaniak, 1997; Samaržija *et al.,* 2012). *Pseudomonas* spp. are particularly predominant in aerobically packaged refrigerated and protein rich aliments (Gram *et al.,* 2002).

Thanks to their favourable nutrient composition and the ability of *Pseudomonas* to degrade pectin, *Pseudomonas* strains find an optimal environment in ready-to-eat vegetables. A great variety of *Pseudomonas* species have been isolated from prepared vegetables, with a clear predominance of *P. fluorescens* and *P. fluorescens* group strains (Caldera and Franzetti, 2014; Andreani *et al.,* 2014), but with other subgroups also represented. The main reported species in Minimally Processed Vegetables (MPV) are usually *P. fluorescens*, *P. putida*, *P. chicorii*, *P. maltophilia* and *P. marginalis* (Franzetti and Scarpellini, 2007).

High-protein content and neutral pH foods, like tofu, are also advantageous matrices for *Pseudomonas* strains (Stoops *et al.,* 2012).

Pseudomonas is commonly part of the microflora of aerobically stored fish (Gram and Huss, 1996) and a specific spoiler of fresh and marine water tropical fish (Gram *et al.,* 1990; Gram, 1992) and its growth should usually be inhibited by the use of Modified Atmosphere Packages (MAPs). Actually, *Pseudomonas* and H_2S (hydrogen sulphide gas) producing bacteria were reported to dominate some products such as sea bass both in the presence of air and under MAP conditions (Parlapani *et al.,* 2015). Some species are also fish opportunists (e.g. *P. aeruginosa* and *P. fluorescens*) or fish pathogens (such as *P. putida, P. fluorescens*) as reported by different studies (Altinok *et al.,* 2006; Franzetti and Scarpellini, 2007; Zhang *et al.,* 2014; Sun and Sun, 2015).

Meat represents another favourable environment for the growth of *Pseudomonas*. *P. fluorescens*, *P. lundensis* and *P. fragi* were the most represented groups in poultry, both in processed meat, on the skin of slaughtered broilers and in processing plants (Arnaut-Rollier *et al.,* 1999; Mellor *et al.,* 2011; Oakley *et al.,* 2013; Grewal *et al.,* 2015). *Pseudomonas* spp., and especially *P. lundensis*, have also been isolated from goat and lamb slaughterhouse surfaces. Most of them were demonstrated to be resistant to at least one antimicrobial compound (Lavilla Lerma *et al.,* 2012; Lavilla Lerma *et al.,* 2014; Lavilla Lerma *et al.,* 2015). *Pseudomonas* strains have also been isolated from beef: Doulgeraki and Nychas (2013) reported *P. fragi* to be the predominant species during the storage of minced beef, especially at 15 °C and in presence of oxygen; at lower temperatures (5 and 10 °C), *P. fragi* prevails, while under a MAP environment at 15 °C *P. fragi* and *P. fluorescens* dominate the microflora. Mastromatteo and colleagues reported *Pseudomonas* to be responsible for unacceptability of pork sausage if no MAP was applied in the packaging of the meat product (2011). Finally, a gene expression investigation conducted by Mohareb and co-workers identified specific genes involved in meat spoilage, specifically regulated by glucose concentration and storage temperature (Mohareb *et al.,* 2015).

A great advantage for several *Pseudomonas* strains is the ability to compete with food microflora for iron (Cornelis, 2010). For example, milk is characterised by a low free iron content: due to the production of siderophores, *Pseudomonas* strains are able to obtain iron from lactoferrin (Mcphee and Griffiths, 2002). Due to their ubiquitous presence,

Pseudomonas spp. (and primarily *P. fluorescens*) have been often choose as model organisms to investigate spoilage mechanisms of psychrotrophs in milk and dairy-products and to evaluate control measures for reducing food contamination (de Oliveira *et al.,* 2015). Milk and dairy products are optimal matrices for the growth of *Pseudomonas* spp., thanks to their nutritional value, water content and pH close to neutrality. *Pseudomonas* strains have been demonstrated to be the majority of bacteria in milk silos during a monitoring study in Scotland, comprising 70% of all bacteria isolated (Mcphee and Griffiths, 2002). In milk, *P. lundensis*, *P. fragi*, *P. fluorescens* and *P. gessardii* are the most commonly found species (Mcphee and Griffiths, 2002; Marchand *et al.,* 2009b). The great ability of this bacterial group to spoil food is not only related to its ability to grow at refrigeration temperatures, but also its aptitude to adapt to several different environmental conditions (De Jonghe *et al.,* 2011).

Several spoilage phenotypes of *Pseudomonas* seem to be related to quorum sensing, the complex system of communication among cells, based on the production of signalling molecules (Nychas *et al.,* 2009). It should be remembered that spoilage activity is often the consequence of a complex population, whose interactions determine the alteration of the final products.

Pseudomonas is considered a Specific Spoilage Organism (SSO) for several types of food, mainly pork, poultry and seafood (Gram and Dalgaard, 2002; Raab *et al.,* 2008). The designation SSO is largely applied for the purpose of building predictive models for refrigerated food, because of these organisms direct involvement in spoilage. Generally, it is assumed that a level of around 7 Log_{10} CFU as a threshold corresponds to the end of the shelf life (Raab *et al.,* 2008).

The main mechanisms through which *Pseudomonas* implements spoilage activity are:

- Production of pigmented molecules;
- Production of enzymes;
- Production of other spoilage molecules (organoleptic spoilage).

1.5.1. Pigment production

Pseudomonas strains are well-known producers of pigmented molecules that can be involved in several spoilage events; however, not all of the known pigments have been reported in food. Few scientific papers are available except for a few technical reports of official laboratories responsible for food control. Table 1.1 reports cases of bacterial discolouration caused by *Pseudomonas* species.

Table 1.1 - Discolouration caused by Pseudomonas in food products.
Summary of recent reports of discolouration caused by Pseudomonas strains. The table reports the identified pigment, if available, the colour, the food matrix of isolation, the identified species and the reference.

Colour	Pigment	Food	Species	Reference
Blue	Unknown	Rabbit meat	*P. gessardii* *(P. fluorescens subgroup)*	Sarale *et al.,* 2011
Blue	Indigoidine	Mozzarella cheese Fresh, low-acid cheese	*P. lemonnieri* now *P. fluorescens* biovar IV (*P. corrugata* subgroup)	Caputo *et al.,* 2015 Martin *et al.,* 2011
Blue	Melanin	Pork and beef	*P. fluorescens* (*P. fluorescens* group)	Kröckel, 2009
Blue-black	Unknown (Indigo-derivative)	Mozzarella cheese Pork Beef Ricotta cheese	*P. fluorescens subgroup* (*P. fluorescens* group)	Cantoni *et al.,* 2006 Andreani *et al.,* 2014 Andreani *et al.,* 2015
Dark orange-red	Unknown	Mozzarella cheese	*P. brassicacearum* (*P. fluorescens* group)	Cantoni *et al.,* 2003
Yellow-purple	Unknown	Mozzarella cheese	*P. gessardii* (*P. fluorescens* subgroup)	Cantoni *et al.,* 2006
Green-blue	Pyoverdine & indigoidine	Mozzarella cheese	*P. putida* *P. fluorescens*	Cantoni *et al.,* 2006
Green	Pyoverdine	Mozzarella cheese	*P. fluorescens* biovar I	Cantoni *et al.,* 2006

It is quite evident that a more in depth investigation is necessary to clearly understand the chemical nature of the pigments involved in such unpleasant spoilage effects. The most outstanding event was represented by a discolouration reported in 2010 in mozzarella cheese. Official reports of the RASFF (Rapid Alert System for Food and Feed, the system that comes into play when a quick notification of presumed dangerous food contaminations is necessary to defend human health), of 2010 identified the cause in *Pseudomonas* strains (and specifically *P. tolasii* and *P. libanensis*). In the preliminary study conducted by Caputo and colleagues (2015), the pigment was identified as indigoidine (as already reported by Martin *et al.,* 2011 reporting a case of blue discolouration in a Latin-style fresh cheese) and the strains responsible for the spoilage defect were suggested to be *P. fluorescens* biovar IV (previously

P. lemonnieri). Actually, these results differ from the investigation conducted by Andreani and collaborators that placed the blue-pigmenting strains into a well-separated branch of the *P. fluorescens* subgroup (Andreani *et al.,* 2014; Andreani *et al.,* 2015) based on the construction and application of a Multilocus Sequence Typing (MLST) scheme. They also concluded that the blue pigment could not be indigoidine, but an indigo-derivative. In all of these studies, only a high microbiological load was able to induce the blue discolouration (about 10^7 CFU/gram). Interestingly, the pigment seemed to turn blue in the presence of oxygen (meaning that it is derived from an uncoloured parent compound).

Blue discolouration has also been reported in other food products, such as ricotta cheese, bacon and beef (Andreani *et al.,* 2014), as a result of the growth of strains belonging to the *P. fluorescens* subgroup.

1.5.2. Enzyme production

Food alteration defects can also be induced by *Pseudomonas* strains through the production of a wide range of enzymes. As a matter of fact, not only do the resident and living microflora produce enzymes, but due to the thermoresistent nature and activity in low A_w environments of some enzymes, they can also retain their activity in finished products. Hydrolytic enzymes, namely proteases, lipases and lecithinases, may maintain up to the 70% of their activity after heat treatments (Samaržija *et al.,* 2012), contributing to the shortening of the shelf life of food products by altering the physicochemical characteristics of the products, or by affecting food functionality and organoleptic qualities (Teh *et al.,* 2014).

The major spoilage enzymes are proteases. One of the most outstanding examples of this type of spoilage is visible in UHT milk and dairy products, where proteases are active against caseins (the most represented proteins in this type of fresh products). Proteolysis of UHT milk during shelf life causes different changes in organoleptic characteristics, such as gelation of milk, that causes a rise in viscosity, an unpleasant grey discolouration and flavour changes that induce a bitter, unclean, fruity, yeasty and metallic taste (Datta and Deeth, 2003; Arslan *et al.,* 2011; Samaržija *et al.,* 2012). *Pseudomonas* proteases showed activity against κ-, α_{s1}- and β- caseins, with the subsequent coagulation of the newly formed complexes, forming a gel-structure in milk (Datta and Deeth, 2003). It should be mentioned that this alteration

might be also induced by the activity of an endogenous milk proteinase, namely plasmin (Datta and Deeth, 2001). Production of proteinases affects cheese yield by destabilizing casein, as demonstrated by several studies (Mitchel and Marshall, 1989). In particular, plasmin and plasminogen are released from casein micelles, changing the cheese yield, as plasmin is a well-known actor in cheese manufacture, as well as affecting the flavour and texture of the final product (Samaržija *et al.,* 2012).

Marchand and colleagues reported *P. fragi*, *P. lundensis* and other minor species of the *P. fluorescens* group as prolific producers of proteases that are in most cases heat-stable (2009a; 2009b).

A recent study based on the phenotypic investigation of 87 *P. fluorescens* species complex strains revealed that almost all the strains (94%) were able to induce proteolysis on Nutrient Agar Plates with 2% UHT Milk at 22 °C, while about 72% were able to induce the defect at refrigeration temperatures (Andreani *et al.,* 2014), emphasising the high prevalence of proteolytic strains within the *P. fluorescens* group.

The most reported family of thermostable proteases within the genus *Pseudomonas* is the serralysin protease family, a highly conserved protein group whose main representatives are members of the AprX family, an alkaline zinc metalloprotease family with molecular masses between 39.2±0.7 and 45.3±1.3 kDa (Dufour *et al.,* 2008; Marchand *et al.,* 2009b; Teh *et al.,* 2014). Recent advantages in molecular biology have allowed the investigation of genetic and regulation mechanisms underlying protease production. The *aprX* gene was detected in almost all *P. fragi* and several reference strains of *Pseudomonas* species (such as *P. fluorescens, P. rhodesiae, P. tolaasii, P. chlororaphis* and *P. gessardii*), but not in *P. lundensis*. Furthermore, a high degree of heterogeneity among amino acid sequences of AprX proteins was reported (Marchand *et al.,* 2009b). It must be noted that the presence of the gene is not synonymous with certain expression: most protease production depends on several environmental parameters, such as temperature, oxygen concentration, quorum sensing, nutrient composition of the media (i.e. presence of iron) and phase variation (Marchand *et al.,* 2009b; Teh *et al.,* 2014). Interestingly, high storage temperatures seem to inhibit protease production in *P. fragi* (Marchand *et al.,* 2009b); however, strong heat

treatments, such as boiling of milk for 30 minutes, seem to induce a massive release of proteases, inducing a more palpable spoilage effect (Samaržija *et al.,* 2012). In some strains of *Pseudomonas* spp., AprX has been identified as the sole protease involved in food spoilage (Woods *et al.,* 2001).

Proteases are mainly produced at the end of the exponential phase, when cell density is substantially high, highlighting the involvement of quorum sensing mechanisms in spoilage activity (Bai and Rai, 2011) although some other studies (Liu *et al.,* 2007; Pinto *et al.,* 2010) failed to detect any signalling molecules when investigating spoiled milk.

Lipolytic spoilage is less common than the proteolytic type. Lipases, triacylglycerol hydrolases, act on the fat constituents of food and induce the release of fatty acids and glycerol. Free short-chain fatty acids induce unpleasant flavours, usually described as rancid, while medium-chain fatty acids are responsible for unclean, soapy or bitter flavours (Samaržija *et al.,* 2012). No particular unpleasant effect seems to be caused by the production of long-chain fatty acids. It has been extensively reported that lipolytic activity is more remarkable at refrigeration temperatures (low-temperature regulation; Woods *et al.,* 2001; Rajmohan *et al.,* 2002).

In cheese, lipases are adsorbed in fat globules and persist in the cheese, inducing spoilage effects during the ripening of semi-hard and hard cheeses (Samaržija *et al.,* 2012). The heat-stability of lipases seems to be enhanced in presence of multiple lipases (Teh *et al.,* 2014).

Interestingly, in *P. fluorescens* B52, genes coding for the thermostable protease (*aprX*) and the lipase (*lipA*) are located in the same operon, separated by other genes involved in secretion of the extracellular protease (Woods *et al.,* 2001; McCarthy *et al.,* 2004). Therefore, gene expression of both hydrolases is correlated, dependent on low temperature and inhibited by iron (Woods *et al.,* 2001).

Another important class of extracellular enzymes is constituted by lecithinases and other phospholipases. These enzymes disrupt the fat globules of milk and make fat content available for further lipase activity (Koka and Weimer, 2000; Samaržija *et al.,* 2012). Their final products are diglycerides and substituted phosphoric acids. The most commonly represented enzyme family is phospholipase C. In milk, spoilage defects related to the

production of phospholipases are sweet curdling, bittiness and feathering (Koka and Weimer, 2000). As these kinds of enzymes are not usually present as endogenous enzymes in milk, phospholipase mediated spoilage is usually uniquely related to psychrotrophs such as *Pseudomonas* spp. strains.

Some strains have been reported to be able to produce the whole range of spoilage enzymes (proteases, lipases and lecithinases) in a plate assay (Andreani *et al.,* 2014).

Table 1.2 reports the most common spoilage defects related to enzyme production as an effect of *Pseudomonas* growth in different food matrices.

Table 1.2 - Enzymatic spoilage caused by Pseudomonas.
Summary of the main spoilage activities in food products involving enzyme production. The table reports the enzymes responsible of the spoilage, the food involved in the defect and the characteristics of the defect. Finally, responsible species and references are reported. Adapted and implemented from Samaržija et al., 2012.

Enzyme	Food	Alteration	Species	Reference
Lipases	Sterilized milk	Rancidity, off-flavours, bitterness, soapy off-flavours	*Pseudomonas* spp. *P. fluorescens*	Samaržija *et al.,* 2012
Lipases	Cheese	Enhance of coagulation time due to high amount of free fatty acids	*Pseudomonas* spp.	Samaržija *et al.,* 2012
Lipases	Cheese	Undesirable lipolysis at the end of ripening	*Pseudomonas* spp.	Samaržija *et al.,* 2012
Lipases and proteases	Cream Butter	Rancidity, off-flavours, fruity bitterness, soapy off-flavours	*P. fragi* *P. putrefaciens* *Pseudomonas* spp.	Jay, 2000 Mcphee and Griffiths, 2002 Samaržija *et al.,* 2012
Proteases	Pasteurized Milk	Gelation due to enhanced viscosity of milk	*P. fluorescens* *P. fragi*	Datta and Deeth, 2003 Arslan *et al.,* 2011 Samaržija *et al.,* 2012
Proteases	Pasteurized Milk	Fouling in heat exchangers	*Pseudomonas* spp.	Samaržija *et al.,* 2012
Proteases	Cheese	Reduction of cheese yield due to destabilization of casein fraction Reduction of cheese yield due to a higher heat sensitivity of milk proteins	*Pseudomonas* spp.	Mitchell and Marshall, 1989 Samaržija *et al.,* 2012
Proteases	Cheese	Reduction in coagulation time due to high amount of free amino acids	*Pseudomonas* spp.	Samaržija *et al.,* 2012
Proteases	Yogurt	Firmer texture, higher viscosity and greater syneresis Enhanced growth of microflora due to higher free amino acid contents Bitter, rancid, unclean and fruity off-flavours	*Pseudomonas* spp.	Gassem and Frank, 1991 Sørhaug and Stepaniak, 1997
Proteases	Semi-hard and hard cheese	Atypical flavour during ripening due to the persistence of spoilage proteases	*Pseudomonas* spp.	Fox, 1989 Samaržija *et al.,* 2012
Proteases	Fresh cheese	Gelation and slimy texture due to the activity of enzymes	*P. fluorescens* *P. fragi* *P. putida*	Fox, 1989 Samaržija *et al.,* 2012
Proteases	Soft cheese	Poor texture due to the activity of enzymes	*P. fluorescens* *P. fragi* *P. putida*	Samaržija *et al.,* 2012

The detection of these enzymes in the final products might help in monitoring the spoilage effect related to microbial contamination. The most common methods are the use of caseinate agar (to detect proteolytic activity), but also electrophoresis, HPLC, UV-Vis spectrometry, fluorimetric and immunological methods to detect enzymes directly in food (Teh *et al.,* 2014).

In fact, *Pseudomonas* should not be the only target of the screening: Teh and colleagues (2012) demonstrated that enzymatic activity of *P. fragi* BC5 was enhanced in co-culture with other strains, such as *Bacillus licheniformis*.

1.5.3. Other types of spoilage

Besides enzymatic and pigment spoilage, the growth of *Pseudomonas* spp. on several food matrices can induce further quality modifications making the product undesirable for human consumption (organoleptic spoilage). In these cases, the spoilage involves the ability of bacterial strains to use nutrients in food and to produce molecules that induce modification of the food quality.

As an example, several studies have reported that the growth of *Pseudomonas* spp. on iced fish is related to fruity, rotten, and sulphydryl odours and flavours as a result of the synergy of newly produced acetate, butyrate and hexanoate (Miller *et al.,* 1973; Gram, 1992). Furthermore, strains of the genus *Pseudomonas* produce several volatile aldehydes, ketones, esters and sulphides (Dainty *et al.,* 1984; Miller *et al.,* 1973). According to Gram and Huss (1996), the fruity off-odours produced by *P. fragi* originate from monoamino-monocarboxylic amino acids. Moreover, *P. fragi* develops apple-like esters inducing rancidity in butter (Samaržija *et al.,* 2012). Ercolini and co-workers revealed through a challenge test that the volatile compounds produced after fifteen days of storage of inoculated beef with a *P. fragi* strain were highly heterogeneous and showed their highest diversity in ketones and higher-molecular weight alcohols, while only a few esters were detected (Ercolini *et al.,* 2009). Recently, Bhadra and colleagues (2015) correlated volatile compounds such as trimethylamine, dimethylamine and ammonia with the enhancement of total viable count that after twelve hours is completely represented by *Pseudomonas*. *P. putrefaciens* is able to release a wide range of organic acids, and mainly isovaleric acid, inducing a putrid odour in

butter (Jay, 2000; Samaržija *et al.,* 2012). It seems clear that the production of volatile compounds is a strain-dependent characteristic and a common pattern for all of the spoiler *Pseudomonas* cannot be identified, as has already been reported in single-species investigations (Ercolini *et al.,* 2009).

Some strains are able to produce a surface film: for example, strains of *P. fluorescens* have been detected on the surface of cottage cheese during its shelf life at 7 °C (Brocklehurst and Lund, 1985).

Pseudomonas spp. also induce putrefactive odours and slime on fresh chilled meat in the presence of a population with at least 10^7 CFU/cm^2 (Sofos, 1994).

In 2010, Motoyama and co-workers reported *Pseudomonas* spp. and specifically *P. fragi* as responsible of beef reddening in raw meat due to the conversion of metmyoglobin into deoxymyoglobin, together with slime production.

1.6. Related genera: *Xanthomonas* and *Shewanella*

Other genera belonging to *Gammaproteobacteria* and involved in food spoilage, such as *Shewanella* and *Xanthomonas*, were previously considered as related genera to *Pseudomonas* (Liao, 2006). Nowadays, *Xanthomonas* is considered as a monophyletic lineage and a distinct genus from the other family members of *Xanthomonadaceae*. Species belonging to the genus *Xanthomonas* are reported as causative agents of disease in plants. Proteases and toxins are involved in host colonization (having mainly cellulolytic and pectolytic activities) while the presence of Lipopolysaccharide (LPS) or the release of extracellular polysaccharides could be involved in the pathogenicity of different pathovars and species. The maintenance of the genus in the environment seems to be related to seed transmission or persistence in plant tissues. However, some species also exhibit a saprophytic life style and are related to the postharvest decay of fruits and vegetables.

On the other hand, bacteria ascribed to the genus *Shewanella* show heterogeneous phenotypic characteristics, mainly related to the origin habitat of each species. The ecological niches occupied by *Shewanella* range from aquatic sediments to animal gut, demonstrating

their capability to adapt to anoxic environments (Hau and Gralnick, 2007). Despite the heterogeneity of these environments, water (marine and fresh water) can be considered as the preferred environment for *Shewanella*. These waterborne bacteria are involved in food contamination, especially of seafood, but also of poultry and other meat products (Parker and Levin, 1983; Nychas *et al.,* 2007; Dainty *et al.,* 1989). Moreover, Janda recently reported some species of *Shewanella* as human pathogens (i.e. *S. algae*; Janda, 2014).

Due to their ability to induce changes in the odour profile (off-odours) and in relation to the wide range of metabolites produced, in the last decade some members of this genus have been proposed as SSOs of food and have been used as shelf life predictors (e.g. in seafood products; Gram and Dalgaard, 2002).

1.6.1. *Shewanella* spp.

Shewanella is the type genus and, at the present, the sole genus of the family Shewanellaceae ascribed to the order Alteromonadales, with around 50 described species (Satomi, 2014). Taking into account the phenotypic variability of the genus, species characterisation and reclassification is based on DNA/DNA hybridization and 16S rRNA gene sequencing (Hau and Gralnick, 2007). The sequence of additional genes, such as *gyrB,* confirmed the species delineation (Dehaut *et al.,* 2014; Satomi *et al.,* 2003). Moreover, molecular typing of strains can reduce misinterpretation and has allowed the development of intra-species studies through different approaches such as Random Amplification of Polymorphic DNA (RAPD), Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF), MLST and Whole Genome Sequencing (WGS; Serio *et al.,* 2014; Satomi, 2014; Deng *et al.,* 2014). As for other bacterial taxa, NGS techniques have allowed a deeper investigation of the genomic information related to its phenotypes. To date (December 2015), 25 complete genomes and 25 draft genomes are available on GenBank. Annotations conducted by Dikow (2011) revealed that *Shewanella* possesses about 5,000 genes and that its genome consists of approximately 5 Mbp.

These Gram-negative bacteria are oxidase positive, non-endospore forming bacteria characterised by rod-shaped morphology. All the members are motile (e.g. swimming motility) due to a unique polar flagellum (Wu *et al.,* 2011; Satomi, 2014). Generally, a first

phenotypic separation among psychrotrophic and mesophilic species can be delivered by testing the growth at 4, 37 and 42 °C (Fonnesbech Vogel *et al.,* 2005). Careful attention is required to the isolation temperature in order to differentiate the three major phenotypic lineages: i) psychrotolerant non-halophilic bacteria, ii) psychrophilic species requiring salt for growth and iii) mesophilic species (Satomi, 2014). The use of different salt concentrations in combination with the use of different carbon sources and enzymatic activities (e.g. amino acid decarboxylation) have been widely proposed for strain characterisation, taxonomic keys and numerical taxonomy purposes (Fonnesbech Vogel *et al.*, 2005; Satomi *et al.,* 2006; Satomi *et al.,* 2007; Dehaut *et al.,* 2014; Serio *et al.,* 2014). Basically, the aforementioned phenotypic traits, together with the production of H_2S and non-fermentative metabolism on oxidative-fermentative (OF) medium are indicative of the genus. The excellent work of Satomi (2014) retained the most useful phenotypic analyses for species delineation. The species are defined as aerobes/facultative anaerobes. Moreover, one of the evolutionary keys to the worldwide diffusion of *Shewanella* is related to the wide number of electron acceptors and metabolic pathways adopted under limited oxygen conditions (Hau and Gralnick, 2007). The use of several compounds such as trimethylamine-N-oxide (TMAO), sulphur and other organic compounds under anaerobic conditions and their psychrotrophic habits make *Shewanella* one of the major spoilers of refrigerated food such as seafood products. The spoiler species of *Shewanella* can typically be described as H_2S producers and media containing thiosulphate (such as Iron Agar (IA) plates) are commonly applied for their first isolation and enumeration (Fonnesbech Vogel *et al.,* 2005; Dehaut *et al.,* 2014; Serio *et al.,* 2014). On the other hand, the use of common media such as Plate Count Agar (PCA) could lead to a lack of recovery of species or strains that prefer salt (e.g. some strains of *S. frigidimarina*; Broekaert *et al.,* 2011).

1.6.2. Spoilage potential of *Shewanella* spp.

From the first isolation on the surface of taint butter in the 1931, the genus *Shewanella* was associated with food spoilage (Derby and Hammer, 1931). Despite their relevance as SSO in aquatic food products, some species are also involved in the deterioration of meat and poultry. The spoilage potential of *Shewanella* has been extensively reviewed in products

stored in aerobic, but also in low oxygen conditions such as MAP and vacuum packaging (Russel *et al.,* 1995; Nychas *et al.,* 2007; Vihavainen and Björkroth, 2010). In refrigerated food rich in amino acid and other nitrogen sources, such as fish or flesh stored at low temperature, some members of the genus *Shewanella* can dominate the microbiota and produce several compounds according to their flexible metabolism. However, dominance in the spoilage population is not necessary in order to exert spoilage effects. The genome sequence of *Shewanella oneidensis* revealed that *Shewanella* are able to metabolise a wide range of different substrates as the sole carbon sources (Fredrickson *et al.,* 2008). For this reason, these bacteria are among the most important microorganisms involved in the degradation of organic matter. The catabolic pathways described in *Shewanella oneidensis* revealed the ability to use substrates commonly present in food (e.g. fatty acids, amino acids and several carbohydrates). Moreover, the study of functional properties through genomic approaches could lead to a new comprehension of the spoilage activities of *Shewanella* species. The *in silico* analysis of *S. putrefaciens* genomes has suggested an alternative functional pathway involved in the production of amines (Remenant *et al.,* 2015). According to this comparative analysis, putrescine production seems related to the ability to transform L-ornithine derived from glutamate, while some strains also have the potential ability to convert putrescine to spermidine. Clearly, the production of foetid amines and diamines is strain-dependent and this approach could elucidate specific strains food spoilage ability (Remenant *et al.,* 2015). The decarboxylation of amino acids such as lysine and ornithine is a phenotypic marker for strains involved in the production of cadaverine and putrescine, while the use of histidine seemed to be less common, with low risk of histamine production (Serio *et al.,* 2014). The role of *Shewanella* as an SSO of fish, especially marine fish, is related to the production of two spoilage markers involved in the classical alteration of odour: trimethylamine (TMA) and H_2S. The use of TMAO as a final electron acceptor during anaerobic metabolism would be consistent with fishy odour production due to its conversion to TMA (Fonnesbech Vogel *et al.,* 2005), while the putrid odour caused by the H_2S formation is related to the use of sulphite and other molecules such as polysulphide and thiosulphate (Hau and Gralnick, 2007). In fresh meat of warm-blooded animals, especially in vacuum

packaged flesh at high pH values, the production of H_2S can induce the greening discolouration of muscle due to the formation of a pigment complex called sulphmyoglobin. This is probably due to the catabolism of sulphur-containing amino acids with the formation of sulphide (Garcia-Lopez *et al.,* 1998; Nychas *et al.,* 2007; Nychas *et al.,* 2008). Other additional enzymatic activities of *Shewanella* can induce spoilage: for example, extracellular DNAse activity is involved in nucleotide degradation (Satomi, 2014; Serio *et al.,* 2014). The breakdown of hypoxanthine (Hx) or the conversion of Inosine MonoPhosphate (IMP) and inosine into Hx coincide with bitter flavours and the related decay of fish freshness (Haard, 2002; Serio *et al.,* 2014). Finally, proteolytic activities could also favour deterioration and the spread of bacteria into the muscles due to the hydrolysis of collagen and other structural proteins (Gennari *et al.,* 1999; Fonnesbech Vogel *et al.,* 2005; Serio *et al.,* 2014). On the basis of their metabolism, the spoilage potential of *Shewanella* is then related to the substantial production of chemical compounds with low odour thresholds. H_2S, TMA and Hx and other volatile compounds such as alcohols and aldehydes contribute to the formation of the volatilome of food during its shelf life (Casaburi *et al.,* 2015).

These malodourous molecules are common indicators of spoilage; however, not all the *Shewanella* species are able to produce this wide range of compounds. Up to ten years ago, the major species described during the deterioration of food, especially seafood, was *S. putrefaciens* (Fonnesbech Vogel *et al.,* 2005). Fonnesbech Vogel and co-workers (2005) on the basis of phenotypic taxonomy, G-C content, DNA/DNA hybridization and phylogenetic analysis of 16S rRNA highlighted that a dominant species among the H_2S producers is *Shewanella baltica*. Recent studies have confirmed that *S. baltica* plays a key role in the deterioration of finfish and freshwater fish, together with *S. putrefaciens* (Beaz-Hidalgo *et al.,* 2014; Dehaut *et al.,* 2014; Serio *et al.,* 2014).

Several other species are H_2S producers such as *S. algae*, *S. colwelliana* (Fonnesbech Vogel *et al.,* 2005) and other well-characterised species as reported in the works of Satomi and co-workers (Satomi *et al.,* 2006, 2007), although only *S. putrefaciens* and *S. baltica* are widely isolated and used as SSOs, as documented by recent studies on H_2S producing bacteria (see Table 1.3 for details).

Table 1.3 summarises some recent works related to *Shewanella* contamination, reporting both studies cited above and some minor ones. Nearly all studies applied Iron Agar as a first isolation medium followed by different characterisation methods. The use of H_2S producing bacteria as markers is also commonly applied in predictive microbiology particularly for finfish (Giuffrida *et al.,* 2013; Carrascosa *et al.,* 2014).

Recent findings have suggested a certain heterogeneity of the phenotypic traits of *S. baltica*. Some strains are unable to produce H_2S and this could lead to misinterpretations when applying common media used for *Shewanella* enumeration (Dehaut *et al.,* 2014). On the other hand, other genera with morphologies similar to *Shewanella* species, such as *Serratia,* are able to grow in Iron Agar (Serio *et al.,* 2014). The same authors suggested the application of additional selective media for the enumeration of *Shewanella*. An increase in salt concentration could enhance the isolation and differentiation of halophilic species. At the same time, a shift from the media with thiosulphate to the media that highlight TMAO reduction or pigment synthesis (e.g. salmon pink colour) could improve the detection of different *Shewanella* species (Dehaut *et al.,* 2014; Serio *et al.,* 2014). For instance, other additional substrates such as cysteine in substitution or in addition to thiosulphate could better elucidate the H_2S production pattern (Macé *et al.,* 2014; Serio *et al.,* 2014). Moreover, species identification requires the complementary use of classical phenotypic keys in combination with different genetic targets (Tryfinopoulou *et al.,* 2007). As previously mentioned, some species, such as *S. algae,* have been also reported as putative human pathogens (Janda and Abbot, 2015). Furthermore, new insights suggested that *S. algae* was also involved in the production of tetrodotoxin during cases of food poisoning (Wang *et al.,* 2013).

1.6.3. *Xanthomonas* spp.

Species belonging to the genus *Xanthomonas* are able to colonise a broad range of hosts (400 species of plants); however, these bacteria show a high level of specialization according to the host species and tissues (Ryan *et al.,* 2011). Whole-genome analyses have revealed that this specialization is related to a reduction in genome size, where the species able to colonise seed

Table 1.3 - Shewanella in food products.
Overview of some recent articles (2007-2015) concerning the isolation and characterisation of H_2S producing bacteria (or putative Shewanella spp.) from food matrices.

Food	**Storage condition**	**Media of isolation/ enumeration**	**Method of characterisation**	**TMAO/H_2S**	**Species**	**References**
Sea bream skin and flesh Gills	Not reported	Iron Agar Lyngby	Phenotypic	+/+	H_2S producing bacteria/putative *S. putrefaciens*	Carrascosa *et al.*, 2015
Whole, ungutted sea bass (*Dicentrarchus labrax*)	Not reported	Iron Agar Lyngby	Phenotypic	+/+	H_2S producing bacteria/putative *S. putrefaciens*	Carrascosa *et al.*, 2015
Spoiled salmon fillets	MAP	Iron Agar with 0.04% of L-cysteine	16S rRNA	Not reported	*S. baltica*	Macé *et al.*, 2014
Spoiled cooked whole tropical shrimps	MAP	Iron Agar	16S rRNA gene	Not reported	*S. baltica*	Macé *et al.*, 2014
Red tuna and swordfish steaks	Refrigerated in aerobiosis	Kligler Iron Agar (KIA)	16S rDNA RAPD and REP-PCR	+/+	*S. baltica*, *S. putrefaciens*, *S. morhuae*	Serio *et al.*, 2014
Atlantic horse mackerel (*Trachurus trachurus*)	MAP	Kligler Iron agar Long and Hammer plate	16S rRNA	Not reported	Putative *S. baltica*, *S. glacialipiscicola*, *Shewanella* spp.	Alfaro and Hernandez, 2013
King scallop (*Pecten maximus*)	Aerobic, vacuum packed MAP	Iron Agar	16S rRNA	Not reported	*S. frigidimarina*, *S. baltica*, *S. putrefaciens*	Coton *et al.*, 2013
North American catfish fillets (*Ictalurus punctatus*)	Air	DNase test agar	16S rRNA	Not reported	*S. baltica*	Hickey *et al.*, 2013
Pacific White Shrimp (*Litopenaeus vannamei*)	MAP	Plate Count Agar and Triple Sugar Iron	Phenotypic	Not reported	*Shewanella* spp.	Qian *et al.*, 2013

Whole ungutted sea bream	On ice	Iron Agar	culture independent by 16S rRNA cloning procedure, culture dependent biochemical identification	+/+	*S. putrefaciens*-like	Parlapani *et al.,* 2013
Restaurant knife, marinated beef, poisoned patients	Epidemiological survey after tetrodotoxin intoxication	WS Salmonella Agar, Salmonella Shigella Agar, Eosin Methylene Blue Agar, Thiosulfate Citrate Bile Salts Sucrose Agar	16S rDNA	Not reported	*S. algae*, *Shewanella* spp.	Wang *et al.,* 2013
Broiler carcasses	Air-packaged	Not reported	PCR-DGGE	Not reported	*Shewanella* spp.	Zhang *et al.,* 2013
Surface of the salmon pieces	MAP	Nutrient Agar Long and Hammer Agar	16S rRNA gene	Not reported	*S. baltica*, *S. morhuae* *Shewanella* spp.	Powell and Tamplin, 2012
Marine finfish	Not reported	Iron Agar with 0.5% NaCl (L-cysteine and sodium thiosulphate)	REP-PCR and phenotypic, 16S rRNA, *gyrB* gene	Not reported	*S. frigidimarina* *S. vesiculosa* *S. baltica* *S. glacialipiscicola*	Broekaert *et al.,* 2011
Mediterranean hake (*Merluccius merluccius*)	Not reported	Iron Agar Lyngby	API 20NE	Not reported	Presumptive *S. putrefaciens*	Baixas-Nogueras *et al.,* 2009
North- Atlantic cod	air or MAP, superchilled temperatures, brined	Iron Agar	16S rRNA clone analysis	Not reported	*Shewanella* spp., *S. benthica*	Reynisson *et al.,* 2009
Atlantic cod (*Gadus morhua*)	MAP	Iron Agar Lyngby	DGGE	Not reported	*S. putrefaciens*	Hovda *et al.,* 2007
Sparus aurata	Air, MAP	Iron agar	SDS-PAGE 16S rRNA phenotypic	+/+	*S. baltica* (phenotypic clusters: *S. baltica*, *S. oneidensis* and *S. putrefaciens*)	Tryfinopoulou *et al.,* 2007

surfaces (*X. oryzae* pv. *oryzae*) differ from species associated with xylem invasion (e.g. *X. fastidiosa*).

Xanthomonas can be a cause of postharvest diseases and was reported as one of the genera isolated from spoiled fruits and vegetables (Barth *et al.,* 2009). As for other Gammaproteobacteria, the taxonomy of *Xanthomonas* has been progressively rebuilt during the last two decades. Particularly, *X. campestris* pathovars were split into new species taking into account their host range (Vorhölter *et al.,* 2008). One of the main characteristics that identifies the genus is the production of yellow pigments (xanthomonadins), conferring the name to the genus: *xanthos* is yellow in ancient Greek (Rajagopal *et al.,* 1997). Despite their diverse and adaptable pathogenicity, the genus shows heterogeneous phenotypic and physiological traits that lead to difficult phenotypic classification (Meyer and Bogdanove, 2009). The bacteria are motile due to the presence of a single polar flagellum, catalase positive and some species are able to produce the exopolysaccharide xanthan that is largely applied in the food industry as emulsifier (Vorhölter *et al.,* 2008; Palaniraj and Jayaraman, 2011). Several genotyping methods were proposed for a polyphasic approach classification of *Xanthomonas* together with genome sequencing (Meyer and Bogdanove, 2009). However, the study of full metabolic profiles using different carbon sources (e.g. the BIOLOG system) could be applied routinely in order to classify common species in large datasets (Stoyanova *et al.,* 2014).

1.6.4. Spoilage potential of *Xanthomonas* spp.

Liao and Wells (1987) provided experimental evidence of the pectolytic activity of some strains of *Xanthomonas campestris*. Some strains showed the capacity to liquefy pectate gels in *in vitro* tests; moreover, in fresh plant material the strains were able to macerate vegetable tissue suggesting that *X. campestris* plays an active role in the postharvest rot of plant crops (e.g. potato tubers, carrot, cucumber, cauliflower). *Xanthomonas* species are also involved in alterations to fruits that are directly associated with their pathogenic behaviour such as cankers and necrotic lesions. *Xanthomonas arboricola* pv. *pruni* is the disease agent of the bacterial spot of stone fruit (BSSF) that was spread around world due to human migrations (Boudon *et al.,* 2005). The lesions on fruits depend on early infections after petal fall, with

different aspects ranging from large open-deep lesions into the pulp to the coalescent spotting on the fruit surface (Ritchie, 1995). In this case, as for other fruits, each specific pathovar or species was involved in the bacterial spot on both leaves and fruits (e.g. *Xanthomonas axonopodis* pv. *passiflorae* on the passion fruit); outbreaks can lead to economic loss due to direct damage to the fruit and plant (Muhnoz *et al.,* 2011).

1.7. Conclusion and future perspectives: biofilm formation and control of spoilage defects

Besides the taxonomic history of *Pseudomonas*, *Shewanella* and *Xanthomonas*, that often groups the three genera altogether along with their spoilage activity, these taxa are also associated because of their biofilm formation and their psychrotrophic nature.

For these reasons, maintenance of the cold chain might not be the solution to all of the issues related to their growth and spoilage. It has been reported that a further reduction of storage temperature from 4-6 °C to 2 °C might be helpful to reduce the spoilage activity of psychrotrophs during longer and longer shelf life times (Kumarsan *et al.,* 2007).

As already reported, the three genera are able to produce biofilms: for this reason, the identification of safe and uncontaminated raw materials and final products is not synonymous with non-spoiled food. Indeed, working surfaces, packaging and transport and storage equipment might be contaminated by bacterial biofilms, which are very difficult to remove during sanitization. Particular attention must be paid to post-process contaminations. Adequate sanitization is fundamental, taking into account the need to deal with complex structures as biofilms. Because of these structures, bacteria are able to resist a wide range of stresses, especially sanitisers applied for the decontamination of processing surfaces. Furthermore, biofilms might act as a reservoir of secreted enzymes, protecting them from the activity of detergents and sanitization methods and enhancing enzyme activity (Teh *et al.,* 2014).

The management of packaging processes, taking into account the aerobic nature of *Pseudomonas* and *Xanthomonas* can be helpful in controlling proliferation. MAP, with a low

or null percentage of oxygen, should reduce their count; however, recently Stoops and colleagues (2012) revealed that a strain of *Pseudomonas* isolated from the microflora of tofu, was not completely inhibited by packaging in 100% CO_2.

Interestingly, natural compounds have been applied to food products. For example, Quintieri and co-workers applied bovine lactoferrin B and its pepsin-digested hydrolysate to control *Pseudomonas* in governing liquid and on mozzarella cheese to reduce growth and pigment production, demonstrating the efficacy of the hydrolysate in the control of spoilage strains (Quintieri *et al.,* 2012). Similarly, an innovative active packaging system was proposed combining lemon extract, brine and a gel solution made of sodium alginate, that demonstrated a significant enhancement of the shelf life of mozzarella cheese (Conte *et al.,* 2007). A natural compound from the essential oil of *S. thymbra* is particularly active against *Pseudomonas* biofilms, being more effective than several acid-based disinfectants (Chorianopoulos *et al.,* 2008). In a similar way, recently Zhu and colleagues reported the effective activity of green tea polyphenols against quorum sensing and biofilm formation in *Shewanella baltica,* substantially reducing its food spoilage potential (Zhu *et al.,* 2015).

Recently, bacteriophages have emerged as new and safe options for the treatment of pathogens and spoilers in a wide range of foods. In 2010, Sillankorva and colleagues reported the high efficacy of a phage against *P. fluorescens* in mono and dual species biofilms.

The scenario described in this chapter is complex and reveals a large variety of mechanisms through which *Pseudomonas*, *Xanthomonas* and *Shewanella* induce spoilage effects. It is evident that only a complete understanding of all of these systems can lead to the identification of targets for the effective control of all spoilage species, with the ultimate aim of reaching high microbiological quality standards in all food products.

1.8. References

- Alfaro, B., Hernandez, I., 2013. Evolution of the indigenous microbiota in modified atmosphere packaged Atlantic horse mackerel (*Trachurus trachurus*) identified by

conventional and molecular methods. International Journal of Food Microbiology, 167, 117-123.

- Altinok, I., Kayis, S., Capkin, E., 2006. *Pseudomonas putida* infection in rainbow trout. Aquaculture, 261, 850-855.
- Andreani, N.A., Martino, M.E., Fasolato, L., Carraro, L., Montemurro, F., Mioni, R., Bordin, P., Cardazzo, B., 2014. Tracking the blue: a MLST approach to characterise the *Pseudomonas fluorescens* group. Food Microbiology, 39, 116-126.
- Andreani, N.A., Carraro, L., Martino, M.E., Fondi, M., Fasolato, L., Miotto, G., Magro, M., Vianello, F., Cardazzo, B., 2015. A genomic and transcriptomic approach to investigate the blue pigment phenotype in *Pseudomonas fluorescens*. International Journal of Food Microbiology, 213, 88-98.
- Andrews-Polymenis, H.L., Santiviago, C.A., McClelland, M., 2009. Novel genetic tools for studying food borne *Salmonella*. Current opinion in Biotechnology, 20, 149-157.
- Anzai, Y., Kim, H., Park, J.-Y., Wakabayashi, H., Oyazu, H., 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequences. International Journal of Systematic and Evolutionary Microbiology, 50, 1563-1589.
- Arnaut-Rollier, I., Vauterin, L., De Vos, P., Massart, D.L., Devriese, L.A., De Zutter, L., Van Hoof, J., 1999. A numerical taxonomic study of the *Pseudomonas* flora isolated from poultry meat. Journal of Applied Microbiology, 87, 15-28.
- Arslan, S., Eyi, A., Özdemir, F., 2011. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. Journal of Dairy Science, 94, 5851-5856.
- Baader, A., Garre, C., 1887. Über Antagonisten unter den Bacterien. Corresp. Bl. Schweiz. Ärzte, 13, 385-392.
- Bai, A.J., Rai, V.R., 2011. Bacterial quorum sensing and food industry. Comprehensive Reviews in Food Science and Food Safety, 10, 183-193.
- Baixas-Nogueras, S., Bover-Cid, S., Veciana-Nogués, T., Vidal-Carou, M.C.M., 2009. Effect of gutting on microbial loads, sensory properties, and volatile and biogenic amine contents of European hake (*Merluccius merluccius* var. *mediterraneus*) stored in ice. Journal of Food Protection, 72, 1671-1676.

- Barth, M., Hankinson, T.R., Zhuang, H., Breidt, F., 2009. Microbiological Spoilage of Fruits and Vegetables. W.H. Sperber, M.P. Doyle (eds.), Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Food Safety.
- Beaz-Hidalgo, R., Agüeria, D., Latif-Eugenín, F., Yeannes, M.I., Figueras, M.J., 2015. Molecular characterization of *Shewanella* and *Aeromonas* isolates associated with spoilage of Common carp (*Cyprinus carpio*). FEMS Microbiology Letters, 362, 1-8.
- Bergey's Manual of Determinative Bacteriology. American Journal of Public Health (New York, NY : 1912). 1923, 13(12), 1042.
- Bergey's Manual of Systematic Bacteriology: The Proteobacteria; The Alpha-, Beta-, Delta-, and Epsilonproteobacteria: Garrity, G., Brenner, D.J., Krieg, N.R., Staley, J.T., Boone, D.R., 2005.
- Bhadra, S., Narvaez, C., Thomson, D.J., Bridges, G.E., 2015. Non-destructive detection of fish spoilage using a wireless basic volatile sensor. Talanta, 134, 718-723.
- Boudon, S., Manceau, C., Nottéghem, J.L., 2005. Structure and Origin of *Xanthomonas arboricola* pv. *pruni* Populations Causing Bacterial Spot of Stone Fruit Trees in Western Europe. Phytopathology, 95, 1081-1088.
- Brocklehurst, T.F., Lund, B.M., 1985. Microbiological changes in cottage cheese varieties during storage at +7°C. Food Microbiology, 2, 207-233.
- Broekaert, K., Heyndrickx, M., Herman, L., Devlieghere, F., Vlaemynck, G., 2011. Seafood quality analysis: Molecular identification of dominant microbiota after ice storage on several general growth media. Food Microbiology, 28, 1162-1169.
- Caldera, L., Franzetti, L., 2014. Effect of storage temperature on the microbial composition of ready-to-use vegetables. Current Microbiology, 68, 133-139.
- Cantoni, C., Iacumin, L., Comi, G., 2003. Alterazione giallo-arancio di Mozzarella. Industrie Alimentari, 42, 134-136.
- Cantoni, C., Soncin, G., Milesi, S., Cocolin, L., Iacumin, L., Comi, G., 2006. Colorazioni anomale e rigonfiamento di formaggi fusi e mozzarelle. Industrie alimentari, 45, 276-281.

- Caputo, L., Quintieri, L., Bianchi, D.M., Decastelli, L., Monaci, L., Visconti, A., Baruzzi, F., 2015. Pepsin-digested bovine lactoferrin prevents Mozzarella cheese blue discoloration caused by *Pseudomonas fluorescens*. Food Microbiology, 46, 15-24.
- Carrascosa, C., Millán, R., Saavedra, P., Jaber, J.R., Raposo, A., Pérez, E., Montenegro, T., Sanjuán, E., 2015. Microbiological evolution of gilthead sea bream (*Sparus aurata*) in Canary Islands during ice storage. Journal of food science and technology, 52, 1586-1593.
- Casaburi, A., Piombino, P., Nychas, G.J., Villani, F., Ercolini, D., 2015. Bacterial populations and the volatilome associated to meat spoilage. Food Microbiology, 45, 83-102.
- Chorianopoulos, N.G., Giaouris, E.D., Skandamis, P.N., Haroutounian, S.A., Nychas, G.J., 2008. Disinfectant test against monoculture and mixed-culture biofilms composed of technological, spoilage and pathogenic bacteria: bactericidal effect of essential oil and hydrosol of *Satureja thymbra* and comparison with standard acid-base sanitizers. Journal of Applied Microbiology, 104, 1586-1596.
- Conte, A., Scrocco, C., Sinigaglia, M., Del Nobile, M.A., 2007. Innovative Active Packaging Systems to Prolong the Shelf Life of Mozzarella Cheese. Journal of Dairy Science, 90, 2126-2131.
- Cornelis, P., 2010. Iron uptake and metabolism in pseudomonads. Applied Microbiology and Biotechnology, 86, 1637-1645.
- Coton, M., Joffraud, J.J., Mekhtiche, L., Leroi, F., Coton, E., 2013. Biodiversity and dynamics of the bacterial community of packaged king scallop (*Pecten maximus*) meat during cold storage. Food Microbiology, 35, 99-107.
- Dainty, R.H., Edwards, R.A., Hibbard, C.M., 1984. Volatile compounds associated with the aerobic growth of some *Pseudomonas* species on beef. Journal of Applied Bacteriology, 57, 75-81.
- Dainty, R.H., Edwards, R.A., Hibbard, C.M., Marnewick, J.J., 1989. Volatile compounds associated with microbial growth on normal and high pH beef stored at chill temperatures. Journal of Applied Bacteriology, 66, 281-289.

- Datta, N., Deeth, H.C., 2001. Age gelation of UHT milk. Transaction of the institute of Chemical Engineers C. Food and Bioproducts Processing, 79, 197-210.
- Datta, N., Deeth, H.C., 2003. Diagnosing the cause of proteolysis in UHT milk. LWT-Food Science and Technology, 36, 173-182.
- Dehaut, A., Midelet-Bourdin, G., Brisabois, A., Duflos, G., 2014. Phenotypic and genotypic characterization of H_2S-positive and H_2S-negative strains of *Shewanella baltica* isolated from spoiled whiting (*Merlangius merlangus*). Letters in Applied Microbiology, 59, 542-548.
- De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P., Heyndrickx, M., 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Applied and Environmental Microbiology, 77, 460-470.
- den Dooren de Jong, L.E., 1926. Bijdrage tot de kennis van het mineralisatieproces. Rotterdam, The Netherlands: Nijgh & van Ditmar Uitgevers-Mij.
- Deng, J., Brettar, I., Luo, C., Auchtung, J., Konstantinidis, K.T., Rodrigues, J.L., Höfle, M., Tiedje, J.M., 2014. Stability, genotypic and phenotypic diversity of *Shewanella baltica* in the redox transition zone of the Baltic Sea. Environmental Microbiology, 16, 1854-1866.
- De Oliveira, G.B., Favarin, L., Luchese, R.H., McIntosh, D., 2015. Psychrotrophic bacteria in milk: How much do we really know?. Brazilian Journal of Microbiology, 46, 313-321.
- Derby, H.A., Hammer, H.A., 1931. Bacteriology of butter. IV. Bacteriological studies on surface taint butter. Iowa Agricultural Experiment Station Research Bulletin, 145, 389-416.
- Diaz-Sanchez, S., Hanning, I., Pendleton, S., D'Souza, D., 2012. Next-generation sequencing: the future of molecular genetics in poultry production and food safety. Poultry Science, 92, 562-572.
- Dikow, R.B., 2011, Genome-level homology and phylogeny of *Shewanella* (Gammaproteobacteria: Alteromonadales: Shewanellaceae). BMC Genomics, 12, 237.

- Dogan, B., Boor, K.J., 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Applied and Environmental Microbiology, 69, 130-138.
- Doulgeraki, A.I., Nychas, G.J., 2013. Monitoring the succession of the biota grown on a selective medium for pseudomonads during storage of minced beef with molecular-based methods. Food Microbiology, 34, 62-69.
- Dufour, D., Nicodème, M., Perrin, C., Driou, A., Brusseaux, E., Humbert, G., Gaillard, J.L., Dary, A., 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology, 125, 188-196.
- Ercolini, D., Russo, F., Nasi, A., Ferranti, P., Villani, F., 2009. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. Applied and Environmental Microbiology, 75, 1990-2001.
- Fonnesbech Vogel, B., Venkateswaran, K., Satomi, M., Gram, L., 2005. Identification of *Shewanella baltica* as the most important H_2S-producing species during iced storage of Danish marine fish. Applied and Environmental Microbiology, 71, 6689-6697.
- Fonseca, P., Moreno, R., Rojo, F., 2011. Growth of *Pseudomonas putida* at low temperature: global transcriptomic and proteomic analyses. Environmental Microbiology Reports, 3, 329-339.
- Fox, F., 1989. Proteolysis During Cheese Manufacture and Ripening. Journal of Dairy Science, 72, 1379-1400.
- Franzetti, L., Scarpellini, M., 2007. Characterisation of *Pseudomonas* spp. isolated from foods. Annals of Microbiology, 57, 39-47.
- Frapolli, M., Défago, G., Moënne-Loccoz, Y., 2007. Multilocus sequence analysis of biocontrol fluorescent *Pseudomonas* spp. producing the antifungal compound 2,4-diacetylphloroglucinol. Environmental Microbiology, 9, 1939-1955.

- Fredrickson, J.K., Romine, M.F., Beliaev, A.S., Auchtung, J.M., Driscoll, M.E., Gardner, T.S., Nealson, K.H., Osterman, A.L., Pinchuk, G., Reed, J.L., Rodionov, D.A., Rodrigues, J.L., Saffarini, D.A., Serres, M.H., Spormann, A.M., Zhulin, I.B., Tiedje, J.M., 2008. Towards environmental systems biology of *Shewanella*. Nature Reviews Microbiology, 6, 592-603.
- Garcia-Lopez, M.L., Prieto, M., Otero, A., 1998. The physiological attributes of Gram-negative bacteria associated with spoilage of meat and meat products. Blackie Academic and Professional.
- Gassem, M.A., Frank, J.F., 1991. Physical Properties of Yogurt Made from Milk Treated with Proteolytic Enzymes. Journal of Dairy Science, 74, 1503-1511.
- Gennari, M., Tomaselli, S., Cotrona, V., 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) Sea and stored in ice. Food Microbiology, 16, 15-28.
- Gilmour, M.W., Graham, M., Van Domselaar, G., Tyler, S., Kent, H., Trout-Yakel, K.M, Larios, O., Allen, V., Lee, B., Nadon, C., 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC genomics, 11, 120.
- Giuffrida, A., Valenti, D., Giarratana, F., Ziino, G., Panebianco, A., 2013. A new approach to modelling the shelf life of Gilthead seabream (*Sparus aurata*). International Journal of Food Science & Technology, 48, 1235-1242.
- Gram, L., 1992. Spoilage of three Senegalese fish species stored in ice at ambient temperature. In: E.H. Bligh (editor), Seafood Science and Technology. Fishing News Books, Blackwell, Oxford, 225-233.
- Gram L., Dalgaard P., 2002. Fish spoilage bacteria - problems and solutions. Current opinion in Biotechnology, 13, 262-266.
- Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology, 33, 121-137.

- Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002. Food spoilage--interactions between food spoilage bacteria. International Journal of Food Microbiology, 78, 79-97.
- Gram, L., Wedell-Neergaard, C., Huss, H.H., 1990. The bacteriology of fresh and spoiling Lake Victorian Nile perch (*Lates niloticus*). International Journal of Food Microbiology, 10, 303-316.
- Grewal, M.K., Jaiswal, P., Jha, S.N., 2015. Detection of poultry meat specific bacteria using FTIR spectroscopy and chemometrics. Journal of Food Science Scientists & Technologists (India), 52, 3859-3869.
- Haard, N., 2002. The role of enzymes in determining seafood color and texture. In A. H. Bremner, Safety and quality issues in food processing.
- Hau, H.H., Gralnick, J.A., 2007. Ecology and Biotechnology of the Genus *Shewanella*. Annual Review of Microbiology, 61, 237-258.
- Hickey, M.E., Besong, S.A., Kalavacharla, V., Lee, J.-L., 2013. Identification of Extracellular DNase-producing Bacterial Populations on Catfish Fillets during Refrigerated Storage. Food Science and Biotechnology, 22, 87-92.
- Hilario, E., Buckley T.R., Young, J.M., 2004. Improved resolution on the phylogenetic relationships among *Pseudomonas* by the combined analysis of *atpD*, *carA*, *recA* and 16S rDNA. Antoine van Leeuwenhoek, 86, 51-64.
- Holt, K.E., Parkhill, J., Mazzoni, C.J., Roumagnac, P., Weill, F.X., Goodhead, I., Rance, R., Baker, S., Maskell, D.J., Wain, J., Dolecek, C., Achtman, M., Dougan, G., 2008. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. Nature Genetics, 40, 987-993.
- Hovda, M.B., Lunestad, B.T., Sivertsvik, M., Rosnes, J.T., 2007. Characterisation of the bacterial flora of modified atmosphere packaged farmed Atlantic cod (*Gadus morhua*) by PCR-DGGE of conserved 16S rRNA gene regions. International Journal of Food Microbiology, 117, 68-75.
- Janda, J.M., 2014. *Shewanella*: a Marine Pathogen as an Emerging Cause of Human Disease. Clinical Microbiology Newsletter, 36, 25-29.

- Janda, J.M., Abbott, S.L., 2015. The genus *Shewanella*: From the briny depths below to human pathogen (Review). Critical Reviews in Microbiology, 40, 293-312.
- Jay, J.M., 2000. Modern Food Microbiology. Sixth Edition. Aspen Publishers, Inc. Gaithersburg, Maryland.
- Jay, J.M., 2003. A review of recent taxonomic changes in seven genera of bacteria commonly found in foods. Journal of Food Protection, 66, 1304-1309.
- Johnsen, K., Andersen, S., Jacobsen, C.S., 1996. Phenotypic and genotypic characterization of phenanthrene-degrading fluorescent *Pseudomonas* biovars. Applied and Environmental Microbiology, 62, 3818-3825.
- Kisand, V., Lettieri, T., 2013. Genome sequencing of bacteria: sequencing, de novo assembly and rapid analysis using open source tools. BMC Genomics, 14, 211.
- Koka, R., Weimer, B.C., 2000. Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. Journal of Applied Microbiology, 89, 280-288.
- Kröckel, L., 2009. Black spots on adipose tissue of pork and beef caused by melanin producing *Pseudomonas fluorescens*. Fleischwirtschaft, 89, 89-92.
- Kumarsan, G., Annalvilli, R., Sivakumar, K., 2007. Psychrotrophic spoilage of raw milk at different temperatures of storage. Journal of applied science research, 3, 1383-1387.
- Laguerre, G., Rigottier-Gois, L., Lemanceau, P., 1994. Fluorescent *Pseudomonas* species categorized by using polymerase chain reaction (PCR)/restriction fragment analysis of 16S rDNA. Molecular Ecology, 3, 479-487.
- Lavilla Lerma, L., Benomar, N., Gálvez, A., Abriouel, H., 2012. Prevalence of bacteria resistant to antibiotics and/or biocides on meat processing plant surfaces throughout meat chain production. International Journal of Food Microbiology, 161, 97-106.
- Lavilla Lerma, L., Benomar, N., Casado Muñoz, Mdel C., Gálvez, A., Abriouel, H., 2014. Antibiotic multiresistance analysis of mesophilic and psychrotrophic *Pseudomonas* spp. isolated from goat and lamb slaughterhouse surfaces throughout the meat production process. Applied and Environmental Microbiology, 80, 6792-6806.

- Lavilla Lerma, L., Benomar, N., Casado Muñoz, Mdel C., Gálvez, A., Abriouel, H., 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic *Pseudomonas* spp. isolated from slaughterhouse surfaces throughout meat chain production. Food Microbiology, 51, 33-44.
- Liao, C.H., Wells, J.M., 1987. Association of Pectolytic Strains of *Xanthomonas campestris* with Soft Rots of Fruits and Vegetables at Retail Markets. Phytopathology, 77, 418-422.
- Liao, C.-H., 2006. *Pseudomonas* and related genera. In Food Spoilage Microorganisms, 507-540.
- Liu, M., Wang, H., Griffiths, M.W., 2007. Regulation of alkaline metalloprotease promoter by N-acyl homoserine lactone quorum sensing in *Pseudomonas fluorescens*. Journal of Applied Microbiology, 103, 2174-2184.
- Lo, R., Stanton-Cook, M.J., Beatson, S.A., Turner, M.S., Bansal, N., 2015. Draft Genome Sequence of *Pseudomonas fluorescens* SRM1, an Isolate from Spoiled Raw Milk. Genome Announcement, 3, e00138-15.
- Macé, S., Cardinal, M., Jaffrès, E., Cornet, J., Lalanne, V., Chevalier, F., Sérot, T., Pilet M.F., Dousset, X., Joffraud, J.J., 2014. Evaluation of the spoilage potential of bacteria isolated from spoiled cooked whole tropical shrimp (*Penaeus vannamei*) stored under modified atmosphere packaging. Food Microbiology, 40, 9-17.
- Marchand, S., Heylen, K., Messens, W., Coudijzer, K., De Vos, P., Dewettinck, K., Herman, L., De Block, J., Heyndrickx, M., 2009a. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. Environmental Microbiology, 11, 467-482.
- Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., De Jonghe, V., Dewettinck, K., De Vos, P., Devreese, B., Heyndrickx, M., De Block, J., 2009b. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. International Journal of Food Microbiology, 133, 68-77.

- Martin, N.H., Murphy, S.C., Ralyea, R.D., Wiedmann, M., Boor, K.J., 2011. When cheese gets the blues: *Pseudomonas fluorescens* as the causative agent of cheese spoilage. Journal of Dairy Science, 94, 3176-3183.
- Mastromatteo, M., Incoronato, A.L., Conte, A., Del Nobile, M.A., 2011. Shelf life of reduced pork back-fat content sausages as affected by antimicrobial compounds and modified atmosphere packaging. International Journal of Food Microbiology, 150, 1-7.
- McCarthy, C.N., Woods, R.G., Beacham, I.R., 2004. Regulation of the *aprX-lipA* operon of *Pseudomonas fluorescens* B52: differential regulation of the proximal and distal genes, encoding protease and lipase, by *ompR-envZ*. FEMS Microbiology Letters, 241, 243-248.
- McPhee, J.D., Griffiths, M.W., 2002. *Pseudomonas* spp. Encyclopaedia of Dairy Sciences, Vol.4, Edited by Roginski, H., Fuquay, W.J., Fox, F.P., Academic Press, 2340-2350.
- Mellor, G.E., Bentley, J.A., Dykes, G.A., 2011. Evidence for a role of biosurfactants produced by *Pseudomonas fluorescens* in the spoilage of fresh aerobically stored chicken meat. Food Microbiology, 28, 1101-1104.
- Meyer, J.M., 2000. Pyoverdines: pigments, siderophores and potential taxonomic marker of fluorescent *Pseudomonas* species. Archives of Microbiology, 174, 135-142.
- Meyer, D.F., Bogdanove, A.J., 2009. Genomics-driven advance in *Xanthomonas* biology. Plant Pathogenic Bacteria: Genomics and Molecular Biology (Book). Caister Academic Press, Robert W. Jackson, School of Biological Sciences, University of Reading, Whiteknights, Reading, UK.
- Meyer, J.M., Geoffroy, V.A., Baida, N., Gardan, L., Izard, D., Lemanceau, P., Achouak, W., Palleroni, N.J., 2002. Siderophore typing, a powerful tool for the identification of fluorescent and nonfluorescent Pseudomonads. Applied and Environmental Microbiology, 68, 2745-2753.
- Migula, W., 1894. Über ein neues System der Bakterien. Arb Bakteriol Inst Karlsruhe, 1, 235-328.
- Migula, W., 1900. System der Bakterien, Vol. 2, Jena, Germany: Gustav Fischer.

- Miller, A., Scanlan, R.A., Lee, J.S., Libbey, L.M., 1973. Volatile Compounds Produced in Sterile Fish Muscle (*Sebastes melanops*) by *Pseudomonas putrefaciens*, *Pseudomonas fluorescens*, and an *Achromobacter* Species. Applied Microbiology, 26, 18-21.
- Mitchell, S.L., Marshall, R.T., 1989. Properties of Heat-Stable Proteases of *Pseudomonas fluorescens*: Characterization and Hydrolysis of Milk Proteins. Journal of Dairy Science, 72, 864-874.
- Mohareb, F., Iriondo, M., Doulgeraki, A.I., Van Hoek, A., Aarts, H., Cauchi, M., Nychas, G.-J.E., 2015. Identification of meat spoilage gene biomarkers in *Pseudomonas putida* using gene profiling. Food Control, 57, 152-160.
- Moore, E.R.B., Mau, M., Arnscheidt, A., Böttger, E.C., Hutson, R.A., Collins, M.D., Van de Peer, Y., De Wachter, R., Timmis, K.N., 1996. The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (*sensu stricto*) and estimation of the natural intrageneric relationships. Systematic and Applied Microbiology, 19, 478-492.
- Moreno, R., Rojo, F., 2014. Features of pseudomonads growing at low temperatures: another facet of their versatility. Environmental Microbiology Reports, 6, 417-426.
- Motoyama, M., Kobayashi, M., Sasaki, K., Nomura, M., Mitsumoto, M., 2010. *Pseudomonas* spp. convert metmyoglobin into deoxymyoglobin. Meat Science, 84, 202-207.
- Mulet, M., Lalucat, J., Garcìa-Valdes, E., 2010. DNA sequence-based analysis of the *Pseudomonas* species. Environmental Microbiology, 12, 1513-1530.
- Munhoz, C.F., Weiss, B., Hanai, L.R., Zucchi, M.I., Fungaro, M.H., Oliveira, A.L., Monteiro-Vitorello, C.B., Vieira, M.L., 2011. Genetic diversity and a PCR-based method for *Xanthomonas axonopodis* detection in passion fruit. Phytopathology, 101, 416-424.
- Munsch-Alatossava, P., Alatossava, T., 2005. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. Microbiological research, 161, 334-346.

- Nogarol, C., Acutis, P.L., Bianchi, D.M., Maurella, C., Peletto, S., Gallina, S., Adriano, D., Zuccon, F., Borrello, S., Caramelli, M., Decastelli, L., 2013. Molecular characterization of *Pseudomonas fluorescens* isolates involved in the Italian "blue mozzarella" event. Journal of Food Protection, 76, 500-504.
- Nychas, G.J.E., Marshall, D., Sofos, J., 2007. Food microbiology fundamentals and frontiers. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Meat Poultry and Seafood, third ed. ASM Press, 105-140.
- Nychas, G.J., Skandamis, P.N., Tassou, C.C., Koutsoumanis, K.P., 2008. Meat spoilage during distribution. Meat Science, 78, 77-89.
- Nychas, G.J., Dourou, D., Skandamis, P., Koutsoumanis, K., Baranyi, J., Sofos, J., 2009. Effect of microbial cell-free meat extract on the growth of spoilage bacteria. Journal of Applied Microbiology, 107, 1819-1829.
- Oakley, B.B., Morales, C.A., Line, J., Berrang, M.E., Meinersmann, R.J., Tillman, G.E., Wise, M.G., Siragusa, G.R., Hiett, K.L., Seal, B.S., 2013. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. PLoS One, 8, e57190.
- Palaniraj, A., Jayaraman, V., 2011. Production, recovery and applications of xanthan gum by *Xanthomonas campestris*. Journal of Food Engineering, 106, 1-12.
- Palleroni, N.J., Ballard, R.W., Ralston, E., Doudoroff, M., 1972. Deoxyribonucleic acid homologies among some *Pseudomonas* species. Journal of Bacteriology, 110, 1-11.
- Palleroni, N.J., 1984. Genus *Pseudomonas*. In Bergey's Manual of Systematic Bacteriology, Vol. 1. Krieg, N.R., and Holt, J.G. (eds). Baltimore, MD, USA: Williams & Wilkins, 141-199.
- Palleroni, N.J., 2010. The *Pseudomonas* story. Environmental Microbiology, 12, 1377-1383.
- Parker, L.L., Levin, R.E., 1983. Relative incidence of *Alteromonas putrefaciens* and *Pseudomonas putrefaciens* in ground beef. Applied and Environmental Microbiology, 45, 796-799.

- Parlapani, F.F., Meziti, A., Kormas, K.A., Boziaris, I.S., 2013. Indigenous and spoilage microbiota of farmed sea bream stored in ice identified by phenotypic and 16S rRNA gene analysis. Food Microbiology, 33, 85-89.
- Parlapani, F.F., Haroutounian, S.A., Nychas, G.J., Boziaris, I.S., 2015. Microbiological spoilage and volatiles production of gutted European sea bass stored under air and commercial modified atmosphere package at 2 °C. Food Microbiology, 50, 44-53.
- Pecknold, P.C., Grogan, R.G., 1973. Deoxy ribonucleic Acid Homology Groups among Phytopathogenic *Pseudomonas* Species. International Journal of Systematic Bacteriology, 23, 111-121.
- Peix, A., Ramírez-Bahena, M., Velázquez, E., 2009. Historical evolution and current status of the taxonomy of genus *Pseudomonas*. Science Direct, 9, 1132-1147.
- Peladan, F., Monteil, H., 1988. Identification of *Pseudomonas*, *Flavobacterium*, and *Alcaligenes* with the API 20 NE system. Pathologie-biologie, 36, 187-192.
- Pinto, U.M., Costa, E.D., Mantovani, H.C., Vanetti, M.C.D., 2010. The proteolytic activity of *Pseudomonas fluorescens* 07A isolated from milk is not regulated by quorum sensing signals. Brazilian Journal of Microbiology, 41, 91-96.
- Powell, S.M., Tamplin, M.L., 2012. Microbial communities on Australian modified atmosphere packaged Atlantic salmon. Food Microbiology, 30, 226-232.
- Qian, Y.F., Yang, S.P., Xie, J., Xiong, Q., Gao, Z.L., 2013. Impact of the O_2 concentrations on bacterial communities and quality of modified atmosphere packaged Pacific white shrimp (*Litopenaeus vannamei*). Journal of Food Science, 78, M1878-1884.
- Quintieri, L., Caputo, L., Monaci, L., Deserio, D., Morea, M., Baruzzi, F., 2012. Antimicrobial efficacy of pepsin-digested bovine lactoferrin on spoilage bacteria contaminating traditional Mozzarella cheese. Food Microbiology, 31, 64-71.
- Raab, V., Bruckner, S., Beierle, E., Kampmann, Y., Petersen, B., Kreyenschmidt, J., 2008. Generic model for the prediction of remaining shelf life in support of cold chain management in pork and poultry supply chains. Journal on Chain and Network Science, 8, 59-73.

- Rajagopal, L., Sundari, C.S., Balasubramanian, D., Sonti, R.V., 1997. The bacterial pigment xanthomonadin offers protection against photodamage. FEBS Letters, 415, 125-128.
- Rajmohan, S., Dodd, C.E.R., Waites, W.M., 2002. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. Journal of Applied Microbiology, 93, 205-213.
- Rapid Alert System for Food and Feed (RASFF). Annual Report, 2010.
- Remenant, B., Jaffres, E., Dousset, X., Pilet, M.F., Zagorec, M., 2015. Bacterial spoilers of food: behavior, fitness and functional properties. Food Microbiology, 45, 45-53.
- Reynisson, E., Lauzon, H.L., Magnússon, H., Jónsdóttir, R., Olafsdóttir, G., Marteinsson, V., Hreggvidsson, G.O., 2009. Bacterial composition and succession during storage of North-Atlantic cod (*Gadus morhua*) at superchilled temperatures. BMC Microbiology, 9, 250.
- Ritchie, D.F., 1995. Bacterial spot. In: Ogawa JM, Zehr EI, Bird GW, RitchieDF, Uriu K, Uyemoto JK, eds. Compendium of Stone Fruit Diseases. St Paul, MN, USA: American Phytopathological Society, 50-52.
- Russell, S.M., Fletcher, D.L., Cox, N.A., 1995. Spoilage bacteria of fresh broiler chicken carcasses. Poultry Science, 74, 2041-2047.
- Ryan, R.P., Vorhölter, F.J., Potnis, N., Jones, J.B., Van Sluys, M.A., Bogdanove, A.J., Dow, J.M., 2011. Pathogenomics of *Xanthomonas*: understanding bacterium-plant interactions. Nature Reviews Microbiology, 9, 344-355.
- Samaržija, D., Zamberlin, Š., Pogačić, T., 2012. Psychrotrophic bacteria and milk and dairy products quality. Mljekarstvo, 62, 77-95.
- Sarale, A., Grassi, M.A., Civera, T., 2011. *Pseudomonas gessardii* causative agent for blue spot formation on rabbit carcasses. A.I.V.I online, June 2011 1.
- Satomi, M., Oikawa, H., Yano, Y., 2003. *Shewanella marinintestina* sp. nov., *Shewanella schlegeliana* sp. nov. and *Shewanella sairae* sp. nov., novel

eicosapentaenoic-acid-producing marine bacteria isolated from sea-animal intestines. International Journal of Systematic and Evolutionary Microbiology, 53, 491-499.

- Satomi, M., Vogel, B.F., Gram, L., Venkateswaran, K., 2006. *Shewanella hafniensis* sp. nov. and *Shewanella morhuae* sp. nov., isolated from marine fish of the Baltic Sea. International Journal of Systematic and Evolutionary Microbiology, 56, 243-249.
- Satomi, M., Vogel, B.F., Venkateswaran, K., Gram, L., 2007. Description of *Shewanella glacialipiscicola* sp. nov. and *Shewanella algidipiscicola* sp. nov., isolated from marine fish of the Danish Baltic Sea, and proposal that *Shewanella affinis* is a later heterotypic synonym of *Shewanella colwelliana*. International Journal of Systematic and Evolutionary Microbiology, 57, 347-352.
- Satomi, M., 2014. The family *Shewanellaceae*. in: The prokaryotes - Gammaproteobacteria. Rosemberg *et al.,* (eds), 597-619.
- Scales, B.S., Dickson, R.P., LiPuma, J.J., Huffnagle, G.B., 2014. Microbiology, genomics, and clinical significance of the *Pseudomonas fluorescens* species complex, an unappreciated colonizer of humans. Clinical microbiology reviews, 27, 927-948.
- Serio, A., Fusella, G.H., Chaves López, C., Sacchetti, G., Paparella, A., 2014. A survey on bacteria isolated as hydrogen sulfide-producers from marine fish. Food Control, 39, 111-118.
- Silby, M.W., Winstanley, C., Godfrey, S.A.C., Levy, S.B., Jackson, R.W., 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiology Review, 35, 652-680.
- Sillankorva, S., Neubauer, P., Azeredo, J., 2010. Phage control of dual species biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus*. Biofouling, 26, 567-575.
- Sofos, J.N., 1994. Microbial growth and its control in meat, poultry and fish. Chapter in Quality Attributes and their Measurement in Meat, Poultry and Fish Products, Volume 9 of the series Advances in Meat Research, 359-403.
- Solieri, L., Dakal, T.C., Giudici, P., 2012. Next-generation sequencing and its potential impact on food microbial genomics. Annals of Microbiology, 63, 21-37.

- Sørhaug, T., Stepaniak, L., 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends in Food Science & Technology, 8, 35-41.
- Spiers, A.J., Buckling, A., Rainey, P.B., 2000. The causes of *Pseudomonas* diversity. Microbiology, 146, 2345-2350.
- Stanier, R.Y., Palleroni, N.J., Doudoroff, M., 1966. The aerobic pseudomonads: a taxonomic study. Journal of General Microbiology, 43, 159-271.
- Stoops, J., Maes, P., Claes, J., Van Campenhout, L., 2012. Growth of *Pseudomonas fluorescens* in modified atmosphere packaged tofu. Letters in Applied Microbiology, 54, 195-202.
- Stoyanova, M., Vancheva, T., Moncheva, P., Bogatzevska, N., 2014. Differentiation of *Xanthomonas* spp. Causing Bacterial Spot in Bulgaria Based on Biolog System. International Journal of Microbiology, 2014, 495476.
- Sun, Y.Y., Sun, L., 2015. *Pseudomonas fluorescens*: iron-responsive proteins and their involvement in host infection. Veterinary Microbiology, 176, 309-320.
- Teh, K.H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., Lindsay, D., 2012. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. International Journal of Food Microbiology, 157, 28-34.
- Teh, K.H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., Lindsay, D., 2014. Biofilm - An unrecognised source of spoilage enzymes in dairy products?. International Dairy Journal, 34, 32-40.
- Tryfinopoulou, P., Drosinos, E.H., Nychas, G.J., 2001. Performance of *Pseudomonas* CFC-selective medium in the fish storage ecosystems. Journal of Microbiological Methods, 47, 243-247.
- Tryfinopoulou, P., Tsakalidou, E., Vancanneyt, M., Hoste, B., Swings, J., Nychas, G.-J.E., 2007. Diversity of *Shewanella* population in fish *Sparus aurata* harvested in the Aegean Sea. Journal of Applied Microbiology, 103, 711-721.
- Tümmler, B., Wiehlmann, L., Klockgether, J., Cramer, N., 2014. Advances in understanding *Pseudomonas*. F1000prime reports, 6, 9.

- Vihavainen, E.J., Björkroth, J., 2010. Microbial ecology and spoilage of poultry meat and poultry meat products in: Guerrero-Legarreta (Ed.), Handbook of Poultry Science and Technology, Secondary Processing, vol. 2 Blackwell-Wiley, New York, NY (2010), 485-493.
- Vorhölter, F.J., Schneiker, S., Goesmann, A., Krause, L., Bekel, T., Kaiser, O., Linke, B., Patschkowski, T., Rückert, C., Schmid, J., Sidhu, V.K., Sieber, V., Tauch, A., Watt, S.A., Weisshaar, B., Becker, A., Niehaus, K., Pühler, A., 2008. The genome of *Xanthomonas campestris* pv. *campestris* B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. Journal of Biotechnology, 134, 33-45.
- Wang, D., Wang, Y., Huang, H., Lin, J., Xiao, D., Kan, B., 2013. Identification of tetrodotoxin-producing *Shewanella* spp. from feces of food poisoning patients and food samples. Gut Pathogens, 5, 15.
- Wasi, S., Tabrez, S., Ahmad, M., 2013. Use of *Pseudomonas* spp. for the bioremediation of environmental pollutants: a review. Environmental monitoring and assessment, 185, 8147-8155.
- Woods, R.G., Burger, M., Beven, C.A., Beacham, I.R., 2001. The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology, 147, 345-354.
- Wu, L., Wang, J., Tang, P., Chen, H., Gao, H., 2011. Genetic and molecular characterization of flagellar assembly in *Shewanella oneidensis*. PLoS One, 6, e21479.
- Yamamoto, S., Kasai, H., Arnold, D.L., Jackson, R.W., Vivian, A., Harayama, S., 2000. Phylogeny of the genus *Pseudomonas*: intragenic structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology, 146, 2385-2394.
- Zhang, H., Fu, H., Wang, J., Sun, L., Jiang, Y., Zhang, L., Gao, H., 2013. Impacts of nitrate and nitrite on physiology of *Shewanella oneidensis*. PLoS One, 8, e62629.
- Zhang, S.R., Zhang, L., Sun, L., 2014. Identification and analysis of three virulence-associated TonB-dependent outer membrane receptors of *Pseudomonas fluorescens*. Diseases of aquatic organisms, 110, 181-191.

- Zhu, J., Huang, X., Zhanmg, F., Feng, L., Li, J., 2015. Inhibition of quorum sensing, biofilm, and spoilage potential in *Shewanella baltica* by green tea polyphenols. Journal of Microbiology, 53, 829-836.

CHAPTER 2: Tracking the blue: A MLST approach to characterise the *Pseudomonas fluorescens* group

---

*Adapted with the permission of "Elsevier" from: **Andreani Nadia Andrea**, Martino Maria Elena, Fasolato Luca, Carraro Lisa, Montemurro Filomena, Mioni Renzo, Bordin Paola and Cardazzo Barbara

Food Microbiology 39 (2014), 116-126.

Article info:

Article history:

Received 10 July 2013

Received in revised form 18 November 2013

Accepted 20 November 2013

Available online 3 December 2013

Keywords:

Pseudomonas fluorescens group, Multilocus Sequence Typing, Food spoilage, Blue mozzarella.

2.1. Abstract

The *Pseudomonas fluorescens* group comprises several closely related species that are involved in food contamination and spoilage. Specifically, the interest in *P. fluorescens* as a spoiler of dairy products increased after the cases of “blue mozzarella” that occurred in Italy in 2010.

A Multilocus Sequence Typing (MLST) scheme was developed and applied to characterise 136 isolates (reference strains and food borne isolates) at strain level, to reveal the genetic relationships among them and to disclose any possible genetic clustering of phenotypic markers involved in food spoilage (protease, lipase, lecithinase activities and pigmented or fluorescent molecule production). The production of dark blue diffusible pigment was evaluated on several bacterial culture media and directly on mozzarella cheese.

The MLST scheme provided precise genotyping at the strain level, and the population analyses of the concatenated sequences allowed major taxa to be defined. This approach was revealed to be suitable for tracking the strains according to their origin, such as dairy plants or food matrices. The genetic analysis revealed the presence of a connection between the blue pigment production and a specific phylogenetic cluster. The development of the online database specific to the *P. fluorescens* group (http://pubmlst.org/pfluorescens/) will facilitate the application of the scheme and the sharing of the data.

2.2. Introduction

The strains ascribed to the genus *Pseudomonas* are Gram negative, rod-shaped, ubiquitous bacteria, characterised by poor nutritional needs and present in various environments (soil, organic material under decomposition, atmospheric dust, vegetation and water), with a wide range of animal and plant hosts (Anzai *et al.,* 2000; Frapolli *et al.,* 2007). The *Pseudomonas fluorescens* group is strictly connected to food spoilage, and its importance is clearly related to the food trade and hygienic standards. Because of the psychrotrophic and mesophilic characteristics of these bacteria, they can replicate at refrigeration temperatures and are

easily destroyed by heat treatments; however, long periods of shelf life can easily increase the *Pseudomonas* concentration in foods (Marchand *et al.,* 2009; Munsch-Alatossava and Alatossava, 2005). Strains belonging to the *P. fluorescens* group are found in a wide range of foods, such as ready-prepared fresh vegetables, raw fish (especially sushi or sashimi), meat and dairy products (Arnaut-Rollier *et al.,* 1999; Franzetti and Scarpellini, 2007).

Specifically, *Pseudomonas* represents part of the main microflora of raw milk and its products, which can be contaminated via defiled water and soil, inadequately sanitised milking surfaces, storage and transporting equipment (Munsch-Alatossava and Alatossava, 2005). Dairy products are a particularly favourable substrate to grow different bacteria, including *Pseudomonas*, because of their nutritional value, water content and a neutral pH (Marchand *et al.,* 2009). As previously reported, psychrotrophic bacteria are not resistant to heat treatments, but they are able to produce extracellular enzymes, such as different proteases, lipases and lecithinases, which are often heat resistant, responsible for spoilage and instability problems in food, and their production increases in suboptimal storage conditions (Marchand *et al.,* 2009; De Jonghe *et al.,* 2011). Their activity is more remarkable at refrigeration temperatures, and they induce grey colouration, unpleasant bitter off-flavours, gelation, a decrease of cheese-making performance, variation in pH, rancidity and saponification when present in dairy products (Rajmohan *et al.,* 2002; Arslan *et al.,* 2011). Additionally, one of the most outstanding food-altering effects is the capacity of some *Pseudomonas* strains to produce coloured or fluorescent pigments, which cause food discolouration (Gennari and Dragotto, 1992). The interest *in P. fluorescens* as a spoiler of dairy products increased after the cases of “blue mozzarella” that occurred in 2010 in which some European consumers noted discolouration on some mozzarella products. The microbiological analyses on the cheese samples showed high concentrations of *P. fluorescens*, up to 10^6 CFU per gram of cheese (Bogdanova *et al.,* 2010). Little information is available on the nature of the blue pigment that was observed in the mozzarella cheese, and the characteristics must be understood, in contrast with the thorough studies of other pigments, such as pyocyanine, pyoverdine, fluorescein, pyorubin and pyomelanin, that are typically produced by some *P. fluorescens* strains.

Thus, because the role of the *P. fluorescens* group in food spoilage has been established, the interest in methods for correctly identifying the different species of this group has grown. Concerning *Pseudomonas* taxonomy, several different phenotypic traits were chosen for species identification until molecular biology methods, such as DNA/DNA hybridisation (Wayne *et al.,* 1987; Palleroni *et al.,* 1973), RNA/DNA hybridisation (Palleroni *et al.,* 1973; De Vos *et al.,* 1985; De Vos *et al.,* 1989), REP-PCR (Repetitive Extragenic Palindromic Sequence Polymerase Chain Reaction; Johnsen *et al.,* 1996), 16S rRNA sequencing (Woese *et al.,* 1984; Laguerre *et al.,* 1994; Moore *et al.,* 1996; Bennasar *et al.,* 1998), 16S-23S rRNA intergenic spacer (ITS; Gurtler and Stanisich, 1996) and PFGE (Pulsed-Field Gel Electrophoresis, Nogarol *et al.,* 2013) became more frequent. Because of the low level of variability in 16S rRNA and other housekeeping genes and past lateral gene transfer events, a single-gene-based identification approach may not be appropriate for strain typing (Frapolli *et al.,* 2007). Since 1998, MLST has been proposed as a portable and universal method for characterising bacteria by sequence polymorphisms within internal fragments of housekeeping genes (Maiden, 2006). Different approaches have been developed to improve the characterisation of *Pseudomonas* spp.: Yamamoto *et al.,* (2000) studied the genus *Pseudomonas* by analysing the *gyrB* and the *rpoD* genes, whereas Hilario and co-workers developed an approach based on *atpD*, *carA*, *recA* and 16S sequencing (Hilario *et al.,* 2004). In 2010, Mulet and colleagues developed a MLST scheme to disclose the phylogenetic relationships within the *Pseudomonas* genus by 16S rRNA, *gyrB*, *rpoB* and *rpoD* sequencing of 107 *Pseudomonas* Type strains.

Many MLST schemes have been created as tracking instruments of pathogens (i.e., the first MLST scheme was created for *Neisseria meningitidis* by Maiden *et al.,* 1998), but schemes to track food spoilers could be helpful to discover the causes of the spoilage reactions and to solve related problems.

Currently, no studies are available about the nature of the blue pigment involved in blue mozzarella events, whereas only one study has been conducted using PFGE analysis to highlight the phylogenetic relationships of the isolates involved in the case of the mozzarella contamination (Nogarol *et al.,* 2013).

Here, five phenotypic tests were used to characterise strains for specific traits; furthermore, a MLST scheme based on the analysis of seven loci was developed and applied to 18 reference strains, 117 foodborne strains and a human case of infection. The phenotypic and molecular results were compared to highlight a possible correlation between them, and the MLST data were used to reconstruct phylogenetic trees and conduct evolutionary analyses. Additionally, the first MLST online database specific to the *P. fluorescens* group was developed (http://pubmlst.org/pfluorescens).

2.3. Materials and methods

2.3.1. Bacterial strains

A total of eighteen reference and Type strains belonging to the *P. fluorescens* group were selected and included in the MLST scheme from previous studies conducted on the genus *Pseudomonas* (Mulet *et al.,* 2010). Additionally, 118 field strains were isolated from the Istituto Zooprofilattico delle Venezie (IZSve; Legnaro, Italy), the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZStl; Pisa, Italy), the Department of Food Sciences (University of Udine), the School of Veterinary Science (University of Bristol), and the Department of Comparative Biomedicine and Food Science (University of Padova). These isolates were recovered from various food products between 2010 and 2012 from different geographical areas of Italy (North, North-East or the Centre of Italy), three strains were isolated in Germany, and two came from England. Specifically, 70 strains were isolated from dairy products (54 were obtained from mozzarella cheeses coming from plants involved in the blue mozzarella cases, 20 of which came from blue mozzarella cheeses), eighteen from ready-to-eat vegetables, seventeen from pork, eleven from raw fish products (sushi), one from beef, and one from a nosocomial human case of infection. The field isolates had been previously characterised at the genus and species level through API microorganism identification test kits (API20E and API20NE, bioMérieux's), and the suspected *Pseudomonas* spp. were confirmed by 16S sequencing and the subsequent comparison of sequences in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/). The complete list of the

strains that were analysed in this study is reported in Table 2.1. Strains were conserved at -80 °C in Tryptic Soy Broth with 50% v/v glycerol (Sigma-Aldrich).

2.3.2. Phenotypic characterisation

Eighty-seven strains (seventeen Type strains, one reference strain and 69 field strains) were revitalised by a 72-h-preinoculum in Tryptic Soy Broth (TSB) at 22 °C. A dilution of 10^5 CFU/ml was applied for the subsequent tests. The screening of the five phenotypic traits considered (production of pigments, fluorescent emission under UV exposure and proteolytic, lipolytic and lecithinasic activities) was conducted on Petri plates at 4, 7, 10 and 14 days after the inoculums. The tests were performed at 6 °C, 22 °C, and 31 °C.

The evaluation of pigment production was conducted on CFC Pseudomonas Agar Base (CFC PAB; Oxoid Microbiology Products, Thermo Scientific) and on Potato Dextrose Agar (PDA; Oxoid Microbiology Products, Thermo Scientific; Martin *et al.,* 2011). The analysis of fluorescent emission on CFC PAB was conducted using both a Wood Lamp (λ= 320-400 nm) and Gel Doc XR™ (Biorad), exposing the colonies to 320 nm of UV radiation (Johnsen *et al.,* 1996; Arnaut-Rollier *et al.,* 1999). The pigment intensity and the fluorescent emission were assigned values ranging from 0 (strains that did not produce any pigmented or fluorescent molecules) to 4 (high-producers of pigmented or fluorescent molecules). The extracellular protease activity evaluation was conducted on Nutrient Agar (NA; Biokar diagnostics, Solabia Diagnostic) with 2% UHT Milk observing a clear zone around the colonies (Marchand *et al.,* 2009), whereas the evaluation of lipasic and lecithinasic activity was tested on Egg Yolk Agar (EYA; Biokar diagnostics, Solabia Diagnostic) and was identified by the presence of an iridescent layer beyond the edge of the colony and an opaque zone of precipitation (Rossignol *et al.,* 2008). The intensity of the enzyme activity was assigned values ranging from 0 to 3.

To evaluate pyomelanin production, the strains that were characterised by the blue pigment on PDA were examined on Minimal Bacterial Medium Agar (MBM Agar; 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% trisodium citrate, 0.01% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.2% glucose, [Boles *et al.,* 2004]) with or without the addition of 1% L-tyrosine, which is known to enhance pyomelanin production. Additionally, *P. fluorescens* biovar IV (*Pseudomonas lemonnieri*, DSM 50415) was tested on this medium. A hierarchical cluster analysis was conducted considering

Table 2.1 - Origins and typing data of Pseudomonas fluorescens strains analysed in this study.

Sample	Strain	Subgroup [a]	Source	Provenience [b]	ST [c]	Alleles [c]						
						glnS	***gyrB***	***ileS***	***nuoD***	***recA***	***rpoB***	***rpoD***
DSM 17152T	*P. gessardii*	*P. fluorescens*	mineral water	France	7	7	7	7	7	7	7	7
DSM 15294T	*P. brenneri*	*P. fluorescens*	natural mineral water	France	8	8	8	8	8	8	8	8
DSM 17967T	*P. mandelii*	*P. mandelii*	mineral water	France	9	9	9	9	9	9	9	9
DSM 17150T	*P. jessenii*	*P. jessenii*	mineral water	France	10	10	10	10	10	10	10	10
DSM 16610T	*P. koreensis*	*P. koreensis*	agricultural soil	Korea	11	11	11	11	11	11	11	11
DSM 17489T	*P. orientalis*	*P. fluorescens*	spring water	France	12	12	12	12	12	12	12	12
DSM 18928T	*P. synxantha*	*P. fluorescens*	cream	USA	13	13	13	13	13	13	13	13
DSM 18862T	*P. azotoformans*	*P. fluorescens*	paddies	Japan	14	14	14	14	14	14	14	14
DSM 6252T	*P. lundensis*	*P. fragi*	prepacked beef	Sweden	15	15	15	15	15	15	15	15
DSM 14020T	*P. rhodesiae*	*P. fluorescens*	natural mineral water	France	16	16	16	16	16	16	16	16
DSM 11331T	*P. veronii*	*P. fluorescens*	mineral water	France	17	17	17	17	17	17	17	17
DSM 17149T	*P. libanensis*	*P. fluorescens*	spring water	Lebanon	18	18	18	18	18	18	18	18
DSM50415	*P. fluorescens*	*P. fluorescens*	soil	Germany	19	19	19	19	19	19	19	19
CECT124T	*P. corrugata*	*P. corrugata*	tomato: pith necrosis	UK	20	20	20	20	20	20	20	20
CECT229T	*P. marginalis*	*P. fluorescens*	*Cichorium intybus* (endive)	USA	21	21	21	21	21	21	21	21
CECT378T	*P. fluorescens*	*P. fluorescens*	pre-filter tanks, town water works	UK	22	22	22	22	22	22	22	22
CECT446T	*P. fragi*	*P. fragi*	Unknown	Germany	23	23	23	23	23	23	23	23
CECT4470T	*P. chlororaphis*	*P. chlororaphis*	plate contaminant	Germany	24	24	24	24	24	24	24	24
ps_1*	18	*P. fluorescens*	mozzarella cheese	NE Italy	25	25	25	25	25	25	25	25
ps_2*	22	*P. fluorescens*	blue mozzarella cheese	Germany	26	26	26	26	26	26	26	26
ps_3	23	*P. fragi*	mozzarella cheese	Italy	27	27	27	27	27	27	27	27

ps_4	31	*P. fluorescens*	mozzarella cheese	NE Italy	28	28	28	28	28	28	28	28
ps_5*	52	*P. fluorescens*	mozzarella cheese	NE Italy	29	29	29	29	29	29	29	29
ps_6*	57	*P. fluorescens*	blue mozzarella cheese	NE Italy	30	30	30	30	30	26	30	30
ps_7	61	*P. koreensis*	mozzarella cheese	NE Italy	31	31	31	31	31	30	31	31
ps_8	77	*P. fluorescens*	mozzarella cheese	NE Italy	32	28	32	28	32	28	28	28
ps_9	84	*P. fluorescens*	mozzarella cheese	NE Italy	33	32	33	32	33	31	32	32
ps_10	92	*P. fluorescens*	mozzarella cheese	NE Italy	34	32	34	33	34	31	32	32
ps_11	95	*P. fluorescens*	mixed salad	NE Italy	35	21	35	34	35	21	33	21
ps_12	98	*P. fragi*	butter	Germany	36	33	36	35	36	32	34	32
ps_13*	100	*P. fluorescens*	blue mozzarella cheese	Germany	37	34	37	36	37	33	35	33
ps_14	139	*P. koreensis*	pork	NE Italy	38	35	38	37	38	34	36	31
ps_15	3bol	*P. fluorescens*	*Salmo trutta marmoratus* (trout)	NE Italy	39	36	17	38	39	35	37	17
ps_16	6bol	*P. fluorescens*	*Salmo trutta fario* (trout)	NE Italy	40	37	39	39	40	36	38	34
ps_17	33Ae	*P. fragi*	Ricotta	NE Italy	41	38	40	40	41	37	39	35
ps_18	L2-2	*P. fluorescens*	UHT milk	Italy	42	39	41	41	42	38	40	36
ps_19	L3-1	*P. fragi*	UHT milk	Italy	43	40	42	42	43	39	41	37
ps_20	L3-4	*P. fluorescens*	UHT milk	Italy	44	41	30	43	44	40	30	38
ps_21	MOZ2	*P. koreensis*	mozzarella cheese	Italy	45	42	43	44	45	41	42	39
ps_22*	MOZ3	*P. fluorescens*	blue mozzarella cheese	Italy	46	43	44	45	46	26	43	30
ps_23	MOZ5	*P. koreensis*	mozzarella cheese	Italy	47	44	45	46	47	42	44	40
ps_24	PISA 3	*P. fluorescens*	cheese	C Italy	48	45	46	47	48	43	45	41
ps_25*	PISA 4	*P. fluorescens*	UHT milk	C Italy	49	46	26	48	49	26	30	42
ps_26	H6Ae	*P. koreensis*	human	NE Italy	50	47	47	49	50	44	46	43

(it continues in the following pages)

Sample	Strain	Subgroup [a]	Source	Provenience [b]	ST [c]	Alleles [c]						
						glnS	*gyrB*	*ileS*	*nuoD*	*recA*	*rpoB*	*rpoD*
ps_27	602a	*P. fluorescens*	mixed salad	NE Italy	51	48	41	50	42	45	47	44
ps_28	602b	*P. koreensis*	mixed salad	NE Italy	52	49	48	51	51	46	48	45
ps_29	602c	*P. fluorescens*	mixed salad	NE Italy	53	50	49	52	52	47	49	46
ps_30	602d	*P. fluorescens*	mixed salad	NE Italy	54	51	50	53	53	48	50	47
ps_31	602e	*P. fluorescens*	mixed salad	NE Italy	55	50	51	54	54	49	51	48
ps_32	602f	*P. chlororaphis*	mixed salad	NE Italy	56	52	52	55	55	50	52	49
ps_33	602h	*P. fluorescens*	mixed salad	NE Italy	57	53	53	56	56	51	53	50
ps_34	602i	*P. fluorescens*	mixed salad	NE Italy	58	50	54	52	52	52	49	46
ps_35	602l	*P. fluorescens*	mixed salad	NE Italy	59	54	51	54	54	49	51	48
ps_36	602m	*P. fluorescens*	mixed salad	NE Italy	60	55	52	57	57	53	54	51
ps_37	602qc	*P. chlororaphis*	mixed salad	NE Italy	61	56	55	58	58	54	48	52
ps_38	602qi	*P. chlororaphis*	mixed salad	NE Italy	62	56	56	59	58	3	55	3
ps_39	602r	*P. fluorescens*	mixed salad	NE Italy	63	57	57	60	59	21	21	21
ps_40	603g	*P. fluorescens*	ricotta	NE Italy	64	58	58	30	60	55	56	53
ps_41	Pse11	*P. fragi*	pork	NE Italy	65	59	59	61	61	56	57	54
ps_42	Pse12	*P. fragi*	pork	NE Italy	66	60	60	62	62	57	58	55
ps_43	Pse13	*P. fluorescens*	pork	NE Italy	67	61	61	63	63	58	59	56
ps_44	Pse15	*P. fragi*	pork	NE Italy	68	62	62	64	64	59	58	55
ps_45	Pse16	*P. fragi*	pork	NE Italy	69	63	63	15	65	60	60	15
ps_46	Pse19	*P. fragi*	pork	NE Italy	70	60	64	65	66	61	58	57
ps_47	Pse21	*P. fragi*	pork	NE Italy	71	64	23	66	67	62	23	58
ps_48	Pse24	*P. fragi*	pork	NE Italy	72	62	60	64	66	63	58	59

ps_49	Pse26	*P. fragi*	pork	NE Italy	73	65	27	67	68	64	27	27
ps_50	Pse29	*P. fragi*	pork	NE Italy	74	66	60	62	66	65	58	59
ps_51	Pse30	*P. chlororaphis*	pork	NE Italy	75	67	65	68	69	66	61	3
ps_52	Pse38	*P. koreensis*	pork	NE Italy	76	68	66	69	70	67	62	43
ps_53	Pse39	*P. fragi*	pork	NE Italy	77	62	62	64	70	59	58	55
ps_54	Pse44	*P. fragi*	pork	NE Italy	78	60	67	70	71	68	58	60
ps_55	Pse46	*P. koreensis*	pork	NE Italy	79	69	68	71	72	69	63	61
ps_56	761 5	*P. koreensis*	sashimi	NE Italy	80	70	69	72	73	70	64	62
ps_57	762 1	*P. fluorescens*	sashimi	NE Italy	81	39	41	73	42	38	65	36
ps_58	762 2	*P. corrugata*	sashimi	NE Italy	82	71	70	74	74	71	66	63
ps_59	762 3	*P. fluorescens*	sashimi	NE Italy	83	72	71	75	75	72	67	64
ps_60	762 5		sashimi	NE Italy		73	70		76	73	68	65
ps_61	763 1	*P. fluorescens*	sashimi	NE Italy	84	74	25	76	77	74	69	25
ps_62	763 2		sashimi	NE Italy		75	72		78	75	70	66
ps_63	763 3		sashimi	NE Italy		76	73		79	76	71	67
ps_64	763 4	*P. fluorescens*	sashimi	NE Italy	85	77	74	77	80	77	72	68
ps_65	815b	*P. koreensis*	rocket	NE Italy	86	78	75	78	81	78	73	45
ps_66	815 m	*P. koreensis*	rocket	NE Italy	87	79	76	77	82	79	64	69
ps_67	816d	*P. koreensis*	valerian	NE Italy	88	80	77	79	83	80	66	70
ps_68	816o	*P. corrugata*	valerian	NE Italy	89	81	70	80	74	81	74	71
ps_69	Pse48	*P. mandelii*	dairy-product	NE Italy	90	82	78	81	84	9	75	72
ps_70	Pse49	*P. fragi*	dairy-product	NE Italy	91	83	79	82	85	82	76	73
ps_71	Pse50	*P. fragi*	UHT milk	NE Italy	92	84	80	83	86	83	77	74

(it continues in the following page)

Sample	Strain	Subgroup [a]	Source	Provenience [b]	ST [c]	Alleles [c]						
						glnS	*gyrB*	*ileS*	*nuoD*	*recA*	*rpoB*	*rpoD*
ps_72	Pse51	*P. fragi*	dairy-product	NE Italy	93	85	81	84	87	84	78	75
ps_73	Pse52	*P. fluorescens*	dairy-product	NE Italy	94	86	82	85	88	85	79	76
ps_74	Pse54	*P. fragi*	dairy-product	NE Italy	95	62	83	86	62	63	58	59
ps_75*	62	*P. fluorescens*	blue mozzarella cheese	NE Italy	29	29	29	29	29	29	29	29
ps_76*	PISA 1	*P. fluorescens*	mozzarella cheese	C Italy	26	26	26	26	26	26	26	26
ps_77*	uk1	*P. fluorescens*	blue beef	England	96	87	5	5	89	86	80	77
ps_78*	uk4	*P. fluorescens*	blue mozzarella cheese	England	97	88	26	87	44	26	81	78
ps_79*	bb	*P. fluorescens*	blue pork	NE Italy	98	89	5	5	90	86	82	5

[a] The subgroup names (i.e., complexes, which are shown for field isolates) were assigned according to the attribution provided by Mulet *et al.,* (2010).

[b] The geographic position is reported for the Italian samples (NE: North-East, C: Central).

[c] STs and alleles were determined by MLST.

[*] Strains that produced the blue pigment on Potato Dextrose Agar and MBM Agar.

extracellular enzyme production, reporting the presence or the absence of the trait and the related degree. The matrix of the results was analysed with the program R (http://www.r-project.org). The dissimilarity distance matrixes for the variables were based on Gower's coefficient (Gower, 1971), and the identification of phenotypic clusters was conducted (Gonçalves *et al.,* 2008).

2.3.3. Primer design

Seven housekeeping genes (*glnS*, *gyrB*, *ileS*, *nuoD*, *recA*, *rpoB* and *rpoD*) were chosen for the MLST analysis using the following criteria: present as a single copy in all strains, rate of conservation of sequence and wide distribution across the chromosome. The full-length sequences of the seven genes available for the six *P. fluorescens* complete genomes (SBW25, Pf0-1, Pf-5, *Pseudomonas brassicacearum*, A506 and F113 [NC_012660.1, NC_007492.2, NC_004129.6, NC_015379.1, NC_017911.1, NC_016830.1]) were downloaded from GenBank and aligned using ClustalW (http://www.ebi.ac.uk). Primers were designed from the most conserved regions using Primer3 software (http://frodo.wi.mit.edu/primer3/), with a length of 18-25 nucleotides and similar annealing temperatures. The primer pair used to amplify the *recA* gene was obtained from a previous study (Frapolli *et al.,* 2007). The complete list of the primers used for PCR amplification and sequencing is reported in Table 2.2.

2.3.4. DNA extraction and PCR amplification

For DNA extraction, a single colony from a fresh culture on CFC PAB was suspended in 100 mL of nuclease-free water, vortexed at high speed for 5 s and incubated at 95 °C for 10 min. The tube was vortexed again and centrifuged for 2 min at 14,000 rpm. The supernatant was transferred to a fresh tube and stored at -20 °C (Martino *et al.,* 2011). The PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler in a final volume of 20 mL of amplification mix containing 1U of GoTaq polymerase (Promega, Madison, WI), 1U GoTaq Buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 250 nM each primer and 5 ng of genomic DNA as the template. The reaction mixture was subjected to the following thermal cycle: an initial step at 94 °C for 2 min to activate the polymerase and 35 cycles each of denaturation at 94 °C for 20 s, annealing of the primers at 60 °C for 30 s and

Table 2.2 - Primers used for amplification and sequencing.

Primer	**Sequence (5'-3')**	**Gene Product**	**Size of PCR amplicon (bp)**	**Size of the target sequence (bp)**	**Annealing temp (°C)**	**Reference**
glnS_F	ACCAACCCGGCCAAGAAGACCAGG(24)	glutaminyl-tRNA synthetase	710 bp	501 bp	64.7	this study
glnS_R	TGCTTGAGCTTGCGCTTG(18)				56.0	
gyrB_F	GGTGGTCGATAACTCCATCG(20)	DNA gyrase subunit B	704 bp	492 bp	59.4	this study
gyrB_R	CGCTGAGGAATGTTGTTGGT(20)				57.3	
ileS_F	TTCCCAATGAARGCCGGCCTGCC(23)	isoleucyl-tRNA synthetase	633 bp	552 bp	66.9	this study
ileS_R	GGGGTGGTGGTCCAGATCACG(21)				65.7	
nuoD_F	GAAGTCCTGACCTTCCTGC(19)	NADH dehydrogenase subunit D	771 bp	600 bp	59.9	this study
nuoD_R	GAAGAACTCGGCCATCATG(19)				59.7	
recA_F	TGGCTGCGGCCCTGGGTCAGATC(23)	recombinase A	573 bp	435 bp	69.9	Frapolli *et al.*, 2007
recA_R	ACCAGGCAGTTGGCGTTCTTGAT(23)				62.4	
rpoB_F	TGGCCGGTCGTCACGGTAACA(21)	DNA-directed RNA polymerase subunit beta	610 bp	477 bp	64.8	this study
rpoB_R	CCGAAACGCTGACCACCGAAC(21)				64.3	
rpoD_F	CTGATCCAGGAAGGCAACATCGG(23)	RNA polymerase sigma factor	631 bp	480 bp	64.5	this study
rpoD_R	ACTCGTCGAGGAAGGAGCG(19)				65.6	

extension at 72 °C for 1 min and a final step of extension at 72 °C for 7 min. The amplified products were analysed by electrophoresis on 1.8% agarose-Tris-acetate-EDTA (TAE) gels, stained with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) and visualised on a UV transilluminator (Gel Doc XR, Biorad). The templates were sent to Macrogen Inc. (Amsterdam, the Netherlands) for direct Sanger sequencing with the respective primer pairs used for PCR amplification as sense and antisense sequencing primers.

The entire sequences were checked for quality, edited and aligned to obtain an identical sequence length for each of the seven genes.

2.3.5. MLST data treatment and phylogenetic analyses

The visualisation, analysis and editing of the chromatograms obtained for the seven genes were performed with FinchTV 1.4.0 software (Geospiza). The sequences were confirmed by the alignment of both forward and reverse sequences using ClustalW (http://www.ebi.ac.uk). All sequences were read in frame. Strains with identical allelic profiles were identified with the same Sequence Type (ST). The concatenated sequences were obtained following the alphabetical order of the seven loci (*glnS*, *gyrB*, *ileS*, *nuoD*, *recA*, *rpoB* and *rpoD*). All the concatenated sequences were of identical length (3,453 nt). The diversity parameters for each locus and for the concatenated sequences, such as G-C content, number of polymorphic sites, number of parsimony informative sites, rates of synonymous and non-synonymous sites, Tajima's D statistic, nucleotide diversity per site (π), the standard deviation and the average number of nucleotide differences per site (θ), were obtained with DnaSP 5.10.01 software (Librado and Rozas, 2009).

The phylogenetic analysis was conducted using the online software PhyML (http://www.phylogeny.fr), which creates a phylogenetic tree based on the Maximum Likelihood (ML) algorithm; this analysis was conducted both for the single loci and the concatenated sequences. All the DNA sequences were uploaded to the *P. fluorescens* MLST database (http://pubmlst.org/pfluorescens/) and to GenBank, with the following accession numbers: KC806141-KC806220, KC923442-KC923904 and KF294515-KF294526.

2.3.6. Clonal and population analyses

ClonalFrame software (Didelot and Falush, 2007) was used to investigate the clonal relationships and the presence of recombination. ClonalFrame is a computer package that uses multilocus sequence data to infer the clonal relationships of bacteria and estimates the probability that a nucleotide changes because of recombination or a point mutation.

Structure software (v.2.3.3) was used to assign individuals to populations; this software assumes that the individuals of the dataset come from several populations (K), characterised by a set of allele frequencies specific to each *locus* (Falush *et al.,* 2003). Each strain is assigned to a specific population or more than one if it has an admixed origin. The number of groups was chosen after repeated analyses (5 iterations, following a burn-in period of 100,000 iterations and Markov Chain Monte Carlo [MCMC] value equal to 50,000) with a K between 1 and 20.

The final number of subgroups was assessed according the L(K) and Δ(K) methods (Evanno *et al.,* 2005).

2.3.7. Pigment production on mozzarella cheese

“Cherry Size” fresh mozzarella cheeses that were bought in a retail store were inoculated with specific strains as proposed by Martin *et al.,* (2011). Each mozzarella was transferred into a sterile stomacher bag with 50 mL of their governing liquid. The liquid of each stomacher bag was inoculated with a preinoculum at a final concentration of 10^3 CFU/mL Tryptic Soy Broth of all strains belonging to the phylogenetic cluster that were identified as containing all the “blue strains” (ps_1, ps_2, ps_5, ps_6, ps_13, ps_22, ps_25, ps_75, ps_76, ps_77, ps_78, and ps_79 produced the blue diffusible pigment in Potato Dextrose Agar, whereas ps_20, ps_33, ps_40, and ps_61 did not produce any pigment). The bags were maintained at 6 °C for 7 days, and the presence of pigmentation was monitored. The bacterial count was conducted after one week to detect the presence of *P. fluorescens* in mozzarella cheese.

2.4. Results

2.4.1. Phenotypic characterisation

The results of the phenotypic characterisation are summarised in Table 2.3.

Table 2.3 - Phenotypic traits of the Pseudomonas fluorescens strains.

The percent values are reported in brackets.

Phenotypic traits			
	6 °C	**22 °C**	**31 °C**
Pigment production on CFC PAB			
Yellow	27 (31.03%)	47 (54.02%)	11 (12.64%)
Ochre	0 (0%)	10 (11.49%)	16 (18.39%)
Blue (diffusible)	3 (3.45%)	5 (5.75%)	0 (0%)
Other	0 (0%)	4 (4.60%)	4 (4.60%)
No colour	57 (65.52%)	21 (24.14%)	56 (64.37%)
Not grown	0 (0%)	0 (0%)	0 (0%)
Pigment production on PDA			
Yellow	1 (1.15%)	7 (8.05%)	3 (3.45%)
Ochre	0 (0%)	2 (2.30%)	10 (11.49%)
Blue (diffusible)	10 (11.49%)	12 (13.79%)	0 (0%)
Light blue (not diffusible)	1 (1.15%)	4 (4.60%)	1 (1.15%)
No colour	75 (86.21%)	62 (71.26%)	64 (73.56%)
Not grown	0 (0%)	0 (0%)	9 (10.35%)
Fluorescent emission on CFC PAB			
Yes	60 (68.97%)	55 (63.22%)	43 (49.43%)
No	27 (31.03%)	32 (36.78%)	44 (50.57%)
Not grown	0 (0%)	0 (0%)	0 (0%)
Proteolytic activity on NA with 2% UHT Milk			
Yes	63 (72.41%)	82 (94.25%)	36 (41.38%)
No	24 (27.59%)	5 (5.75%)	50 (57.47%)
Not grown	0 (0%)	0 (0%)	1 (1.15%)
Lipolytic activity on EYA			
Yes	52 (59.77%)	64 (73.56%)	25 (28.74%)
No	35 (40.23%)	23 (26.44%)	61 (70.11%)
Not grown	0 (0%)	0 (0%)	1 (1.15%)
Lecithinasic activity on EYA			
Yes	52 (59.77%)	50 (57.47%)	34 (39.08%)
No	35 (40.23%)	37 (42.53%)	52 (59.77%)
Not grown	0 (0%)	0 (0%)	1 (1.15%)

To standardise the data collection, a cut-off value was selected to verify the phenotypic traits. At 6 °C, the data were collected on the 10th day, when the traits were completely evident; at

22 °C and 31 °C, the cut-off was fixed on the 7th day, when the maximum expression of the traits was observed.

All strains grew on CFC PAB, whereas 9 (10.98%) strains did not grow on PDA at 31 °C. Several strains did not show any pigment at 6 °C and 31 °C, whereas most of the strains (75.86%) produced pigments at 22 °C (yellow was the most common). The pigment produced by ps_1, ps_2, ps_5, ps_6, ps_13, ps_22, ps_25, ps_75, ps_76, ps_77, ps_78, and ps_79 on PDA was light blue at the beginning of the colour production, and its intensity increased turning deep grey, dark blue and deep brown or black and the pigment diffused throughout the entire agar plate. Additionally, *P. fluorescens* biovar IV (*P. lemonnieri,* DSM 50415), ps_58, ps_62 and ps_68 produced a non-diffusible light blue pigment on PDA. *Pseudomonas libanensis* (DSM 17149T), which was suggested as the strain responsible for the discolouration of mozzarella cheeses in the Annual Report of RASFF, did not produce any blue pigment on PDA.

The production of fluorescent molecules was tested both on CFC PAB and PDA. Because of the higher fluorescent emission detected on CFC PAB, the results of the test on this medium are reported in Table 2.3; more than half of the strains produced fluorescent molecules at 6 °C, 22 °C and 31 °C. Only 1 strain did not grow on EYA at 31 °C, and another one did not grow on NA with 2% of UHT Milk at the same temperature. Proteolytic activity was observed in 82 (94.25%) strains at 22 °C, underlining that protease production is a common spoilage mechanism of the *P. fluorescens* group.

Approximately 75% of the strains showed lipolytic activity in EYA at 22 °C, whereas half of the strains showed lecithinasic activity. These phenotypes and the related degree of manifestation were strictly connected with the growth temperature; thus, all the information was summarised by a hierarchical cluster analysis.

The statistical analysis of the enzyme production, representing the spoilage potential, allowed the definition of four major groups within the considered population (Figure 2.1). The group showing the maximum spoilage potential was characterised by high enzymatic activity, especially at 22 °C; the high spoilage activity group included all the strains with high proteasic and lipasic potential and low amounts of lecithinase production; the group showing

medium activity comprised strains with different levels of enzymatic production (high at 22 °C, medium at 6 °C and low at 31 °C), whereas the low activity group included strains with low levels of enzyme excretion.

With the aim to verify pyomelanin as the cause of the blue discolouration on mozzarella cheese, the strains that were able to produce the blue pigment on PDA (ps_1, ps_2, ps_5, ps_6, ps_13, ps_22, ps_25, ps_77, ps_78 and ps_79) were tested on MBM Agar with or without 1% L-tyrosine. No differences were found, and the dark pigment was visible in both media; this suggested that the pigmented molecule could not be pyomelanin. Conversely, *P. fluorescens* biovar IV (*P. lemonnieri*, DSM 50415) was characterised by no pigment production on MBM Agar. Overall, the blue pigment production was preponderant on PDA and MBM Agar, whereas the trait manifestation was lower on CFC PAB and was visible later.

2.4.2. MLST scheme, allelic diversity and the nucleotide substitution analysis of housekeeping genes

A total of 142 strains were included in the MLST analysis: a portion of seven housekeeping loci was sequenced for seventeen Type strains, one reference strain and 118 isolates, whereas the homologue sequences that were obtained from six completely sequenced strains ascribed to the *P. fluorescens* group were downloaded from GenBank. Thirty-nine strains were excluded from the analysis as redundant strains that were isolated from the same sample matrices and originated at the same dairy plant.

The amplification and sequencing were performed for all the selected genes, except for *ileS*, which was not amplified for three field strains (ps_60, ps_62 and ps_63). The length of the sequences ranged from 435 bp (*recA*) to 552 bp (*ileS*). The number of alleles per gene varied from 78 (*rpoD*) to 90 (*nuoD*). The G-C content was between 0.559 (*gyrB*) and 0.617 (*nuoD*), and the nucleotide diversity (the average number of nucleotide differences per site from two randomly selected sequences) ranged from 0.044 ± 0.0019 (*rpoD*) to 0.108 ± 0.0022 (*gyrB*; Table 2.4). The genetic equilibrium of alleles was analysed using the Tajima's D test (Tajima,

Table 2.4 - Nucleotide diversity observed within the Pseudomonas fluorescens strains characterised in this study.

Loci	Fragment size (bp)	No. of alleles	G+C content	No. (%) of variable sites	No. (%) of parsimony informative sites	Synonymous changes	Non Synonymous changes	d_S [a]	d_N [a]	d_N/d_S	Tajima's D test	θ [a]	π [a] (SD)
glnS	501	89	0.616	213 (42.51)	184 (36.73)	159	17	0.416717	0.03855	0.092509	0.740866	0.084018	0.107162 (0.00503)
gyrB	492	83	0.559	185 (37.60)	163 (33.13)	201	11	0.55511	0.02085	0.037560	1.457315	0.075354	0.107562 (0.00223)
ileS	552	87	0.592	217 (39.31)	182 (32.97)	175	26	0.44172	0.02862	0.064792	0.813253	0.078040	0.096687 (0.00325)
nuoD	516	90	0.617	179 (34.69)	144 (27.91)	156	13	0.37838	0.02760	0.072943	0.849773	0.068402	0.085599 (0.00241)
recA	435	86	0.604	153 (35.17)	142 (32.64)	193	7	0.48489	0.02040	0.042071	1.605872	0.069985	0.103200 (0.00486)
rpoB	477	82	0.575	141 (29.60)	113 (23.69)	122	7	0.23619	0.02143	0.090732	0.097412	0.059383	0.061088 (0.00377)
rpoD	480	78	0.563	105 (21.86)	79 (16.46)	129	4	0.21797	0.00310	0.014222	-0.024674	0.044394	0.044070 (0.00187)
concatenated sequence	**3,453**	**98**	**0.589**	**1189 (34.43)**	**1031 (29.86)**	**1132**	**85**	**0.38700**	**0.02413**	**0.062351**	**1.049536**	**0.066770**	**0.087297 (0.00284)**

[a] **π,** nucleotide diversity per site; **SD,** standard deviation reported in brackets; **θ**, average number of nucleotide differences per site; $\mathbf{d_S}$, number of synonymous changes per synonymous site; $\mathbf{d_N}$, number of nonsynonymous changes per nonsynonymous site.

1989). All the D values ranged from -2 to 2, supporting the complete fitting of the population in the coalescent model. The ratio between the number of non-synonymous substitutions (d_N) and the number of synonymous substitutions (d_S), which is a measure of the selection level acting on the genes, ranged from 0.014 (*rpoD*) to 0.093 (*glnS*). All of the dN/dS (ω) were lower than 1, indicating that each locus was subjected to purifying selection.

The concatenated sequence was 3,453 nucleotides, among which 2,264 (65.57%) sites were conserved and 1,031 (29.86%) were parsimony-informative. The ratio between the number of non-synonymous substitutions (d_N) and the number of synonymous substitutions (d_S) was 0.062.

The allelic number was assigned for each locus giving a progressive number to each newly found allele, and the combination of the alleles generated 98 different STs (Table 2.1).

2.4.3. Phylogeny of *P. fluorescens*

Initially, phylogenetic trees were inferred for each housekeeping gene separately using Maximum Likelihood (ML), including sequence information from the six *P. fluorescens* complete genomes, reference and field strains. The tree topologies of each locus were similar to one another (Figures 2.4-2.10 in the Supplementary data, paragraph 2.8, reported at the end of Chapter 2), except for the position of a few branches; however, the phylogenetic trees clearly delineated the clusters defined by the concatenated sequence tree which had higher bootstrap values and is shown in Figure 2.1.

Two major phylogroups were identified: the first corresponds to the *Pseudomonas fragi* subgroup (containing the type strains of *Pseudomonas lundensis* and *P. fragi*), and the second is divided into five subgroups: *Pseudomonas jessenii*, *Pseudomonas corrugata*, *Pseudomonas koreensis* (containing the reference strain DSM 50415 *P. fluorescens* subsp. *lemonnieri* and *Pseudomonas mandelii*), *Pseudomonas chlororaphis* and *P. fluorescens* (containing *Pseudomonas rhodesiae*, *Pseudomonas brenneri*, *Pseudomonas gessardii*, *Pseudomonas veronii*, *Pseudomonas azotoformans*, *P. fluorescens*, *Pseudomonas marginalis*, *Pseudomonas synxantha*, *P. libanensis* and *Pseudomonas orientalis*).

As highlighted in Figure 2.1, the twelve strains producing the blue pigment in PDA clustered in a distinct phylogenetic group (the “blue branch” including ps_1, ps_2, ps_5, ps_6, ps_13, ps_22,

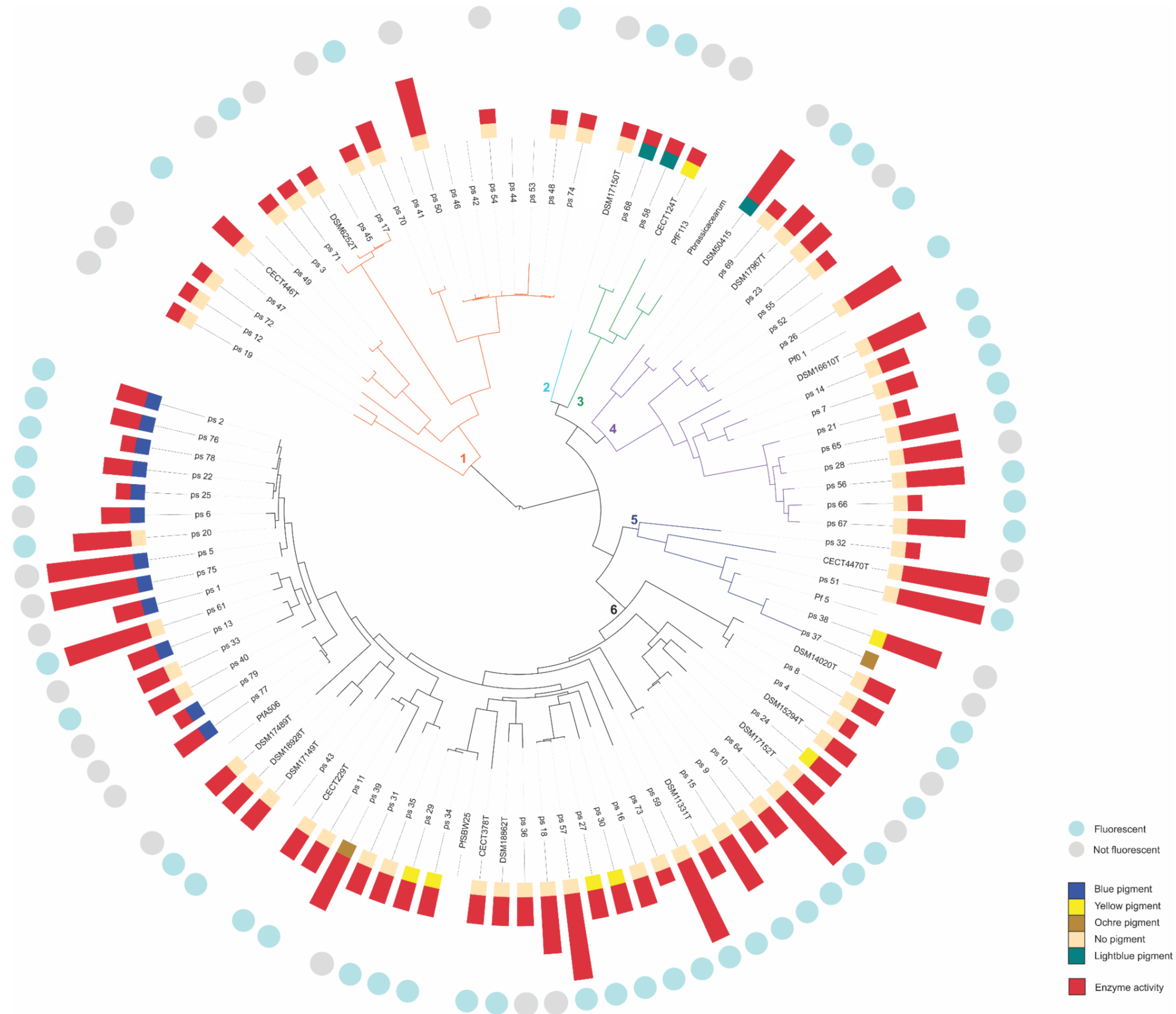


Figure 2.1 - Maximum Likelihood (ML) tree of the dataset.

Phylogenetic reconstruction of the 100 Pseudomonas strains based on the seven MLST genes. The subgroups identified by the phylogenetic analysis are numbered as follows: (1) P. fragi subgroup; (2) P. jessenii subgroup; (3) P. corrugata subgroup; (4) P. koreensis subgroup; (5) P. chlororaphis subgroup; (6) P. fluorescens subgroup. Coloured rectangles (internal ring) represent pigment production on PDA. The length of the red bars (middle ring) indicates the enzymatic activity level according to the hierarchical cluster analysis. Coloured circles (external ring) represent the fluorescent emission ability on CFC PAB at 22 °C.

ps_25, ps_75, ps_76, ps_77, ps_78, and ps_79) that also contains four non-pigmented strains (ps_20, ps_33, ps_40, and ps_61).

The completely sequenced strain *P. fluorescens* A506 and the type strain *P. orientalis* DSM 17489 were closely related to the blue-pigmented strains. The *P. orientalis* DSM 17489 strain showed no blue pigment production in PDA. With the aim to evaluate the genetic diversity between groups of strains that were isolated from distinct ecological niches, the topology obtained through the phylogenetic reconstruction of the 79 field strains, excluding the reference and the completely sequenced strains (most of them isolated from the soil and the environment), was compared with the one that was previously obtained (Figure 2.11, reported at the end of Chapter 2); the tree had higher bootstrap values, although some branches were located in different positions. However, the main cluster composition was maintained.

2.4.4. Recombination analysis of the population structure

ClonalFrame allowed the investigation of clonal relationships and recombination events for the concatenated sequences. The genes *glnS* and *recA* were revealed to be the most recombinant, whereas *nuoD*, *rpoB* and *rpoD* were not affected by recombination. The ML and ClonalFrame trees were compared, and the topologies were found to be very similar to one another, supporting the main cluster and sub-cluster division (data not shown).

The ratio of recombination to mutation (r/m) was calculated for the entire population and was equal to 0.710 (a low value according to Vos and Didelot, 2009), demonstrating the predominance of mutation events. However, when the field strains were analysed separately, ClonalFrame detected a slightly higher recombination rate with an r/m value equal to 1.228 (an intermediate value according to Vos and Didelot, 2009).

Structure software was used to investigate the population structure of the *Pseudomonas* population: the program allows to detect the presence of subpopulations that are different in terms of allelic frequencies and also to visualise the occurrence of genetic transfer within the subgroups. The software identified two major groups (K = 2; Figure 2.2).

Sixty-nine strains were homogeneous, whereas the remaining strains were of a mixed origin, indicating that some sequences could have been imported from the other population.

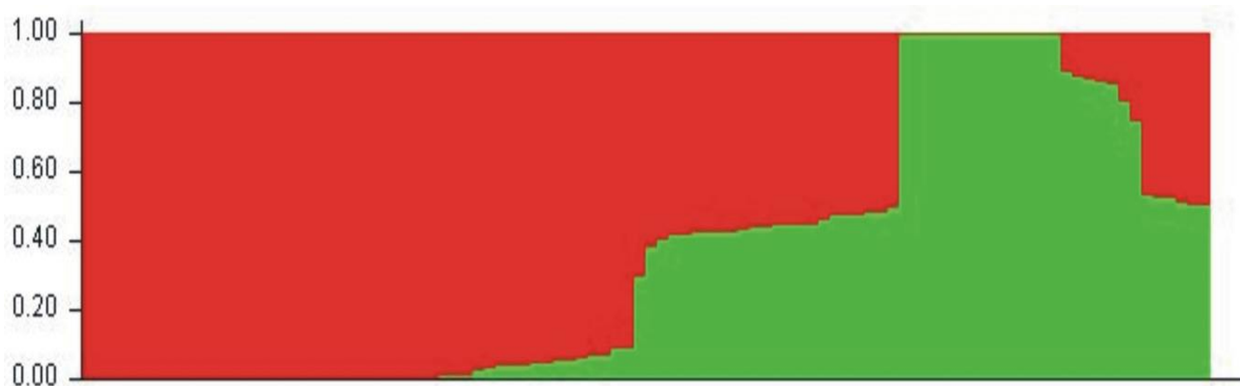


Figure 2.2 - Structure analysis.

K=2 analysis of the six completely sequenced strains, seventeen Type strains, one reference strain and 79 isolates obtained from Structure software. Each different colour corresponds to a distinct population. Mixed colours represent high genetic homology with the other group or import of DNA from the other population.

2.4.5. Pigment production on mozzarella cheese

The sixteen strains belonging to the “blue cluster” that were previously identified by phylogenetic analysis (among which twelve produced the blue pigment on PDA and MBM Agar, and four were non-pigmenting strains and were used as negative controls, see Figure 2.1), the reference strains previously reported as causing the “blue mozzarella” events (*P. lemonnieri* and *P. libanensis*) and the Type strain *P. orientalis*, which was very close to the blue branch, were selected for the inoculum test on mozzarella cheese.

Only the twelve strains that produced the blue pigment on PDA and MBM Agar generated a visible alteration on mozzarella cheese, which changed from white to light blue (Figure 2.3); specifically, the surface became light blue, as described in cases of “blue mozzarella”, when a large portion of the “Cherry Size” fresh mozzarella was exposed to air.

The concentration of *Pseudomonas* in mozzarella cheese reached at least 10^8 CFU/mL by the end of the examination.

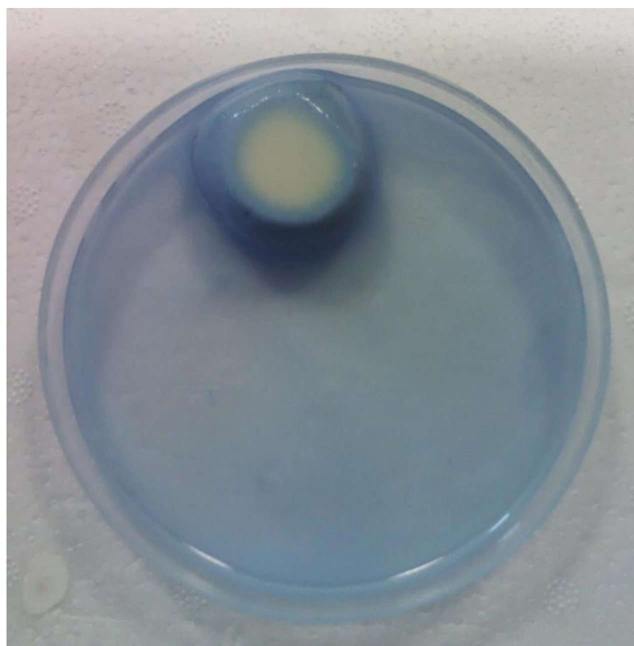


Figure 2.3 - Pigment production on mozzarella cheese.

Mozzarella cheese involved in blue discolouration after the inoculum of 10^3 CFU/ml of strains belonging to the P. fluorescens group.

2.5. Discussion

Pseudomonas spp. are among the most common bacteria involved in food spoilage (Martin *et al.,* 2011), and the interest in their role in food grew after the "blue mozzarella" events that occurred in 2010. Health authorities and official laboratory analyses connected the outbreak to the contamination of processing water with strains of *P. fluorescens* (Marro *et al.,* 2011). Specifically, in the Annual Report of RASFF, scientists confirmed the involvement of two species belonging to *P. fluorescens* group: *Pseudomonas tolaasii* and *P. libanensis*. *P. libanensis* was also reported as a producer of blue pigment in unripe cheese products (Rapid Alert System for Food and Feed, [RASFF], Annual Report, 2010). However, additional data to precisely identify these strains were not available and, to date, information about the health risks related to the ingestion of blue mozzarella are lacking because the chemical structure of the pigment has not yet been investigated.

Improving the identification and tracking methods for the *P. fluorescens* group strains in food processing systems is important to understand and reduce the frequency of contamination.

The MLST approach is a greatly advantageous typing method as it allows rapid and cheap sequence-based strain identification that could be easily shared between different laboratories.

The aim of this study was the creation and implementation of a MLST scheme and public database to genetically type the strains belonging to the *P. fluorescens* group that were isolated from food products to understand genetic differences among them and to identify a potential connection with specific phenotypic traits.

The strain discrimination power resulting from the MLST was high, the method was robust, practical and allowed the discrimination of all the taxa that were analysed. Among the initial 142 strains, 98 different STs were identified, demonstrating the presence of high heterogeneity even within the same *taxon*. Only two STs (ST26 and ST29) were found simultaneously in different strains that were isolated from samples of blue mozzarella cheese originating from different dairy plants.

The ML tree showed a similar topology to that obtained from 16S rRNA, *gyrB*, *rpoB* and *rpoD* sequences in the study conducted by Mulet *et al.,* (2010), except for the *P. fragi* subgroup that was not well-separated from all the others. A similar clustering was obtained by Matthijs *et al.,* (2012): *P. fragi* belonged to a distinct cluster, whereas *P. mandelii* and *P. koreensis* were in two different subgroups. However, the tree obtained through this double-loci approach was characterised by lower bootstrap values (Matthijs *et al.,* 2012).

A low level of recombination (r/m: 0.710) was detected by the ClonalFrame analysis, demonstrating that mutation could be the predominant driving force in the diversification of the *P. fluorescens* group that was considered. The r/m value was lower than the one reported for *Pseudomonas viridiflava* (r/m = 2.0) and *Pseudomonas syringae* (r/m = 1.5) by Vos and Didelot (2009). This difference is most likely because of two factors: first, recombination rates depend on the ecological characteristics of the strains, and the populations considered by Vos and Didelot are both plant pathogens, suggesting that a higher occurrence of recombination could be detected among strains sharing an identical ecological niche. Actually, when the field strains were analysed separately, excluding the Type and reference strains and the six completely sequenced genomes obtained from GenBank, a higher r/m

value (1.228) was found. Second, *P. viridiflava* and *P. syringae* are plant pathogens that could enhance their virulence and pathogenic power through recombination events.

Despite the high genetic heterogeneity of the strains in analysis, the Structure software identified only two major populations, corresponding to the clusters identified in the phylogenetic analysis. The result shows high genetic diversity primarily between *P. fragi* and all the other *Pseudomonas* spp., with some strains exhibiting a mixed genotype. The genetic diversity among the different subgroups was not high enough to support a higher value of subpopulations that were detected. However, this result might depend on the number of strains that were considered in this study.

To understand the spoilage potential of *P. fluorescens* strains that were isolated from food matrices and the environmental conditions (i.e. different nutritional resources or incubation temperatures, which could enhance or reduce their phenotypic behaviour), a screening of the five major factors involved in food alteration was performed (Munsch-Alatossava and Alatossava, 2005). The spoiler reactions of *P. fluorescens* are mainly because of their ability to produce and release different molecules, such as pigments or enzymes. The genus *Pseudomonas* produces an extended range of coloured molecules, such as pyocyanine, pyoverdine, fluorescein, pyorubin and pyomelanin, but the blue pigment observed on mozzarella cheese has different features than the pigments that are classically produced.

The blue pigment was produced on PDA, on MBM Agar and on CFC PAB, although on this medium the manifestation had a lower intensity. No blue pigment was observed on NA with 2% UHT Milk and EYA, most likely because of the composition of these media; however, more studies are necessary to better understand this phenomenon.

Additionally, the phenotypic traits may be influenced by the temperature and the medium composition, in addition to the size of inoculums and the phase of inoculum growth (Rajmohan *et al.,* 2002).

As previously reported, the nature of the blue pigment observed on PDA that was produced by the “blue branch” strains is unknown, and a chemical-physical analysis is in progress. According to the pigment production on PDA, it was excluded that this pigment could be identical to that produced by *P. fluorescens* biovar IV (e.g. subsp. *lemonnieri*, DSM 50415) or

another pigment, such as pyomelanin. Actually, *P. fluorescens* biovar IV, a strain isolated from soil, could also produce a deep-blue pigment on PDA (Hugo and Turner, 1957), which has been described by Starr *et al.,* (1967) as a blue insoluble intracellular pigment. In 2011, Martin and collaborators simulated the contamination of a fresh low-acid cheese with the *P. fluorescens* biovar IV: the isolates produced a blue pigment and fluoresced under UV light when re-inoculated onto fresh, low-acid cheese. However, the phenotypic analysis of the *P. fluorescens* biovar IV in this study demonstrated important differences in the pigment produced from the mozzarella borne strains. The *P. fluorescens* biovar IV produced a blue water insoluble pigment that could be observed at 31 °C on PDA and a fluorescent emission after UV light exposure (Martin *et al.,* 2011), whereas the blue pigment produced by the strains in this study was diffusible, not fluorescent and was produced only at 6 °C and 22 °C and not at 31 °C; additionally, *P. lemonnieri* did not produce any pigment on MBM Agar or on mozzarella cheese.

Pyomelanin was also excluded from the hypothesis as the cause of the mozzarella discolouration because the addition of lysine did not enhance the pigment production in MBM Agar (Boles *et al.,* 2004). Thus, the matched use of both PDA and MBM Agar is suggested to highlight this phenotypic trait.

Concerning enzymatic alteration, *P. fluorescens* is responsible for the highest depredation of milk because of its capacity to synthesise extracellular enzymes (Dufour *et al.,* 2008).

As reported by Marchand *et al.,* (2009), protease production is more frequent than other observed phenotypic traits.

Lipolytic and lecithinasic spoilage is less frequently reported in food, most likely because of the lower percentage of strains bearing this adulteration ability. Nevertheless, all the traits were temperature-dependent. The *P. fragi* strains were characterised by no lecithinasic activity, confirming the results that were obtained by Franzetti and Scarpellini (2007).

The strain clustering based on the phenotypic traits underlined the clear distinction within the considered population, which, however, could not be directly linked to the phylogenetic grouping, except for the blue pigment production. Indeed, it is interesting to highlight that all the blue-pigmented strains belong to a unique phylogenetic cluster, as shown in Figure 2.1.

Moreover, the only two strains isolated from meat (ps_77 and ps_79) were clustered together in a clearly independent group, very close to *P. fluorescens* A506, even though isolated from different geographical area (England and Italy) and from different sources (beef and pork). No evident clusterization based on other phenotypic traits (i.e. degree of enzymatic activity) was apparent.

In 2013, Nogarol and colleagues applied a PFGE protocol to 181 strains of *P. fluorescens* that were isolated from mozzarella cheese samples from Italian and German plants involved in the blue mozzarella events, but they could not identify any phylogenetic clusters that were responsible for the blue discolouration (Nogarol *et al.,* 2013). Most likely this is because of the lack of phenotypic tests that confirmed the real ability of the strains to produce the blue pigment. It has been demonstrated here that not all the strains that were isolated from the spoiled blue mozzarella cheeses have been able to produce the pigment on PDA (see Table 2.1).

The pigment production test on the cheese demonstrated that the *P. fluorescens* strains (ps_1, ps_2, ps_5, ps_6, ps_13, ps_22, ps_25, ps_75, ps_76, ps_77, ps_78 and ps_79) were able to reproduce the blue colouration under controlled experimental conditions in an *in vitro* system on mozzarella cheese, confirming that the mozzarella cheese is a substrate that allowed the pigment production after oxygen exposure. The phylogenetic relatedness of the blue-pigmenting strains represents an outstanding result, indicating that this characteristic is clearly related to the core genome, although phenotypic properties are often linked to the accessory genes. No previous studies were able to connect this phenotypic trait to a specific genetic cluster and this discovery may help focus on a specific group for controlling the strains involved in the blue discolouration of dairy products.

In conclusion, this study was characterised by thorough comparative work of microbiological and molecular approaches to investigate the nature of the genetic relationships between the isolates and to characterise the blue pigmented producers together with the main spoilage traits. The production of the blue pigment seemed to be related to the presence of a specific gene or genomic regions in the core genome of the strains, as they all clustered in the same phylogenetic group.

Additional studies are required to understand the molecular and biochemical pathways involved in inducing the pigment production and to highlight the propitious condition in which *P. fluorescens* is able to induce food discolouration.

The data obtained through the MLST analysis have been collected and uploaded in the single expanding central Multilocus Sequence Database pubMLST available online at pubmlst.org/pfluorescens/. This system may be used by food factories to track the presence of *P. fluorescens* on working surfaces, raw materials and final products to avoid unpleasant spoilage events and correlated economic losses.

2.6. Acknowledgements

The study was supported by the University of Padova (Progetto di Ricerca di Ateneo 2011 to L.F. - Development of a molecular typing system and evaluation of phenotype characters involved in food colouring and spoilage in *Pseudomonas* spp. strains. CPDA115333). Also supported by the PhD school of Veterinary Science of the University of Padova to support the education of N.A.A. The authors are grateful to the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, to Dr. M. Marino and M. Maifreni (Department of Food Sciences, University of Udine), and to Dr. Janet Corry (School of Veterinary Science, University of Bristol) for providing *Pseudomonas* strains.

2.7. References

- Anzai, Y., Kim, H., Park, J.-Y., Wakabayashi, H., Oyazu, H., 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequences. International Journal of Systematic and Evolutionary Microbiology, 50, 1563-1589.
- Arnaut-Rollier, I., Vauterin, L., De Vos, P., Massart, D.L., Devriese, L.A., De Zutter, L., Van Hoof, J., 1999. A numerical taxonomic study of the *Pseudomonas* flora isolated from poultry meat. Journal of Applied Microbiology, 87, 15-28.
- Arslan, S., Eyi, A., Özdemir, F., 2011. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. Journal of Dairy Science, 94, 5851-5856.

- Bennasar, A., Guasp, C., Lalucat, J., 1998. Molecular methods for the detection and identification of *Pseudomonas stutzeri* in pure culture and environmental samples. Microbial Ecology, 35, 22-53.
- Boles, B.R., Thoendel, M., Singh, P.K., 2004. Self-generated diversity produces "insurance effects" in biofilm communities. Proceeding of the National Academy of Sciences, 101, 16630-16635.
- Bogdanova, T., Flores Rodas, E.M., Greco, S., Tolli, R., Bilei, S., 2010. Indagine microbiologica su campioni di mozzarella in occasione dell'allerta "Mozzarella blu". XII Congresso Nazionale S.I.Di.L.V., Volume Atti, 117-118.
- De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P., Heyndrickx, M., 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Applied and Environmental Microbiology, 77, 460-470.
- De Vos, P., Goor, M., De Gillis, M., De Ley, J., 1985. Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. International Journal of Systematic bacteriology, 35, 169-184.
- De Vos, P., Van Landschoot, A., Segers, P., Tytgat, R., Gillis, M., Bauwens, L.M., De Ley, J., 1989. Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxyribonucleic acid: ribosomal ribonucleic acid hybridizations. International Journal of Systematic Bacteriology, 39, 35-49.
- Didelot, X., Falush, D., 2007. Inference of bacterial microevolution using Multilocus sequence data. Genetics, 175, 1251-1266.
- Dufour, D., Nicodème, M., Perrin, C., Driou, A., Brusseaux, E., Humbert, G., Gaillard, J.L., Dary, A., 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology, 125, 188-196.

- Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology, 14, 2611-2620.
- Falush, D., Stephens, M., Pritchard, J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics, 164, 1567-1587.
- Franzetti, L., Scarpellini, M., 2007. Characterisation of *Pseudomonas* spp. isolated from foods. Annals of Microbiology, 57, 39-47.
- Frapolli, M., Défago, G., Moënne-Loccoz, Y., 2007. Multilocus sequence analysis of biocontrol fluorescent *Pseudomonas* spp. producing the antifungal compound 2,4-diacetylphloroglucinol. Environmental Microbiology, 9, 1939-1955.
- Gennari, M., Dragotto, F., 1992. A study of the incidence of different fluorescent *Pseudomonas* species and biovars in the microflora of fresh and spoiled meat and fish, raw milk, cheese, soil and water. Journal of Applied Microbiology, 72, 281-288.
- Gonçalves, L.S.A., Rodrigues, R., Amaral Júnior, A.T., Karasawa, M., Sudré, C.P., 2008. Comparison of multivariate statistical algorithms to cluster tomato heirloom accessions. Genetics and Molecular Research, 7, 1289-1297.
- Gower, J.C., 1971. A general coefficient of similarity and some of its properties. Biometrics, 27, 623-637.
- Gurtler, V., Stanisich, V.A., 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology, 142, 3-16.
- Hilario, E., Buckley T.R., Young, J.M., 2004. Improved resolution on the phylogenetic relationships among *Pseudomonas* by the combined analysis of *atpD*, *carA*, *recA* and 16S rDNA. Antoine van Leeuwenhoek, 86, 51-64.
- Hugo, W.B., Turner, M., 1957. A soil bacterium producing an unusual blue pigment. Journal of Bacteriology, 73, 154-157.
- Johnsen, K., Andersen, S., Jacobsen, C.S., 1996. Phenotypic and genotypic characterization of phenanthrene-degrading fluorescent *Pseudomonas* biovars. Applied and Environmental Microbiology, 62, 3818-3825.

- Johnson, J.M., Weagant, S.D., Jinneman, K.C., Bryant, J.L., 1995. Use of Pulsed-Field Gel Electrophoresis for Epidemiological Study of *Esherichia coli* O157:H7 during a Food-Borne Outbreak. Applied and Environmental Microbiology, 61, 2806-2808.
- Laguerre, G., Rigottier-Gois, L., Lemanceau, P., 1994. Fluorescent *Pseudomonas* species categorized by using polymerase chain reaction (PCR)/restriction fragment analysis of 16S rDNA. Molecular Ecology, 3, 479-487.
- Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25, 1451-1452.
- Maiden, M.C.J., Bygraves J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., Spratt, B.G., 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proceeding of the National Academy of Sciences, 95, 3140-3145.
- Maiden, M.J.C., 2006. Multilocus sequence typing of bacteria. Annual Reviews of Microbiology, 60, 561-588.
- Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., De Jonghe, V., Dewettinck, K., De Vos, P., Devreese, B., Heyndrickx, M., De Block, J., 2009. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. International Journal of Food Microbiology, 133, 68-77.
- Marro, S., Griglio, B., Testa, A., Piovesan, F., Civera, T., 2001. Alterazioni organolettiche negli alimenti causate da Pseudomonadaceae e possibili ricadute per la sanità pubblica. Rapporto tecnico scientifico 1/2011 Ce.I.R.S.A.
- Martin, N.H., Murphy, S.C., Ralyea, R.D., Wiedmann, M., Boor, K.J., 2011. When cheese gets the blues: *Pseudomonas fluorescens* as the causative agent of cheese spoilage. Journal of Dairy Science, 94, 3176-3183.
- Martino, M.E., Fasolato, L., Montemurro, F., Rosteghin, M., Manfrin, A., Patarnello, T., Novelli, E., Cardazzo, B., 2011. Determination of microbial diversity of *Aeromonas* strains on the basis of multilocus sequence typing, phenotype and presence of putative virulence genes. Applied and environmental microbiology, 77, 4986-5000.

- Matthijs, S., Coorevits, A., Gebrekidan, T.T., Tricot, C., Wauven, C.V., Pirnay, J.P., De Vos, P., Cornelis, P., 2012. Evaluation of *oprI* and *oprL* genes as molecular markers for the genus *Pseudomonas* and their use in studying the biodiversity of a small Belgian River. Research in Microbiology, 164, 254-261.
- Moore, E.R.B., Mau, M., Arnscheidt, A., Böttger, E.C., Hutson, R.A., Collins, M.D., Van de Peer, Y., De Wachter, R., Timmis, K.N., 1996. The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (*sensu stricto*) and estimation of the natural intrageneric relationships. Systematic and Applied Microbiology, 19, 478-492.
- Mulet, M., Lalucat, J., Garcìa-Valdes, E., 2010. DNA sequence-based analysis of the *Pseudomonas* species. Environmental Microbiology, 12, 1513-1530.
- Munsch-Alatossava, P., Alatossava, T., 2005. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. Microbiological research, 161, 334-346.
- Nogarol, C., Acutis, P.L., Bianchi, D.M., Maurella, C., Peletto, S., Gallina, S., Adriano, D., Zuccon, F., Borrello, S., Caramelli, M., Decastelli, L., 2013. Molecular characterization of *Pseudomonas fluorescens* isolates involved in the Italian "blue mozzarella" event. Journal of Food Protection, 76, 500-504.
- Palleroni, N.J., Kunisawa, R., Contopoulou, R., Dourdoroff, M., 1973. Nucleic acid homologies in the genus *Pseudomonas*. International Journal of Systematic and Evolutionary Bacteriology, 23, 333-339.
- Rapid Alert System for Food and Feed (RASFF). Annual Report, 2010.
- Rajmohan, S., Dodd, C.E.R., Waites, W.M., 2002. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. Journal of Applied Microbiology, 93, 205-213.
- Rossignol, G., Merieau, A., Guerillon, J., Veron, W., Lesouhaitier, O., Feuilloley, M.G.J., Orange, N., 2008. Involvement of a phospholipase C in the hemolytic activity of a clinical strain of *Pseudomonas fluorescens*. BMC microbiology, 30, 189.
- Starr, M.P., Knackmuss, H., Cosens, G., 1967. The intracellular blue pigment of *Pseudomonas lemonnieri*. Archiv für Mikrobiologie, 59, 287-294.

- Tajima, F., 1989. Statistical methods to testing the neutral mutation hypothesis by DNA polymorphism. Genetics, 123, 585-595.
- Vos, M., Didelot, X., 2009. A comparison of homologous recombination rates in bacteria and archaea. The ISME Journal, 3, 199-208.
- Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P., Trüper, H.G., 1987. Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. International Journal of Systematic Bacteriology, 37, 463-464.
- Woese, C.R., Strackebrandt, E., Weisburg, W.G., Paster, B.J., Madigan, M.T., Fowler, V.J., Hahn, C.M., Blanz, P., Gupta, R., Nealson, K.H., Fox, G.E., 1984. The phylogeny of purple bacteria: the alpha subdivision. Systematic and Applied Microbiology, 5, 315-326.
- Yamamoto, S., Kasai, H., Arnold, D.L., Jackson, R.W., Vivian, A., Harayama, S., 2000. Phylogeny of the genus *Pseudomonas*: intragenic structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology, 146, 2385-2394.

2.8. Supplementary data

Figure 2.4 - ML tree based on glnS sequencing.

Phylogenetic reconstruction of six completely sequenced strains, seventeen Type strains, one reference strain and 79 isolates based on glnS sequencing.

Figure 2.5 - ML tree based on gyrB sequencing.

Phylogenetic reconstruction of six completely sequenced strains, seventeen Type strains, one reference strain and 79 isolates based on gyrB sequencing.

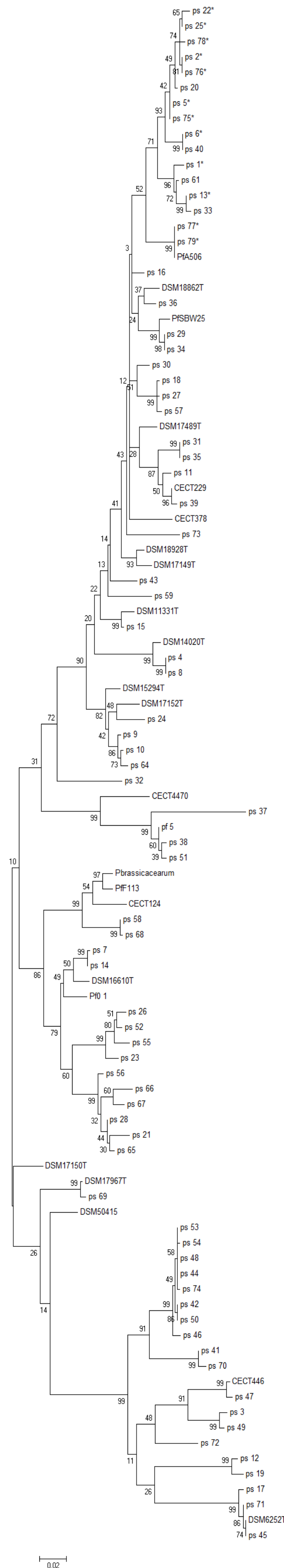


Figure 2.6 - ML tree based on ileS sequencing.

Phylogenetic analysis of six completely sequenced strains, seventeen Type strains, one reference strain and 76 isolates based on ileS sequencing.

Figure 2.7 -ML tree based on nuoD sequencing.

Phylogenetic reconstruction of six completely sequenced strains, seventeen Type strains, one reference strain and 79 isolates based on nuoD sequencing.

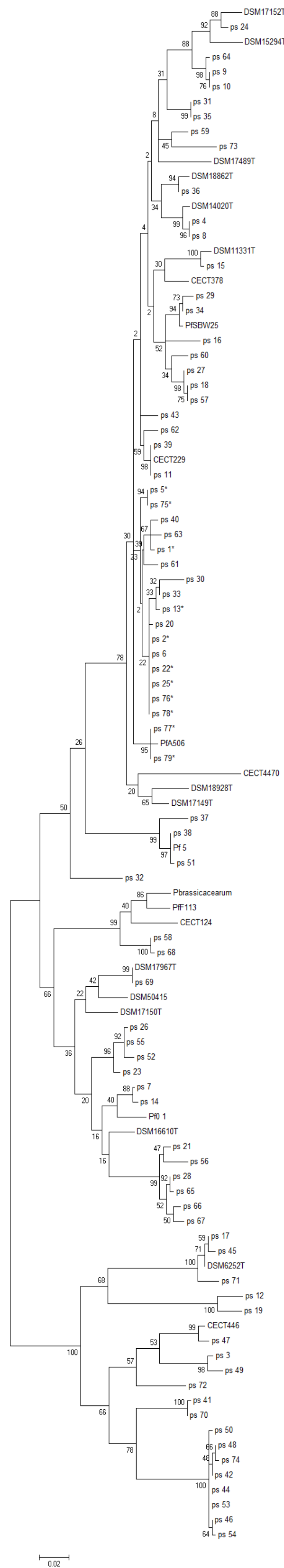


Figure 2.8 - ML tree based on recA sequencing.

Phylogenetic reconstruction of six completely sequenced strains, seventeen Type strains, one reference strain and 79 isolates based on recA sequencing

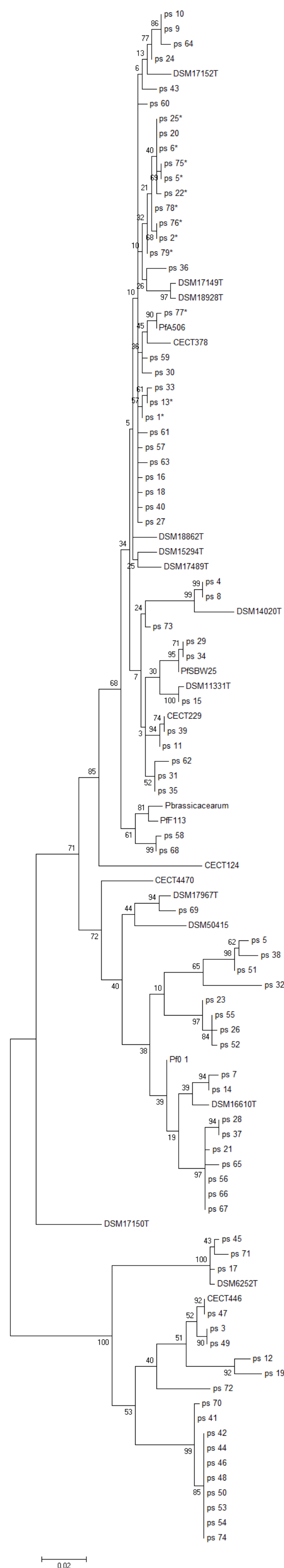


Figure 2.9 - ML tree based on rpoB sequencing.

Phylogenetic reconstruction of six completely sequenced strains, seventeen Type strains, one reference strain and 79 isolates based on rpoB sequencing.

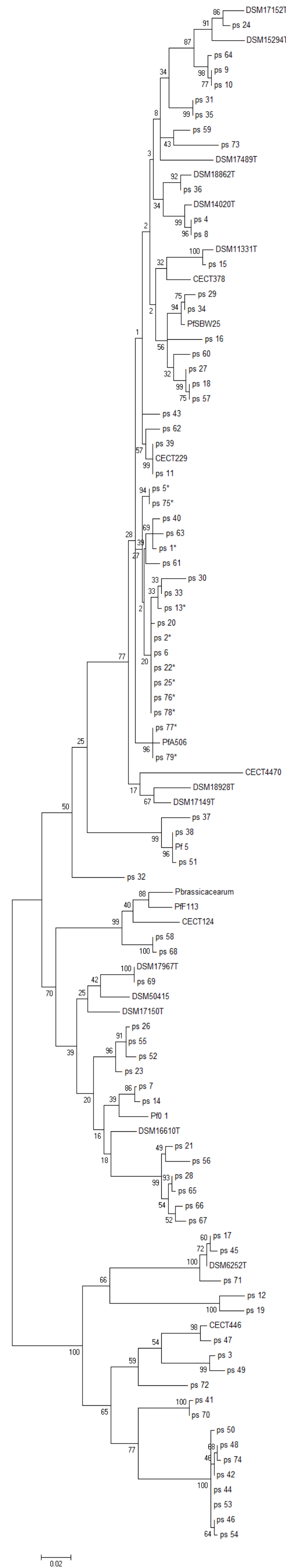


Figure 2.10 - ML tree based on rpoD sequencing.

Phylogenetic reconstruction of six completely sequenced strains, seventeen Type strains, one reference strain and 79 isolates based on rpoD sequencing.

Figure 2.11 - ML tree of the field strains of the dataset.

*Phylogenetic reconstruction of 76 isolates based on the concatenated analysis of glnS, gyrB, ileS, nuoD, recA, rpoB e rpoD. Blue pigmenting strains are marked with * symbol.*

2.9. Appendix to Chapter 2

After the publication of the paper "Tracking the blue: A MLST approach to characterise the *Pseudomonas fluorescens* group", other *P. fluorescens* strains were analysed through the phenotypic and genotypic approach proposed in the paper.

The approach was applied to further 7 *Pseudomonas fluorescens* strains. Table 2.5 reports the strains investigated.

The phenotypic test, namely the growth on PDA and MBM, allowed the identification of the 6 blue- and 1 non-pigmenting strains.

Subsequently, the MLST scheme was applied to verify the ability to cluster in the blue branch all the strains characterised by the production of the blue pigment.

As previously demonstrated with the strains analysed in the paper, the blue strains clustered together in the "blue branch" (Figure 2.12).

These results confirmed the strength of the proposed method and confirmed the belonging of the strains that are able to induce the spoilage effect through the production of the pigment to a specific phylogenetic group. The previously reported subgroups were identified by the phylogenetic reconstruction.

Table 2.5 - Origins and typing data of Pseudomonas fluorescens strains analysed in this study.

Sample	Strain	Subgroup [a]	Source	Provenience [b]	ST [c]	Alleles [c]						
						glnS	***gyrB***	***ileS***	***nuoD***	***recA***	***rpoB***	***rpoD***
ps_80*	**SAL1**	*P. fluorescens*	salami	NE Italy	99	34	37	88	37	33	35	33
ps_81*	**BU3**	*P. fluorescens*	butter	NE Italy	100	90	84	29	29	29	29	29
ps_82*	**PCA3**	*P. fluorescens*	butter	NE Italy	37	34	37	36	37	33	35	33
ps_83*	**EL1**	*P. fluorescens*	ricotta cheese	NE Italy	101	91	85	43	91	26	83	79
ps_84*	**ML1**	*P. fluorescens*	mozzarella cheese	NE Italy	102	92	5	5	92	86	82	78
ps_85*	**811/2**	*P. fluorescens*	mozzarella cheese	NE Italy	103	93	25	89	93	87	69	25
ps_86	**811/1**	*P. fragi*	mozzarella cheese	NE Italy	104	94	27	90	94	64	84	27

[a] The subgroup names (i.e., complexes, which are shown for field isolates) were assigned according to the attribution provided by Mulet *et al.,* (2010).

[b] The geographic position is reported for the Italian samples (NE: North-East, C: Central).

[c] STs and alleles were determined by MLST.

[*] Strains that produced the blue pigment on Potato Dextrose Agar and MBM Agar.

Figure 2.12 - ML tree of the expanded dataset.

Phylogenetic reconstruction of the 110 Pseudomonas strains based on the sequences seven MLST genes. The blue branch is highlighted in blue.

CHAPTER 3: A genomic and transcriptomic approach to investigate the blue pigment phenotype in *Pseudomonas fluorescens*

---

*Adapted with the permission of "Elsevier" from: **Andreani Nadia Andrea**, Carraro Lisa, Martino Maria Elena, Fondi Marco, Fasolato Luca, Miotto Giovanni, Magro Massimiliano, Vianello Fabio and Cardazzo Barbara.

International Journal of Food Microbiology 213 (2015), 88-98.

Article info:

Article history:

Received 29 December 2014

Received in revised form 14 May 2015

Accepted 29 May 2015

Available online 1 June 2015

Keywords:

Pseudomonas fluorescens, Comparative genomics and transcriptomics, Blue mozzarella.

3.1. Abstract

Pseudomonas fluorescens is a well-known food spoiler, able to cause serious economic losses in the food industry due to its ability to produce many extracellular, and often thermostable, compounds. The most outstanding spoilage events involving *P. fluorescens* were blue discolouration of several food stuffs, mainly dairy products. The bacteria involved in such high-profile cases have been identified as belonging to a clearly distinct phylogenetic cluster of the *P. fluorescens* group. Although the blue pigment has recently been investigated in several studies, the biosynthetic pathway leading to the pigment formation, as well as its chemical nature, remain challenging and unsolved points.

In the present paper, genomic and transcriptomic data of four *P. fluorescens* strains (two blue-pigmenting strains and two non-pigmenting strains) were analysed to evaluate the presence and the expression of blue strain-specific genes. In particular, the pangenome analysis showed the presence in the blue-pigmenting strains of two copies of genes involved in the tryptophan biosynthesis pathway (including *trpABCDF*). The global expression profiling of blue-pigmenting strains versus non-pigmenting strains showed a general up-regulation of genes involved in iron uptake and a down-regulation of genes involved in primary metabolism. Chromogenic reaction of the blue-pigmenting bacterial cells with Kovac's reagent indicated an indole-derivative as the precursor of the blue pigment. Finally, solubility tests and MALDI-TOF mass spectrometry analysis of the isolated pigment suggested that its molecular structure is very probably a hydrophobic indigo analogue.

3.2. Introduction

The genus *Pseudomonas* comprises several rod-shaped, Gram-negative, aerobic, mesophilic and psychrotolerant microorganisms, characterised by simple nutritional requirements (Anzai *et al.,* 2000; Frapolli *et al.,* 2007). Recent taxonomic issues (Palleroni, 2010) led to the identification of multiple phylogenetic clusters, among which the *Pseudomonas sensu stricto* group (rRNA group I) stands out. It comprises several species, such as *Pseudomonas*

aeruginosa, the well-known human opportunistic pathogen, often observed in nosocomial infections, especially in immunodeficient patients (Tümmler *et al.,* 2014), and *Pseudomonas fluorescens*, which causes food spoilage (Gennari and Dragotto, 2012; Samaržija *et al.,* 2012). *P. fluorescens* is commonly part of the microflora of a wide range of food matrices, including ready-to-eat vegetables (Caldera and Franzetti, 2014), raw fish (especially sushi or sashimi), meat and dairy products (Arnaut-Rollier *et al.,* 1999; Franzetti and Scarpellini, 2007). This is due to its extreme adaptability and versatility (Silby *et al.,* 2011) and its ability to replicate at refrigeration temperatures (Decimo *et al.,* 2014). Specifically, *P. fluorescens* can cause undesirable modifications in food products as a result of enzymatic reactions or pigmented molecule production (Andreani *et al.,* 2014). A well-known example is represented by the "blue mozzarella cheese events" which occurred in Europe in 2010 (Annual Report of RASFF), when consumers noticed blue discolourations on mozzarella cheese after opening the packages. About 70,000 balls of mozzarella cheese were seized and the competent control authorities identified strains belonging to the *P. fluorescens* group as responsible of this particular spoilage event.

Recently, the genotypic and phenotypic characterisation of 139 strains belonging to the *P. fluorescens* group revealed the presence of a genetic cluster (the so called "blue branch") including all the blue-pigmenting strains, together with a limited number of non-pigmenting strains (Andreani *et al.,* 2014). This result suggested a close correlation between the blue phenotype and the genetic information coded by a chromosomal gene or gene cluster.

The chemical nature of the blue pigment has recently been investigated on mozzarella cheese directly contaminated with *P. fluorescens* strains by Caputo *et al.,* (2015) using LC-High Resolution-MS (Liquid Chromatography-High Resolution-Mass Spectrometry) analysis. Although both the extraction and the characterisation of the blue pigment were hampered by solubility issues, the presence of the uncoloured parental compound of indigoidine (leuco-indigoidine) was revealed in all samples that showed the blue discolouration. Hence, these results led to the hypothesis that the blue pigment might be indigoidine. Indigoidine is a blue diazadiphenoquinone pigment (chemical formula: $C_{10}H_8N_4O_4$) produced by a large variety of microorganisms (Kuhn *et al.,* 1965; Bartel *et al.,* 1990). The biosynthetic pathway of

indigoidine has been identified in *Erwinia chrysantemi* (now *Dickeya dadantii*; Reverchon *et al.,* 2002; Chu *et al.,* 2010), in *Photorhabdus luminescens* (Brachmann *et al.,* 2012), *Phaeobacter* sp. (Cude *et al.,* 2012), *Streptomyces chromofuscus* (Yu *et al.,* 2013) and *Vogesella indigofera* (van de Loo *et al.,* 1998). Indigoidine synthesis, in *E. chrysanthemi*, depends on a regulator region (*pecS*) and three open reading frames (ORFs) designated *indA*, *indB* and *indC*. In *V. indigofera*, the indigoidine locus is composed of five genes, *igiA*, *igiB*, *igiC*, *igiD* and *igiE* (van de Loo *et al.,* 1998). It has been demonstrated that this pigment plays an important role in tolerance to oxidative stress (Reverchon *et al.,* 2002), but its intrinsic function remains elusive (Brachmann *et al.,* 2012).

In recent years, Next-Generation High-Throughput Sequencing (HT-NGS) has been increasingly used, overtaking classical microbiology and first generation biomolecular technology in several fields, such as diagnostics and biotechnology, but also food microbiology. This is due to the enormous advantages of these techniques, which are less time-consuming, cheaper and more adaptable to several research goals (Diaz-Sanchez *et al.,* 2012; Solieri *et al.,* 2012). In food microbiology, HT-NGS approaches have generally been applied for food safety purposes, investigating food pathogens such as *Salmonella* spp. (Holt *et al.,* 2008; Andrews-Polymenis *et al.,* 2009) and *Listeria monocytogenes* strains isolated from meat products (Gilmour *et al.,* 2010). HT-NGS applications are numerous: including whole-genome sequencing, RNA-sequencing techniques, investigating bacterial expression in different environmental conditions, and the investigation of the microbiota of food products through metagenomics and metatranscriptomics (Diaz-Sanchez *et al.,* 2012; Loman *et al.,* 2012; Solieri *et al.,* 2012). However, poor bibliography is available about food spoilers so far.

In the present study, the draft genomes of four phylogenetically related *P. fluorescens* strains were determined. The strains were previously characterised as having two different phenotypes: two are able to produce the blue pigment and two are non-pigmenting strains. The same strains were also analysed using a transcriptomic approach to identify differential gene expression in blue-producing and not-producing strains. Finally, analytical techniques were applied in order to suggest the molecular structure of the isolated blue pigment.

3.3. Materials and methods

3.3.1. Bacterial strains

The four strains ps_20, ps_40 (non-pigmenting strains), ps_22 and ps_77 (blue-pigmenting strains) chosen in the present study were phenotypically and genotypically characterised in a previous work (Andreani *et al.,* 2014). Ps_20 was isolated from UHT-milk, ps_40 was isolated from ricotta cheese, ps_22 from mozzarella cheese showing the blue discolouration, and ps_77 from a blue slice of beef. The choice of the strains was conducted with the aim of selecting two strains of the blue branch for each phenotype (blue- and no-pigment production) isolated from different food products. Ps_22 and ps_77 produce a dark-blue pigment on Potato Dextrose Agar (PDA; Oxoid Microbiology Products, Thermo Scientific), Minimal Bacterial Medium Agar (MBM Agar; 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% tri-sodium citrate, 0.01% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.2% glucose and 1.5% agar; Boles *et al.,* 2004) and mozzarella cheese. The previous phenotypic characterisation of the two blue-pigmenting strains revealed the production of the same pigment; however, the observation of the pigment at the standardised cut-off (7 days) revealed higher blue pigment production intensity in ps_22. The strains were stored at −80 °C in Tryptic Soy Broth (TSB; Biokar diagnostics, Solabia Diagnostic) with 50% v/v glycerol (Sigma-Aldrich).

3.3.2. Genome sequencing and analysis

A single pure colony of each selected strain was inoculated in 3 mL TSB at 22 °C for 18 h. Genomic DNA was extracted using the commercial kit Invisorb® Spin Tissue Mini Kit (STRATEC Molecular) and it was purified using Amicon Ultra-0.5 mL Centrifugal Filters for DNA and Protein Purification and Concentration (EMD Millipore). DNA quality control was assessed using Qubit® dsDNA HS Assay (Life Technologies) and by 1% w/v agarose gel electrophoresis. Library construction from 1 ng of genomic DNA was performed using Nextera® XT DNA Sample Preparation Kit (Illumina). The quality of the libraries was verified using a 2100 Bioanalyzer (High Sensitivity DNA Assay, Agilent Technologies). Libraries were run on an Illumina MiSeq 300 pair-end (BMR Genomics, Padova, Italy). Raw read sequences

were submitted to the Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra/) with the following accession numbers: SRR1720109; SRR1720183; SRR1720185 and SRR1720188. FASTQ reads were checked for quality using FastQC (Version 0.11.2, Babraham Institute; http://www.bioinformatics.babraham.ac.uk/projects/) and CLCBio GenomicsWorkbench (version 7.5). Trimming of the reads was performed using SolexaQA++ v.3.0 (Cox *et al.,* 2010). De-novo assembly was carried out using Assembl-o-matic (Fondi *et al.,* 2014) with minimum k-value = 20 and maximum k-value = 90. Contig mapping was performed using Contiguator (Galardini *et al.,* 2011) which allowed the mapping of the draft genomes on the reference genome *P. fluorescens* A506 (NC_017911. 1). Finally, the genomes were annotated using Prokka: Prokaryotic Genome Annotation System, which merges Prodigal 2.60, Aragorn and RNAmmer 1.2 to predict open reading frames (ORFs), tRNAs, and rRNAs respectively (Seemann, 2014).

G-C content was obtained using Artemis release 16.0.0 (Carver *et al.,* 2012); the presence of plasmids was investigated using Plasmid finder 1.2 (Carattoli *et al.,* 2014).

The draft genome sequence data are now available in NCBI under the following sequence numbers: LCYA00000000, LCYB00000000, LCYC00000000 and LCYD00000000.

A phylogenomic analysis of the four draft genomes of this study and eighteen complete and draft genomes was performed using Gegenees (Ågren *et al.,* 2012). The list of genomes used for this analysis is reported in Table 3.1.

A preliminary pangenome analysis was performed using the Perl script Parapipe, which uses Multiparanoid and automatically performs paired comparisons within each possible couple of genomes among the ones provided (https://sourceforge.net/projects/parapipe/). Lists of proteins obtained were analysed using Blast2GO® (https://www. blast2go.com/b2ghome; Conesa *et al.,* 2005).

The analysis of the presence of genes involved in indigoidine biosynthesis was conducted using the stand-alone version of Protein-Protein BLAST 2.2.28+ with threshold e-value of 1e-6 (Shiryev *et al.,* 2007). Query was constituted by *indABC* of indigoidine producing species (*D. dadantii*, NC_014500, previously known as *E. chrysanthemi*; *Dickeya zeae*, NC_012912; *P. luminescens*, NC_005126; *Serratia proteamaculans*, NC_009832; *Streptomyces albus*,

Table 3.1 - Complete and draft genomes analysed in the phylogenomic investigation using Gegenees.

Strains	Accession numbers
*P. fluorescens*_A506	NC_017911.1
*P. fluorescens*_BBc6R8	NZ_AKXH00000000.2
*P. fluorescens*_BRIP34879	NZ_AMZW00000000.1
*P. fluorescens*_BS2	NZ_AMZG00000000.1
*P. fluorescens*_CHA0	NC_021237.1
*P. fluorescens*_F113	NC_016830.1
*P. fluorescens*_NCIMB_11764	NZ_CM001560.1
*P. fluorescens*_NZ007	NZ_AKBR00000000.1
*P. fluorescens*_NZ011	NZ_AJXJ00000000.1
*P. fluorescens*_NZ052	NZ_AJXH00000000.1
*P. fluorescens*_NZI7	NZ_AJXF00000000.1
*P. fluorescens*_Pf0_1	NC_007492.2
*P. fluorescens*_Q2_87	NZ_AGBM00000000.1
*P. fluorescens*_Q8r1_96	NZ_AHPO00000000.1
*P. fluorescens*_SBW25	NC_012660.1
*P. fluorescens*_SS101	NZ_CM001513.1
*P. fluorescens*_WH6	NZ_CM001025.1
*P. fluorescens*_Wood1R	NZ_CAFF00000000.1

Table 3.2 - Genes involved in indigoidine biosynthesis used as query for the Blastp investigation of ps_20, ps_22, ps_40 and ps_77 draft genomes.

Gene	Strains	Locus
indA	*Dickeya dadantii* (strain 3937)	Dda3937_00974
	Dickeya zeae	Dd1591_0071
	Photorhabdus luminescens	plu2187
	Serratia proteamaculans	Spro_1699
	Streptomyces albus XNR 5636	XNR_5636
	Streptomyces fulvissimus	SFUL_5937
indB	*Dickeya dadantii* (strain 3937)	Dda3937_00973
	Dickeya zeae	Dd1591_0072
	Photorhabdus luminescens	plu2182
	Serratia proteamaculans	Spro_1697
	Streptomyces albus XNR 5636	XNR_5633
	Streptomyces fulvissimus	SFUL_2478
indC	*Dickeya dadantii* (strain 3937)	Dda3937_00972
	Dickeya zeae	Dd1591_0073
	Photorhabdus luminescens	plu2186
	Serratia proteamaculans	Spro_1698
	Streptomyces albus XNR 5636	XNR_5634
	Streptomyces fulvissimus	SFUL_5936
igiA	*Vogesella indigofera*	AAD54004.1
igiB	*Vogesella indigofera*	AAD54005.1
igiC	*Vogesella indigofera*	AAD54006.1
igiD	*Vogesella indigofera*	AAD54007.1
igiE	*Vogesella indigofera*	AAD54008.1

NC_020990; *Streptomyces fulvissimus*, NC_021177) and *igiABCDE* of *V. indigofera* (AF088856.1). Table 3.2 reports the locus names of the proteins investigated. Blastp was performed using the four draft genomes (ps_20, ps_22, ps_40 and ps_77) as the database.

3.3.3. Assessment of pigment production in MBM broth

Bacterial growth and blue pigment production were monitored in triplicate for the four selected strains in MBM broth (0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% trisodium citrate, 0.01% M_gSO_4, 0.1% $(NH_4)2SO_4$, 0.2% glucose; Boles *et al.,* 2004) in continuous shaking (Mot 1/min = 240; Orbital Shaker Os 5 Basic Yellow Line). MBM broth was chosen because MBM Agar was able to enhance the blue pigment production (Andreani *et al.,* 2014). The growth rate was checked at T_{0h}, T_{24h}, T_{27h}, T_{30h}, T_{32h}, T_{34h}, T_{36h}, T_{48h} and T_{50h} by measuring the Optical Density (O.D.) at 600 nm with a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™) and by verifying real growth plating on CFC Pseudomonas Agar Base (CFC PAB; Oxoid Microbiology Products, Thermo Scientific) at the beginning of growth (T_{0h}), at the moment of pigment production and at the end of the experiment (T_{50h}). Pigment production was revealed optically when the colour of the broth turned from pale yellow (2.5GY9/4 according to Munsell Color System) to a darker shade (2.5PB2/2 according to Munsell Color System). Bacterial count was obtained by incubating the CFC PAB Petri Plates at 22 °C for 48 h in aerobic conditions.

3.3.4. Transcriptome sequencing and analysis

A single pure colony of each strain was inoculated in duplicate (giving 2 biological replicates for each strain) in 3 mL MBM broth and kept at 22 °C for 24 h to reach 10^8 CFU/mL. Then the cultures were diluted 5 times in 40 mL MBM broth and were incubated in continuous shaking (Mot 1/min= 240; Orbital Shaker Os 5 Basic Yellow Line). The growth rate was checked at T_{0h}, T_{10h}, T_{24h}, T_{27h}, T_{29h}, T_{31h}, T_{33h}, T_{34h}, T_{35h}, T_{36h}, T_{37h}, T_{38h}, T_{39h}, T_{40h} and T_{41h} by measuring the O.D. at 600 nm with a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™). When the O.D. reached 0.7, 500 µL of the cultures was transferred into 1 mL of RNAprotect Bacteria Reagent (Qiagen) to stabilize RNA, which was then extracted using the RNeasy mini kit (Qiagen). Library construction was performed from

about 500 ng of RNA using Encore® Complete Prokaryotic RNA-Seq (NuGEN); fragmentation was performed using a Covaris S-Series (duty cycle = 10%; intensity = 5; time = 180 s, intensity/burst = 200). The quality of the libraries was verified using a 2100 Bioanalyzer (RNA 6000 Nano total RNA Kit, Agilent Technologies). Sequencing was performed on HiSeq 2500 50 single-end (UC Davis Genome Center). Raw read sequences were submitted to Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra/) with the following accession numbers: SRR1725678; SRR1725679; SRR1725680; SRR1725681; SRR1725682; SRR1725683; SRR1725684 and SRR1725723.

FASTQ reads were checked for quality using FastQC and CLCBio Genomics Workbench. Mapping of the reads was performed using CLCbio Genomics Workbench using *P. fluorescens* A506 as the reference genome (NC_017911.1).

Preliminary analyses, such as number of reads correctly aligned to *P. fluorescens* A506 and number of reads containing ribosomal RNA, were performed using Rockhopper (default settings: allowed mismatches = 0.15, minimum seed length = 0.33, min reads mapping to a transcript = 20, min transcript length = 50, min count to seed a transcript = 50 and min count to extend a transcript = 5; McClure *et al.,* 2013). Differential expression analysis was performed using the RNAseq tools of CLCBio Genomics Workbench by applying Empirical analysis of DGE (differential gene expression; reference genome = NC_017911.1, minimum length fraction = 0.9, minimum similarity fraction = 0.8, maximum number of hits for a read= 10). Differential expression was considered significant with p-values lower than 0.01 according to the CLCBio Genomics Workbench user manual. Differential expression analysis was performed comparing the transcriptomes of the two blue-pigmenting strains (ps_22 and ps_77; subset “Blue”) and the transcriptomes of the two non-pigmenting strains (ps_20 and ps_40; subset “White”). Finally, the expression profiles of the two blue-pigmenting strains and those of the two non-pigmenting strains (subset “Blue vs White”) were compared, selecting only the genes not differentially expressed (p-value>0.01) in the two previous analyses using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Gene expression of the genes specific for the blue-pigmenting strains (not contained in *P. fluorescens* A506) was evaluated using the draft genome of ps_77 as reference through the RNAseq tools of CLCBio Genomics Workbench as previously described.

3.3.5. Assessment of indole-derivative production test

The indole-derivative production test was carried out using Kovac's reagent (isoamyl alcohol, para-dimethylaminobenzaldehyd in concentrated hydrochloric acid; Sigma-Aldrich). Single pure colonies (n = 4) for each strain analysed (ps_20, ps_22, ps_40 and ps_77) were inoculated in four different tubes containing SIM agar (Sulphur-Indole-Motility agar; Oxoid Microbiology Products, Thermo Scientific) and were incubated at 22 °C; bacterial growth and pigment production were monitored. At 24, 48, 72 and 168 h after the inoculum, a SIM tube for each strain was taken from the incubator and the indole-derivative test was performed, dispensing three drops of Kovac's reagent on the surface of the agar. According to the producer datasheet, positivity of a strain to the test was considered after formation of a cherry-red complex when the reagent was added. Cut-off of measurement was set at 30 s after the addition of Kovac's reagent.

3.3.6. Biochemical characterisation of the blue pigment

Pigmented bacterial cells were grown in MBM broth for 168 h. After growth, cells were pelleted by centrifugation at 14,000 rpm for 30 min. Solubility tests of the pigment were carried out on pelleted cells in the following solvents: H_2O, 1M NaOH, ethanol, chloroform, dimethylsulfoxide (DMSO), trifluoroacetic acid (TFA) and diethyl-ether.

3.3.7. Mass spectrometry

Pigmented bacterial cells were treated with DMSO and subjected to 3 re-suspension cycles. Each cycle comprised 3-hour treatment in an ultrasonic bath (Bransonic, mod.221, 48 kHz, 50 W) and 24-hour end-over-end mixing at room temperature. The resulting extract was analysed by LC-MS/MS using a 6520 Q-TOF mass spectrometer, coupled on-line with a 1200 series HPLC system through a Chip Cube Interface (Agilent Technologies, CA, USA). Each sample (4 µL) was loaded onto a C18 large capacity chip-column, which integrates a 160 nL

capacity trap-column, a RP column (75 µm × 150 mm), connection capillaries, and a nano-spray emitter. Solvent A was water/0.1% formic acid, while solvent B was acetonitrile/0.1% formic acid. Mass spectra were acquired in a data dependent mode: MS/MS spectra were acquired for each MS scan in the range of 350-3000 Da. Scan speed was set to 2 MS spectra s^{-1} and 2 MS/MS spectra s^{-1}. Capillary voltage was set to 1850 V and drying gas to 5 L s^{-1}. Samples were then analysed using a MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems, Canada) operating in Reflex mode with positive polarity. Data were obtained by averaging 2000 laser shots with power set at 5000.

3.4. Results and discussion

3.4.1. Genome sequencing and analysis

A total of 11,812,288 base pairs were generated using Illumina MiSeq 300 pair-end (BMR Genomics, Padova, Italy). The quality of the reads was high, as almost 85% of the reads of each genome showed a PHRED value higher than 30, which corresponds to a very high call accuracy. Reads that showed suboptimal quality were trimmed, substantially reducing their length. Coverage of the completely sequenced genome of *P. fluorescens* A506 was adequate for the subsequent analyses (Kisand and Lettieri, 2013), with a minimum value of coverage of 49-fold (ps_22) and a maximum value of 224-fold (ps_20). Assembly was performed using the software Assembl-o-matic, using different k-values and identifying a best k-value of 44 for ps_22 and of 80 for ps_20, ps_40 and ps_77. Genome size, estimated using Contiguator, varies from 5.1 to 6.3 Mb, in line with genomes of other *P. fluorescens* strains (http://www.pseudomonas.com/). Annotation with Prokka allowed the identification of a variable number of Coding DNA Sequences (CDSs) ranging from 5,281 of ps_20 to 6,459 of ps_40.G-C content ranged from 58.3 to 60.12%. No plasmids were identified using Plasmid finder 1.2.

Draft genome results are summarized in Table 3.3.

Table 3.3 - Summary of genomic data obtained through the analysis of the draft genomes of ps_20, ps_22, ps_40 and ps_77.

strain	number of reads	length (bp)	% of reads with PHRED values ≥30	coverage of *Pseudomonas fluorescens* A 506	Best k-value for the assembly	estimated genome size	% G-C content	CDSs
Ps_20	4,444,758	35-301	88%	224 x	80	5.9 Mb	60.12	5,281
Ps_22	975,544	35-301	85%	49 x	44	5.1 Mb	58.3	6,370
Ps_40	3,613,102	35-301	85%	182 x	80	6.3 Mb	59.23	6,459
Ps_77	2,778,884	35-301	85%	140 x	80	6.1 Mb	59.66	5,523

The phylogenomic analysis (at the row nucleotide level) is reported in Figure 3.1. The analysis confirmed the genomic relatedness of ps_20, ps_22, ps_40 and ps_77 and, in turn, their proximity to *P. fluorescens* A 506 (for which the complete genome sequence is available), as already reported in a previous study (Andreani *et al.,* 2014) and *P. fluorescens* SS101, a root colonizing strain (van de Mortel *et al.,* 2012).

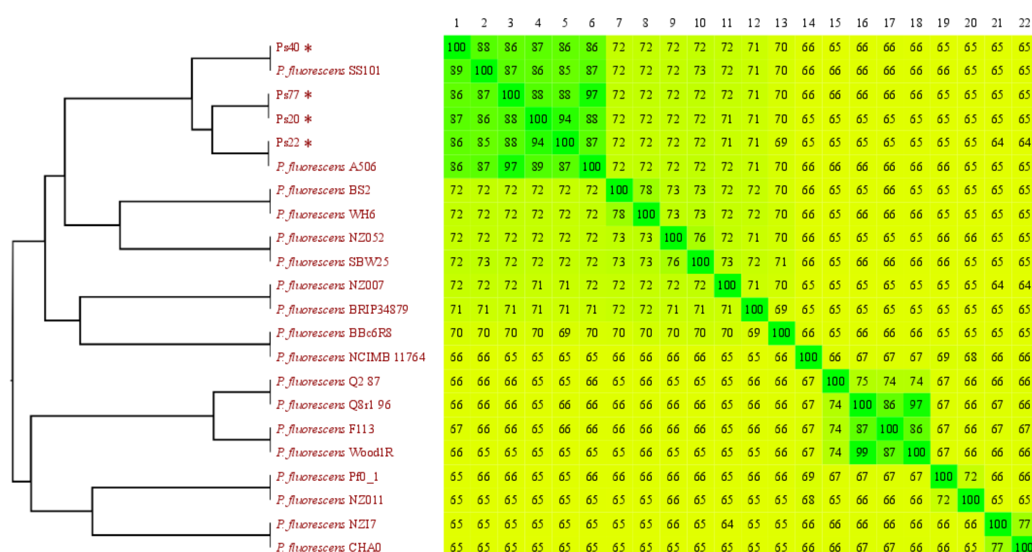


Figure 3.1 - Phylogenomic analysis.

Phylogenomic analysis of the strains analysed in this work (marked with "") and eighteen complete and draft genomes performed using Gegenees (Ågren et al., 2012). On the left, the dendrogram reporting the proximity of the strain is reported. On the right, a heat map reporting similarity values based on the score computed after an all vs. all fragmented BLAST of the strains analysed (the score of each comparison is computed as the average score of all fragments, see Ågren et al., 2012).*

The computed pangenome showed the presence of a core genome represented by 4,065 CDSs. 114 CDSs were accessory genes shared by ps_20 and ps_40 (the non-pigmenting strains), 194 genes were present only in ps_22 and ps_70 (the blue-pigmenting strains),

while 250, 1,526, 1,308 and 391 genes were unique respectively for ps_20, ps_22, ps_40 and ps_77. A summary of pangenome analysis is reported in Figure 3.2.

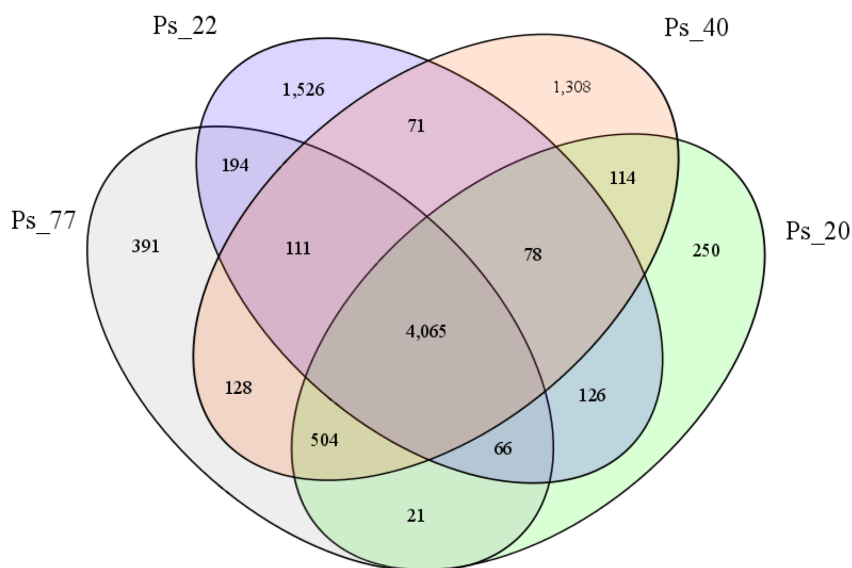


Figure 3.2 - Pangenome analysis.

Euler-Venn chart of computed Pangenome analysis using Parapipe. Each strain is represented by an oval. The number of orthologous coding sequences (CDSs) shared by all strains is reported in the center of the image. Overlapping regions show the number of CDSs conserved only within the specified genomes. Numbers in non-overlapping portions of each oval show the number of CDSs unique to each strain.

The preliminary analysis of the genomic results showed some macroscopic differences between the two phenotypes considered. The 114 genes shared by the non-pigmenting strains and the 194 genes exclusive to the blue-pigmenting strains were classified in 18 COG (Cluster of Orthologous Group) Functional Categories, showing different gene balance between the two phenotypes. The relative abundance of each COG functional category is reported in Table 3.4.

Blastp analysis showed that approximately 50% of the blue pigmenting strains' unique genes are located in the genomic regions that are represented by two contigs of the ps_77 draft genome (contig_0004 and contig_0057 of 1,907,245 and 214,980 bp, respectively).

Contig_0004 is particularly interesting because it contains five clusters that are unique to ps_22 and ps_77 interspersed within clusters of nonexclusive genes. For the sake of simplicity the aforementioned contigs, clusters and genes are reported with the nomenclature referring to ps_77 only. A graphical representation of contig_0004 is reported in Figure 3.3. The major gene cluster with blue-strains exclusive genes is made of loci between

PFLuk1_00663 and PFLuk1_00678 (a total of 16,160 base-pairs) and contains accessory genes coding for enzymes involved in tryptophan biosynthesis: *trpD* (PFLuk1_00664; Anthranilate phosphoribosyltransferase), *trpF* (PFLuk1_00665; N-(5′-phosphoribosyl) anthranilate isomerase), *trpA* (PFLuk1_00666; Tryptophan synthase alpha chain) and *trpC* (PFLuk1_00676; Indole-3-glycerol phosphate synthase). A more in depth analysis of these genes will be given in Section 3.4.4. of the present work.

Table 3.4 - Relative abundance of COG.

Relative abundance of COG (Cluster of Orthologous Group) Functional Categories of genes unique to blue-pigmenting and non-pigmenting strains after Pangenome computation.

COG Functional Category Description	Blue-pigmenting strains (% of 194 genes)	Non-pigmenting strains (% of 114 genes)
Amino acid transport and metabolism	8.7628866	1.754386
Carbohydrate transport and metabolism	2.57731959	1.754386
Cell cycle control, cell division, chromosome partitioning	0.51546392	-
Cell motility	-	3.508772
Cell wall/membrane/envelope biogenesis	3.09278351	4.385965
Coenzyme transport and metabolism	0.51546392	-
Defence mechanisms	0.51546392	-
Energy production and conversion	2.57731959	-
Inorganic ion transport and metabolism	1.54639175	-
Lipid transport and metabolism	-	1.754386
Posttranslational modification, protein turnover, chaperones	3.60824742	0.877193
Replication, recombination and repair	4.63917526	10.52632
Secondary metabolites biosynthesis, transport and catabolism	-	0.877193
Signal transduction mechanisms	2.06185567	4.385965
Transcription	4.63917526	3.508772
Translation, ribosomal structure and biogenesis	-	0.877193
General function prediction only	4.63917526	-
Other	60.3092784	65.78947

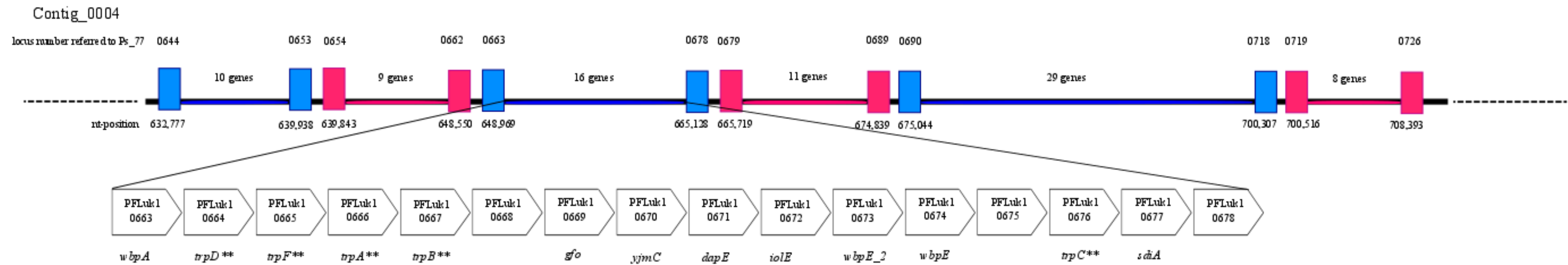


Figure 3.3 - Graphical representation of the 84,616 bp gene clusters in contig_0004 of ps_77 draft genome.

*The cluster of contig_0004 of ps_77 containing accessory trp genes (16,159 bp) is showed in detail. Accessory trp genes are marked with ** symbol. Extremity gene numbers and nt-position for each clusters are reported. The gene clusters unique to the blue-pigmenting strains are highlighted in blue. The clusters shared among pigmenting and non-pigmenting strains are reported in pink.*

The other gene clusters with blue strains exclusive genes (genes located between PFLuk1_00644 and PFLuk1_00653, between PFLuk1_00690 and PFLuk1_00718, between PFLuk1_01176 and PFLuk1_01183 and PFLuk1_01185 and PFLuk1_01195) showed high prevalence of loci annotated as "Hypothetical protein", which hampered a clear understanding of their actual biological function.

The second contig containing blue exclusive genes, contig_0057, enclosed 381 genes, among which two clusters of great interest were identified: the first one is located between PFLuk1_4285 and PFLuk1_4308 and contains genes involved in xenobiotics biodegradation and metabolism; the second cluster is located between PFLuk1_4309 and PFLuk1_4323 and contains several genes involved in mercuric resistance. A Blastn analysis of these genes showed high sequence similarity (99%) with genomic regions contained in *P. aeruginosa* PA7 (CP000744), *P. pseudoalcaligenes* Pseudo_PAC (LK391695) and *P. pseudoalcaligenes* CECT5344 (HG916826). This result, together with the fact this cluster is flanked by transposon sites, suggests the nature of a genomic island, probably involved in survival in polluted environments. Several *Pseudomonas* species are able to survive in these environments by metabolizing industrial waste such as polycyclic aromatic hydrocarbons, pesticides and heavy metals. For this reason, several *Pseudomonas* strains have been employed in bioremediation processes (Wasi *et al.,* 2013).

A previous study suggested that the blue pigment involved in the blue mozzarella events could be the indigoidine molecule (Caputo *et al.,* 2015). For this reason, a Blastp search was conducted on the four strain genomes in order to check the presence of genes encoding for indigoidine biosynthetic pathways. This search failed to discover the indigoidine orthologous genes either in blue-pigmenting or in non-pigmenting strains. These results led to the conclusion that the dark pigment observed is not indigoidine or that the genes involved in the biosynthetic pathway are not the same as those identified in other indigoidine-producing strains. This finding corroborates the hypothesis according to which the blue pigment observed and described by Caputo and colleagues is substantially different from the blue pigment object of the present study. Caputo and colleagues identified strains belonging to the *nomen* species *Pseudomonas lemonnieri* (now renamed *P. fluorescens* biovar IV) as

producers of the blue pigment. However, a previous paper has already demonstrated the blue pigment produced by *P. lemonnieri* is completely different from the one produced by the *P. fluorescens* strains isolated from the blue spoiled food (Andreani *et al.,* 2014). In particular, the reference strain of *P. lemonnieri* (DSM 50415) produces a non-diffusible green-blue pigment (10GY3/8 according to Munsell Color System) on PDA, while no pigment is visible on MBM agar or on mozzarella cheese, which is in contrast to the blue-pigment producing strains under investigation here.

3.4.2. Growth curves and pigment production

No significant differences were noticed between the growth rates of the ps_20, ps_22, ps_40 and ps_77 cultures in MBM broth in the same conditions of incubation (data not shown).

As expected, only ps_22 and ps_77 became darker because of the production of the blue pigment during bacterial growth. After the pigment production, the whole broth took on a darker shade (2.5PB2/2 according to Munsell Color System); the pigment was particularly visible in brown corpuscles on the surface of the broth exposed to air, when the O.D. reached 0.7 at 600 nm, corresponding to about 7×10^8 CFU/mL. As already shown on the agar plates, in liquid medium the pigment seemed to be diffusible, as all the liquid turned from pale yellow to brown. It was evident that the blue pigment production took place in the late logarithmic phase. Based on the present and previously published data (Andreani *et al.,* 2014), MBM broth was selected to study gene expression related to the blue pigment production.

3.4.3. Transcriptome sequencing and analysis

Cells reached an O.D. of 0.7 at 600 nm between 35 and 41 h, showing similar growth rates overall. At the moment of the O.D. measurement, the cultures were in the late logarithmic phase and none of them reached the stationary phase. For this reason each culture can be considered metabolically active.

A total of 127,562,641 base pairs were generated using Illumina 2500 50 single-end (UC Davis Genome Center). The quality of the reads was high, as almost 98% of the reads of each transcriptome had a PHRED value higher than 30. A preliminary evaluation conducted using

the Rockhopper software highlighted the number of reads that correctly mapped to *P. fluorescens* A506, the percentage of rRNA containing reads and the total number of reads used for the RNAseq analysis (Table 3.5).

Table 3.5 - Summary of transcriptomic data.

Summary of transcriptomic data obtained through the analysis of the transcriptomes of ps_20, ps_22, ps_40 and ps_77 in duplicate.

library name	strains	total number of reads	number of correctly aligned reads using Rockhopper	% of correctly aligned reads using Rockhopper	% of rRNA containing reads using Rockhopper	total number of non-rRNA reads	% of reads with PHRED values ≥30
N1	Ps_77.1	17,118,677	10,3385,071	78%	48	6,960,237	98%
N2	Ps_22.1	17,312,408	15,394,084	89%	68	4,926,107	98%
N3	Ps_40.1	14,870,876	9,714,785	65%	65	3,400,175	98%
N4	Ps_20.1	14,684,311	9,139,522	62%	66	3,107,437	99%
N5	Ps_77.2	17,209,997	14,812,204	86%	60	5,924,882	98%
N6	Ps_22.2	16,284,204	9,745,266	60%	67	3,215,938	99%
N7	Ps_40.2	15,276,683	13,074,737	86%	66	4,445,411	99%
N8	Ps_20.2	14,805,485	10,385,957	70%	70	3,115,787	98%
Total		127,562,641				35,095,973	

P. fluorescens A506 was chosen as the reference due to the phylogenetic proximity of its completely sequenced genome to the genetic cluster containing all the blue pigmenting strains (Andreani *et al.,* 2014). Empirical analysis of differential gene expression (DGE) allowed the identification of genes differentially expressed in the 3 experiments (subset "Blue", subset "White" and subset "Blue vs White"). 1,069 genes were differentially expressed between ps_22 and ps_77 (subset "Blue"), 1,238 between ps_20 and ps_40 (subset "White"), while 467 where differentially expressed comparing ps_22 and ps_77 against ps_20 and ps_40 (subset "Blue vs White"). To identify differentially expressed genes in the subset "Blue vs White", an Euler-Venn diagram was created using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). These results are summarized in Figure 3.4.

Subsequently, the investigation focused on the analysis of the 85 genes differentially expressed between the blue- and non-pigmenting phenotypes. The study of their biological roles allowed the identification of seven major pathways characterised by differential regulation between the two phenotypes investigated.

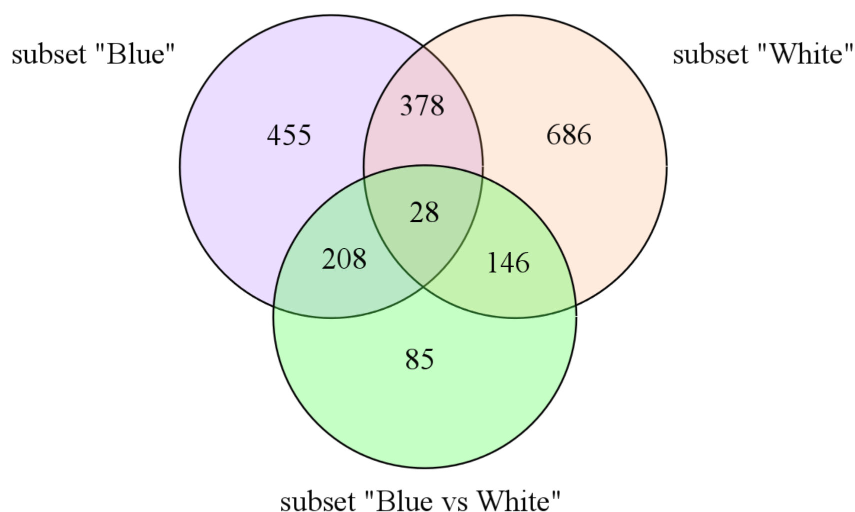


Figure 3.4 - Differential gene expression analysis.

Euler-Venn chart of differentially expressed genes in subset "Blue", subset "White" and subset "Blue vs White". Each subset is represented by a circle in which differentially expressed gene number is reported.

Among the 85 genes, 34 were significantly up-regulated and 51 were significantly down-regulated in the blue-pigmenting strains in comparison with the non-pigmenting strains. Expression results are reported in Table 3.7 (reported at the end of Chapter 3). The up-regulated genes included five genes involved in iron uptake, six genes coding for oxidoreductases, five genes coding for transcriptional regulators and three genes involved in drug resistance. The down-regulated genes included four transcriptional regulators, eight genes involved in amino acid biosynthesis, four genes coding for proteins involved in respiratory chain and three ABC transporters.

Up-regulation of genes involved in iron uptake

Genes involved in the uptake of iron (*efeO* and *efeU*) and in the biosynthesis and uptake of the siderophore pyoverdine (*pvdO*, *pvdI* and *pvdF*) were between 2.2 and 5.6-fold more expressed in the blue-pigmenting strains. The increased co-expression of *efeO* and *efeU* genes reveals a higher iron depletion occurring in the blue-pigmenting strains in comparison to the non-pigmenting ones (Lim *et al.,* 2012). Additionally, the siderophore pyoverdine is a water-soluble yellow fluorescent pigment produced by several *Pseudomonas* species which confers the characteristic fluorescence to many *P. fluorescens* (Cornelis, 2010). Its major function is iron uptake and the fine regulation of gene expression ensures its production only

in states of low iron concentration. The genomes of both blue and non-pigmenting strains carry the genes involved in the biosynthesis of pyoverdine, but only the blue-pigmenting showed the ability to express them and synthesize the yellow pigment to improve iron content.

It is not clear whether the major iron requirement is due to the necessity to produce the blue pigmented molecule or whether the blue pigment produced by the strains could itself be helpful in iron uptake.

Up-regulation of genes encoding for oxidoreductases

Six genes coding for oxidoreductase appear to be up-regulated in the blue-pigmenting strains. This class of enzymes comprises oxygenases (monooxygenases and dioxygenases), and in particular the superoxide dismutase *sodA1*, which is involved in stress resistance and is upregulated also in iron depletion conditions (Lim *et al.,* 2012).

Down-regulation of genes involved in primary metabolism

Genes involved in amino acid biosynthesis (*ilvB*, *asnB*, *pheT*, *aroK*, *nspC*, PflA506_1916, PflA506_4051, PflA506_0784) and cell replication (*recF*, *mdoH*, *fstQ* and *ftsK*) were significantly down-regulated in the blue-pigmenting strains. This means that these strains require a lower primary metabolism activity, leading to a major investment in alternative pathways, as reported for *P. fluorescens* in a state of iron scarcity (Lim *et al.,* 2012).

Down-regulation of genes of the respiratory chains

In aerobic metabolism, the respiratory chain typically uses proteins that require iron cofactors. Down-regulation of genes coding for proteins of the respiratory chain is consistent with the evident status of iron depletion and the up-regulation of genes involved in iron uptake observed in the blue-pigmenting strains (Lim *et al.,* 2012).

The transcriptomic analysis showed the importance of iron metabolism for the production of the blue pigment. However, the link between the blue phenotype and iron depletion remains unclear. No other important differences were observed between blue-pigmenting strains and non-pigmenting strains, which led to the hypothesis that the blue pigment production might

also depend on genes present exclusively in the blue-pigmenting strain genomes, whose differential expression cannot be investigated using *P. fluorescens* A506 as the reference genome.

3.4.4. Analysis of *trp* cluster as putatively involved in the blue pigment biosynthesis

A more in depth analysis of the pangenome showed the presence of a *trp* gene cluster in the core genome (that is composed of genes present in all the four strains). However, additional *trp* genes were present only in the genome of the two blue-pigmenting strains, and in particular in contig_0004 of ps_77. Figure 3.5 shows a schematic representation of the *trp* genes both in the core and in the accessory genomes of the *P. fluorescens* strains analysed in the present study.

The *trp* accessory genes in the two blue-pigmenting strains (ps_22 and ps_77) are identical to each other (100% in nucleotide sequence). The absence of accessory *trpB* (PFLuk1_00667; tryptophan synthase beta chain) in the ps_22 draft genome was caused by the lower coverage in comparison with ps_77 as demonstrated by PCR amplification (data shown in Appendix to Chapter 3). The protein alignment of each core *trp* gene with the corresponding *P. fluorescens* A506 gene showed an identity value higher than 96%. Conversely, the alignment of the two copies of each *trp* gene (core copy with the corresponding accessory copy) showed a low amino acid identity (between 30 and 50.74%). No significant ortholog of the accessory *trp* genes was identified through a Blastp search against the SwissProt database. Preliminary results obtained by PCR amplification demonstrated that these accessory *trp* genes are shared by all the blue-pigmenting strains but not by the non-pigmenting strains contained in the blue branch (data shown in paragraph 3.9.1.). Preliminary results revealed that these paralogous *trp* gene copies might have been acquired by means of horizontal gene transfer (HGT).

Indeed, in a Maximum Likelihood tree, they were shown to form well supported separated (monophyletic) clusters in respect to their “core” counterparts (and also in respect to other *Pseudomonas*-like *trp* genes; data shown in paragraph 3.9.2.). Further analyses will be

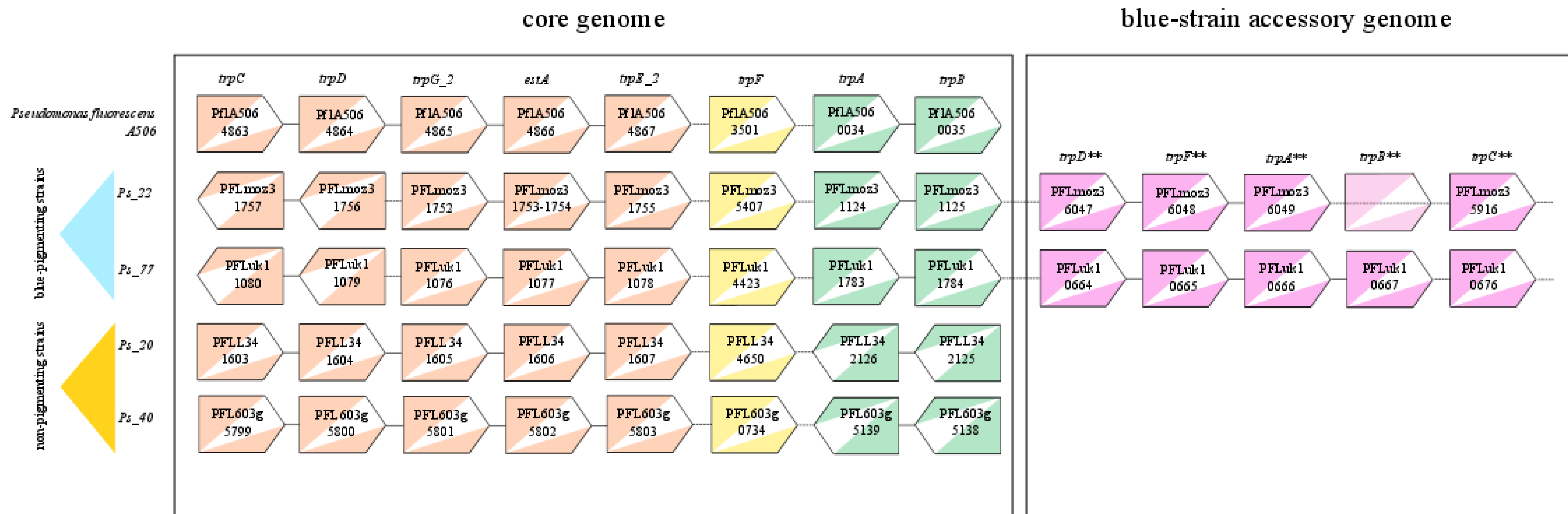


Figure 3.5 - Comparative representation of trp gene cluster.

*Graphical representation of trp gene-containing cluster of P. fluorescens A506, of the blue-pigmenting strains (ps_22 and ps_77) and of the non-pigmenting strains (ps_20 and ps_40). Core genes are reported on the left; the accessory genes unique to the blue-pigmenting strains are shown on the right and marked with ** symbol. Annotated CDS number is reported for each strain.*

necessary to infer the most likely source of this putative HGT event.

Tryptophan is an aromatic amino acid whose synthesis is biochemically the most expensive in bacteria (Bentley, 1990). However, in several bacteria, tryptophan is not only a protein constituent as several bacterial species use the tryptophan biosynthetic pathway for other purposes such as production of quinolone (in *P. aeruginosa*; Xie *et al.,* 2003), indole-3-acetic acid (in *Azospirillum brasilense*; De Troch *et al.,* 1997) and violacein (in *Chromobacterium violaceum*; Antônio and Creczynski-Pasa, 2004).

Because of the absence of accessory *trp* genes in *P. fluorescens* A506, the annotated contig of ps_77 containing both core and accessory copies of *trp* genes (except *trpF*) was used as the reference for the setting up of the RNAseq data analysis. Interestingly, the genes identified as exclusively present in blue-pigmenting strains were highly expressed, especially the *trp* accessory cluster, whose expression was higher than the respective genes present in the core genome (Table 3.6).

Table 3.6 - Read count of core genome and accessory trp genes in blue-pigmenting strains.

Gene	Ps_22		Ps_77	
	Core genome gene	Accessory gene**	Core genome gene	Accessory gene**
trpA	260	11,209	615	15,760
trpB	706	14,289	882	18,410
trpC	270	5,543	785	2,372
trpD	364	12,026	558	20,810
trpF[a]	-	923	-	943

[a]*trpF* core genome gene is not present in contig_0004.

These results suggest the possible involvement of the accessory *trp* genes in the blue pigment production. The knocking down of these genes in the blue-pigmenting strains could be used as a strategy to effectively test their role in synthesizing the pigment.

3.4.5. Biochemical characterisation of the blue pigment

Tryptophan is also involved in indigo biosynthesis (Figure 3.6). Indigo is a dark blue dye, widely used in the textile industry. It was initially extracted from plants of the genus

Indigofera (Adeyanju *et al.,* 2011), but the natural extraction was first replaced by chemical synthesis and more recently by biosynthesis using genetically modified microbes (Ensley *et al.,* 1983; Murdock *et al.,* 1993; Pathak and Madamwar, 2010). Indigo production takes place via indole oxidation. Various enzymes belonging to monoxygenase and dioxygenase classes are capable of indole oxidation to form the unpigmented parental compound of indigo (cisindole-2,3-dihydrodiol; Ensley *et al.,* 1983; O'Connor *et al.,* 1997; O'Connor and Hartmans, 1998; Berry *et al.,* 2002). Several *Pseudomonas* species were reported as synthesizing such enzymes (Eaton and Chapman, 1995; Pathak and Madamwar, 2010; Ma *et al.,* 2012), but so far none of them has been shown to synthesize indole. The analysis of the draft genomes of the four *P. fluorescens* strains showed the absence of the tryptophanase gene (*tnaA*) that encodes for the enzyme responsible of the conversion of tryptophan to indole in indole-producing bacteria. To test the hypothesis that the blue pigment could be indigo, the four strains were tested for indole production, as it is widely accepted that this molecule is the biosynthetic precursor of indigo and an increased tryptophan production could be related to the synthesis of blue pigments through an indole intermediate. A positive result to the Kovac's indole test is typically assessed by observing the formation of a purple compound (7.5R5/18 according to Munsell Color System). The same reagent can produce different coloured substances with different indole derivatives (Ehmann, 1977).

In the present case, the four *P. fluorescens* strains grew similarly in the SIM agar; the straw-yellow colour of the agar inoculated with the blue pigmenting strains started to darken after 24 h, becoming progressively browner, as shown in Figure 3.7. The addition of Kovac's reagent generated no positive reaction at 24 h, while a green-blue halo (2.5B2/4 according to Munsell Color System) was visible in the blue-pigmenting strains at 48 and 72 h. Only pigmented colonies led to the chromogenic reaction in the presence of Kovac's reagent, indicating that the formation of the pigment is unequivocally correlated to the biosynthesis of indole or an indole-derivative. The formation of a green-blue colour demonstrated that an indole-derivative was produced and not indole. It should be noted that the reaction occurred in the same time window as pigment production.

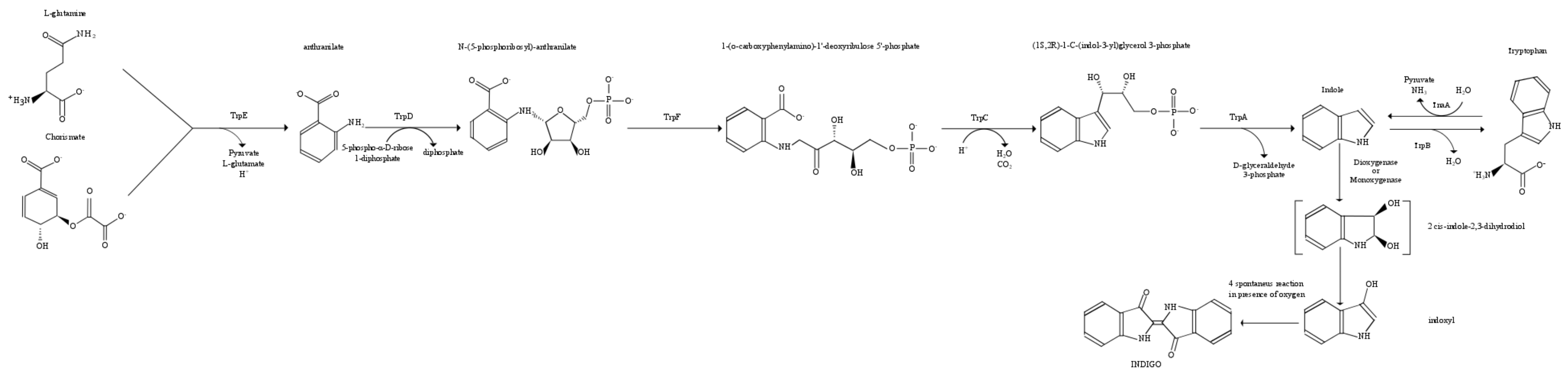


Figure 3.6 - Indigo biosynthetic pathway.

Indigo biosynthetic pathway and role of trp gene-encoding enzymes in the production of indigo-dye in recombinant Escherichia coli showing both indole production and indole-oxidative enzymes. Adapted from Berry et al., 2002.

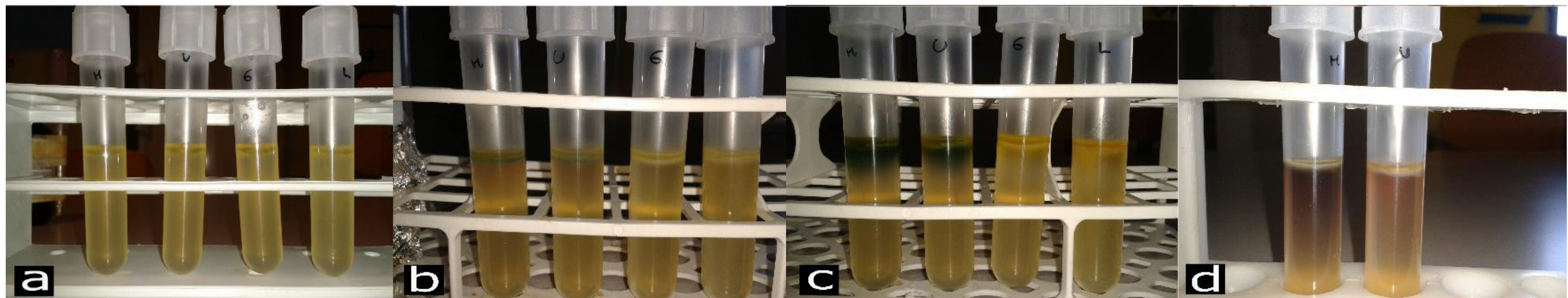


Figure 3.7 - Indole production test.

Reactions observed during indole-test; no positive reaction was visible after 24 hours of growth in SIM agar (a). A green-blue halo was visible in the blue-pigmenting strains at 48 hours (b) and 72 hours (c). No reaction was visible after 168 hours (d).

Interestingly, when the production of the blue pigment by bacterial cells ended, the chromogenic reaction by the Kovac's reagent did not take place anymore, suggesting that the indole-derivative is not present anymore. This indicates that the completion of the blue pigment formation reaction coincides with the depletion of an indole-derivative, evidencing its role as precursor of the pigment in the biosynthetic route. Therefore, as pigmenting strains showed the presence of a second copy of the genes involved in the tryptophan pathway, the activity of this biosynthetic route for the formation of an indole-derivative, which could produce a blue-coloured indigo-derivative, is plausible.

The coloured material was physically separated from the bacterial culture broth and solubility tests were performed in order to characterise the chemical behaviour of the pigment. The pigmented material proved to be insoluble in water, 1 M NaOH, ethanol, methanol, acetonitrile and DMSO. In contrast indigo (Sigma 229296), indigoidine and leuco-indigoidine are freely soluble in water and DMSO (Cude *et al.,* 2012; Kurniawan *et al.,* 2014). The different chemical behaviours can be explained by the presence of organic substituents on the indigo molecule, which are possibly responsible of its hydrophobicity, as is the case with the indigoids studied by Starr *et al.,* (1967), which were soluble in pure TFA as well.

The extract derived from the treatment of the coloured material with DMSO was characterised by LC-MS/MS using a 6520 Q-TOF mass spectrometer as described in the Materials and methods section (paragraph 3.3.7.). In contrast with Caputo *et al.,* (2015), this study did not find any trace of indigoidine or leuco-indigoidine. The pigmented bacterial pellet was only partially soluble in concentrated TFA.

MALDI-TOF mass spectrometry was used to characterise the pigment. As a control, the molar mass of commercial indigo was determined according to the protocol by Sanz *et al.,* (2012), with a result of 263.08 g/mol, which is in agreement with the reported value (262.27 g/mol). MALDI-TOF Mass spectrometry analysis identified a compound with a molar mass of 434.17 g/mol. This result does not agree with the findings by Caputo *et al.,* (2015); the considerably higher value of the molecule confirms the previous hypothesis that it is plausible that an indigo-derivative is formed by coloured *P. fluorescens* strains. Recently, a study reported the involvement of indigo-derivative molecules as modulators in bacterial

signalling pathways (Biswas *et al.,* 2015). This result suggests a potential function for the blue pigment. Additional experiments are required to evaluate the role of the blue pigment acting in cell-cell interactions or as an antimicrobial agent.

Although the chemical formula of the blue pigment is still unknown, these results offer a key for completing the biosynthetic pathway leading to pigment formation from tryptophan.

At the moment it can be concluded that the blue pigment produced by coloured *P. fluorescens* strains is not indigo nor indigoidine, but a heavier molecule, and specifically, an indigo-derivative.

3.5. Conclusions

The spoilage potential of *P. fluorescens* and, in particular, its ability to produce a blue pigment on several food matrices can cause serious economic losses in the food industry. In the present study, the production of the blue pigment was investigated through genomic and transcriptomic techniques. The presence in the blue-pigmenting strains of double copies of *trp* genes indicates the potential involvement of these genes in the blue pigment production. This result is of great interest for the prevention of food discolouration spoilage. In fact, the unique presence of these genes in the pigmenting strains can be useful in designing Real-Time PCR assays to reveal the presence of blue producing strains in different food matrices. The transcriptomic analysis revealed a high state of iron depletion in the blue-pigmenting strains that may be better understood on completion of the pigment chemical structure identification. A hypothesis could be that iron is a co-factor for specific enzymes involved in the pigment production. Again, the *trp* gene cluster has been shown to be potentially related to the blue pigment production, resulting in its being highly expressed only in the blue pigmenting strains.

The results from the chromogenic test, solubility characterisation and mass spectrometry analysis suggest that the blue pigment is a hydrophobic indigo-like molecule. Further chemical analyses of the pigment are still in progress in order to unequivocally identify its molecular structure. In conclusion, the coupling of NGS techniques with biochemical analysis

allowed the narrowing down of the range of the genes potentially involved in the production of the blue pigment by some *P. fluorescens* strains. Tryptophan biosynthesis and iron metabolism seem to be exclusively related to the blue discolouration. In addition, this work demonstrated that indigoidine cannot be the blue pigment, which is more likely to be an indigo-derivative molecule. Further analyses, such as site-directed mutagenesis, coupled with a deeper biochemical characterisation are now needed to investigate these hypotheses.

3.6. Acknowledgments

The study was supported by the University of Padova (Progetto di Ricerca di Ateneo 2011 to L.F.; Grant number CPDA115333) and by the PhD School of Veterinary Science of the University of Padova which has supported the education of N.A.A.. The authors are grateful to the Istituto Zooprofilattico delle Venezie, to Dr. M. Marino and M. Maifreni (Department of Food Sciences, University of Udine), and to Dr. Janet Corry (School of Veterinary Science, University of Bristol) for providing *Pseudomonas* strains.

3.7. References

- Adeyanju, O., Emmanuel, S.E., Akomolafe, S.F., 2011. Extraction of indigo dye (powdered, form) from the leaf of *Indigofera tinctoria*. International Journal of Physical Sciences, 6, 137-143.
- Ågren, J., Sundström, A., Håfström, T., Segerman, B., 2012. Gegenees: fragmented alignment of multiple genomes for determining phylogenomic distances and genetic signatures unique for specified target groups. PLoS ONE, 7, e39107.
- Andreani, N.A., Martino, M.E., Fasolato, L., Carraro, L., Montemurro, F., Mioni, R., Bordin, P., Cardazzo, B., 2014. Tracking the blue: a MLST approach to characterise the *Pseudomonas fluorescens* group. Food Microbiology, 39, 116-126.
- Andrews-Polymenis, H.L., Santiviago, C.A., McClelland, M., 2009. Novel genetic tools for studying food borne *Salmonella*. Current opinion in Biotechnology, 20, 149-157.

- Antônio, R.V., Creczynski-Pasa, T.B., 2004. Genetic analysis of violacein biosynthesis by *Chromobacterium violaceum*. Genetics and molecular research: GMR, 3, 85-91.
- Anzai, Y., Kim, H., Park, J.-Y., Wakabayashi, H., Oyazu, H., 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequences. International Journal of Systematic and Evolutionary Microbiology, 50, 1563-1589.
- Arnaut-Rollier, I., Vauterin, L., De Vos, P., Massart, D.L., Devriese, L.A., De Zutter, L., Van Hoof, J., 1999. A numerical taxonomic study of the *Pseudomonas* flora isolated from poultry meat. Journal of Applied Microbiology, 87, 15-28.
- Bartel, P.L., Zhu, C.B., Lampel, J.S., Dosch, D.C., Connors, N.C., Strohl, W.R., Beale, J.M., Floss, H.G., 1990. Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in Streptomycetes: clarification of actinorhodin gene functions. Journal of Bacteriology, 172, 4816-4826.
- Bentley, R., 1990. The shikimate pathway-A metabolic tree with many branches. CRC Critical Reviews in Biochemistry and Molecular Biology, 25, 307-384.
- Berry, A., Dodge, T.C., Pepsin, M., Weyler, W., 2002. Application of metabolic engineering to improve both the production and use of biotech indigo. Journal of Industrial Microbiology & Biotechnology, 28, 127-133.
- Biswas, N.N., Kutty, S.K., Barraud, N., Iskander, G.M., Griffith, R., Rice, S.A., Willcox, M., Black, D.S., Kumar, N., 2015. Indole-based novel small molecules for the modulation of bacterial signalling pathways. Organic and Biomolecular Chemistry, 13, 925-937.
- Boles, B.R., Thoendel, M., Singh, P.K., 2004. Self-generated diversity produces “insurance effects” in biofilm communities. Proceedings of National Academy of Science of the United States of America, 101, 16630-16635.
- Brachmann, A.O., Kirchner, F., Kegler, C., Kinski, S.C., Schmitt, I., Bode, H.B., 2012. Triggering the production of the cryptic blue pigment indigoidine from *Photorhabdus luminescens*. Journal of Biotechnology, 157, 96-99.
- Caldera, L., Franzetti, L., 2014. Effect of storage temperature on the microbial composition of ready-to-use vegetables. Current Microbiology, 68, 133-139.

- Caputo, L., Quintieri, L., Bianchi, D.M., Decastelli, L., Monaci, L., Visconti, A., Baruzzi, F., 2015. Pepsin-digested bovine lactoferrin prevents Mozzarella cheese blue discoloration caused by *Pseudomonas fluorescens*. Food Microbiology, 46, 15-24.
- Carattoli, A., Zankari, E., Garcia-Fernandez, A., Volby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., Hasman, H., 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrobial Agents and Chemotherapy, 58, 3895-3903.
- Carver, T., Harris, S.R., Berriman, M., Parkhill, J., McQuillan, J.A., 2012. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. Bioinformatics, 28, 464-469.
- Chu, M.K., Lin, L.F., Twu, C.S., Lin, R.H., Lin, Y.C., Hsu, S.T., Tzeng, K.C., Huang, H.C., 2010. Unique features of *Erwinia chrysanthemi* (*Dickeya dadantii*) RA3B genes involved in the blue indigoidine production. Microbiological Research, 165, 483-495.
- Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2go: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 21, 3674-3676.
- Cornelis, P., 2010. Iron uptake and metabolism in pseudomonads. Applied Microbiology and Biotechnology, 86, 1637-1645.
- Cox, M.P., Peterson, D.A., Biggs, P.J., 2010. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinformatics, 11, 485.
- Cude, W.N., Mooney, J., Tavanaei, A.A., Hadden, M.K., Frank, A.M., Gulvik, C.A., May, A.L., Buchan, A., 2012. Production of the antimicrobial secondary metabolite indigoidine contributes to competitive surface colonization by the marine roseobacter *Phaeobacter* sp. strain Y4I. Applied and Environmental Microbiology, 78, 4771-4780.
- De Troch, P., Dosselaere, F., Keijers, V., de Wilde, P., Vanderleyden, J., 1997. Isolation and characterization of the *Azospirillum brasilense trpE(G)* gene, encoding anthranilate synthase. Current Microbiology, 34, 27-32.

- Decimo, M., Morandi, S., Silvetti, T., Brasca, M., 2014. Characterization of Gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. Journal of Food Science, 79, M2081-M2090.
- Diaz-Sanchez, S., Hanning, I., Pendleton, S., D'Souza, D., 2012. Next-generation sequencing: the future of molecular genetics in poultry production and food safety. Poultry Science, 92, 562-572.
- Eaton, R.W., Chapman, P.J., 1995. Formation of indigo and related compounds from indolecarboxylic acids by aromatic acid-degrading bacteria: chromogenic reactions for cloning genes encoding dioxygenases that act on aromatic acids. Journal of Bacteriology, 177, 6983-6988.
- Ehmann, A., 1977. The van urk-Salkowski reagent--a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole-derivatives. Journal of Chromatography, 132, 267-276.
- Ensley, B.D., Ratzkin, B., Osslund, T.D., Simon, M.J., Wackett, L.P., Gibson, D.T., 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science, 222, 167-169.
- Fondi, M., Orlandini, V., Perrin, E., Maida, I., Bosi, E., Papaleo, M.C., Michaud, L., Lo Giudice, A., de Pascale, D., Tutino, M.L., Liò, P., Fani, R., 2014. Draft genomes of three Antarctic *Psychrobacter* strains producing antimicrobial compounds against *Burkholderia cepacia* complex, opportunistic human pathogens. Marine Genomics, 13, 37-38.
- Franzetti, L., Scarpellini, M., 2007. Characterisation of *Pseudomonas* spp. isolated from foods. Annals of Microbiology, 57, 39-47.
- Frapolli, M., Défago, G., Moënne-Loccoz, Y., 2007. Multilocus sequence analysis of biocontrol fluorescent *Pseudomonas* spp. producing the antifungal compound 2,4-diacetylphloroglucinol. Environmental Microbiology, 9, 1939-1955.
- Galardini, M., Biondi, E.G., Bazzicalupo, M., Mengoni, A., 2011. CONTIGuator: a bacterial genomes finishing tool for structural insights on draft genomes. Source Code for Biology and Medicine, 21, 6-11.

- Gennari, M., Dragotto, F., 2012. A study of the incidence of different fluorescent *Pseudomonas* species and biovars in the microflora of fresh and spoiled meat and fish, raw milk, cheese, soil and water. Journal of Applied Bacteriology, 72, 281-288.
- Gilmour, M.W., Graham, M., Van Domselaar, G., Tyler, S., Kent, H., Trout-Yakel, K.M, Larios, O., Allen, V., Lee, B., Nadon, C., 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC genomics, 11, 120.
- Holt, K.E., Parkhill, J., Mazzoni, C.J., Roumagnac, P., Weill, F.X., Goodhead, I., Rance, R., Baker, S., Maskell, D.J., Wain, J., Dolecek, C., Achtman, M., Dougan, G., 2008. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. Nature Genetics, 40, 987-993.
- Kisand, V., Lettieri, T., 2013. Genome sequencing of bacteria: sequencing, de novo assembly and rapid analysis using open source tools. BMC Genomics, 14, 211.
- Kuhn, R., Starr, M.P., Kuhn, D.A., Bauer, H., Knackmuss, H.J., 1965. Indigoidine and other bacterial pigments related to 3,3'-bipyridyl. Archives Microbiology, 51, 71-84.
- Kurniawan, Y.N., Kitani, S., Maeda, A., Nihira, T., 2014. Differential contributions of two SARP family regulatory genes to indigoidine biosynthesis in *Streptomyces lavendulae* FRI-5. Applied Microbiology and Biotechnology, 98, 9713-9721.
- Lim, C.K., Hassan, K.A., Tetu, S.G., Loper, J.E., Paulsen, I.T., 2012. The effect of iron limitation on the transcriptome and proteome of *Pseudomonas fluorescens* Pf-5. PLos ONE, 7, e39139.
- Loman, N.J., Constantinidou, C., Chan, J.Z., Halachev, M., Sergeant, M., Penn, C.W., Robinson, E.R., Pallen, M.J., 2012. High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. Nature Reviews Microbiology, 10, 599-606.
- Ma, Q., Qu, Y., Tang, H., Yu, H., Ma, F., Shi, S., Zhang, X., Zhou, H., Zhou, J., Xu, P., 2012. Genome sequence of a novel indigo-producing strain, *Pseudomonas monteilii* QM. Journal of Bacteriology, 194, 4459-4460.

- McClure, R., Balasubramanian, D., Sun, Y., Bobrovskyy, M., Sumby, P., Genco, C.A., Vanderpool, C.K., Tjaden, B., 2013. Computational analysis of bacterial RNA-Seq data. Nucleic Acids Research, 41, e140.
- Murdock, D., Ensley, B.D., Serdar, C., Thalen, M., 1993. Construction of metabolic operons catalyzing the de novo biosynthesis of indigo in *Escherichia coli*. Biotechnology, 11, 381-386.
- O'Connor, K.E., Dobson, A.D., Hartmans, S., 1997. Indigo formation by microorganisms expressing styrene monooxygenase activity. Applied and Environmental Microbiology, 63, 4287-4291.
- O'Connor, K.E., Hartmans, S., 1998. Indigo formation by aromatic hydrocarbon-degrading bacteria. Biotechnology Letters, 20, 219-223.
- Palleroni, N.J., 2010. The *Pseudomonas* story. Environmental Microbiology, 12, 1377-1383.
- Palmer, G.C., Jorth, P.A., Whiteley, M., 2013. The role of two *Pseudomonas aeruginosa* anthranilate synthases in tryptophan and quorum signal production. Microbiology, 159, 959-969.
- Pathak, H., Madamwar, D., 2010. Biosynthesis of indigo dye by newly isolated naphthalene-degrading strain *Pseudomonas* sp. HOB1 and its application in dyeing cotton fabric. Applied Biochemistry and Biotechnology, 160, 1616-1626.
- Rapid Alert System for Food and Feed (RASFF). Annual Report, 2010.
- Reverchon, S., Rouanet, C., Expert, D., Nasser, W., 2002. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. Journal of Bacteriology, 184, 654-665.
- Samaržija, D., Zamberlin, Š., Pogačić, T., 2012. Psychrotrophic bacteria and milk and dairy products quality. Mljekarstvo, 62, 77-95.
- Sanz, E., Arteaga, A., García, M.A., Caámara, C., Dietz, C., 2012. Chromatographic analysis of indigo from Maya Blue by LC-DAD-QTOF. Journal of Archaeological Science, 39, 3516.

- Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics, 30, 2068-2069.
- Shiryev, S.A., Papadopoulos, J.S., Schäffer, A.A., Agarwala, R., 2007. Improved BLAST searches using longer words for protein seeding. Bioinformatics, 23, 2949-2951.
- Silby, M.W., Winstanley, C., Godfrey, S.A.C., Levy, S.B., Jackson, R.W., 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiology Review, 35, 652-680.
- Solieri, L., Dakal, T.C., Giudici, P., 2012. Next-generation sequencing and its potential impact on food microbial genomics. Annals of Microbiology, 63, 21-37.
- Starr, M.P., Knackmuss, H.J., Cosens, G., 1967. The intracellular blue pigment of *Pseudomonas lemonnieri*. Archiv für Mikrobiologie, 59, 287-294.
- Tümmler, B., Wiehlmann, L., Klockgether, J., Cramer, N., 2014. Advances in understanding *Pseudomonas*. F1000prime reports, 6, 9.
- van de Loo, F.J., Keese, P., Llewellyn, D., 1998. Structural and regulatory genes controlling indigoidine production in *Vogesella indigofera*: involvement of a peptide synthetase homolog. Unpublished. http://getentry.ddbj.nig.ac.jp/getentry/na/AF088856/?filetype=html#cds3
- van de Mortel, J.E., de Vos, R.C., Dekkers, E., Pineda, A., Guillod, L., Bouwmeester, K., van Loon, J.J., Dicke, M., Raaijmakers, J.M., 2012. Metabolic and transcriptomic changes induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. Plant Physiology, 160, 2173-2188.
- Wasi, S., Tabrez, S., Ahmad, M., 2013. Use of *Pseudomonas* spp. for the bioremediation of environmental pollutants: a review. Environmental Monitoring and Assessment, 185, 147-155.
- Xie, G., Keyhani, N.O., Bonner, C.A., Jensen, R.A., 2003. Ancient origin of the tryptophan operon and the dynamics of evolutionary change. Microbiology and Molecular Biology Reviews, 67, 303-342.

- Yu, D., Xu, F., Valiente, J., Wang, S., Zhan, J., 2013. An indigoidine biosynthetic gene cluster from *Streptomyces chromofuscus* ATCC 49982 contains an unusual IndB homologue. Journal of Industrial Microbiology & Biotechnology, 40, 159-168.

3.8. Supplementary data

Table 3.7 - Up- and down- regulated genes obtained through Differential Gene Expression Analysis.

Gene name[a]	Synonym[b]	Annotated Functions[c]	Fold Change[d] (original values)	EDGE test: blue vs white. tagwise dispersions P-value[e]
a. Up-regulated genes				
IRON UPTAKE				
efeO	PflA506_2583	periplasmic iron transport protein EfeO	4.487858112	2.86E-04
efeU	PflA506_2584	ferrous iron permease EfeU	3.681618075	2.05E-03
pvdO	PflA506_3086	chromophore maturation protein PvdO	5.635179619	4.03E-06
pvdF	PflA506_3087	N(5)-hydroxyornithine transformylase PvdF	2.241941643	6.38E-03
pvdI	PflA506_3091	non-ribosomal peptide synthetase PvdI	2.709517532	3.19E-04
OXIDOREDUCTASES				
sodA1	PflA506_0863	superoxide dismutase	3.050019925	8.63E-03
-	PflA506_1293	short chain dehydrogenase/reductase family protein	2.233665347	7.18E-03
-	PflA506_2379	short chain dehydrogenase/reductase family oxidoreductase	2.536304199	1.83E-03
-	PflA506_2528	cupin domain-containing protein	4.24185941	1.62E-07
-	PflA506_3494	short chain dehydrogenase/reductase family oxidoreductase	2.857785569	1.11E-04
-	PflA506_4144	MaoC-like domain protein	4.353851309	8.27E-06
TRANSCRIPTION				
-	PflA506_0211	transcriptional regulator SftR-related protein	2.343291042	4.78E-03
-	PflA506_1949	sigma-54 dependent transcriptional regulator	2.649955405	1.02E-03
-	PflA506_2163	AraC family transcriptional regulator	33.66315143	2.63E-07

Gene name[a]	Synonym[b]	Annotated Functions[c]	Fold Change[d] (original values)	EDGE test: blue vs white. tagwise dispersions P-value[e]
-	PflA506_2968	DNA-binding response regulator	5.867122516	3.65E-07
greA	PflA506_4559	transcription elongation factor GreA	3.183311481	8.22E-05
DRUG RESISTANCE				
acrA	PflA506_2969	acriflavine resistance protein A	2.598557166	2.05E-03
acrB	PflA506_2970	acriflavine resistance protein B	2.498165386	1.38E-03
-	PflA506_4208	drug resistance transporter, EmrB/QacA family	2.536751326	1.20E-03
OTHER FUNCTIONS				
-	PflA506_0160	hypothetical protein	794.2671546	1.17E-13
-	PflA506_0168	lipoprotein	163.3498404	3.39E-28
-	PflA506_0461	hypothetical protein	447.2901811	4.83E-14
-	PflA506_0462	lipoprotein	28.61440038	7.53E-19
-	PflA506_0891	hypothetical protein	3.146866615	8.69E-04
-	PflA506_1096	class III aminotransferase	6.130211791	1.85E-05
dgkA	PflA506_1168	diacylglycerol kinase	2.310586436	9.09E-03
-	PflA506_1594	VacJ family lipoprotein	3.334847267	5.16E-03
-	PflA506_1942	PepSY-associated TM helix domain-containing protein	2.238832209	5.28E-03
-	PflA506_3356	NodT family efflux transporter outer membrane lipoprotein	3.721717155	1.24E-05
arnT_2	PflA506_3380	undecaprenyl phosphate-alpha-4-amino-4-deoxy-L-arabinose arabinosyl transferase	2.311179213	3.37E-03
prpD	PflA506_3931	2-methylcitrate dehydratase	2.388775414	2.75E-03
-	PflA506_4279	hypothetical protein	2.334039481	6.44E-03
rpsQ	PflA506_4813	30S ribosomal protein S17	27.61604053	3.15E-03
-	PflA506_5185	hypothetical protein	2.455557284	2.19E-03

b. Down-regulated genes

AMINO ACIDS BIOSYNTHESIS				
aroK	PflA506_0403	shikimate kinase	-2.086583389	2.96E-03
-	PflA506_0784	tRNA-Thr	-2.255271503	4.73E-03
-	PflA506_1916	tRNA-Val	-1.907614303	1.32E-05
nspC	PflA506_3051	carboxynorspermidine decarboxylase	-1.260451805	9.58E-03
pheT	PflA506_3452	phenylalanyl-tRNA synthetase subunit beta	-2.194101986	1.84E-03
asnB	PflA506_3631	asparagine synthase	-1.83955912	1.23E-03
-	PflA506_4051	tRNA-Arg	-3.721409881	3.05E-04
ilvB	PflA506_4513	acetolactate synthase large subunit	-2.619092295	5.35E-04
RESPIRATORY CHAIN				
-	PflA506_0081	cytochrome c4	-4.591925856	4.38E-06
-	PflA506_3983	electron transfer flavoprotein-ubiquinone oxidoreductase	-3.109137457	6.68E-05
cydB	PflA506_4639	cytochrome d ubiquinol oxidase, subunit II	-1.697009566	1.19E-03
cydA	PflA506_4640	cytochrome d ubiquinol oxidase, subunit I	-2.137704348	1.80E-03
ABC TRANSPORTER				
opuCC	PflA506_0804	glycine betaine/carnitine/choline ABC transporter, periplasmic glycine betaine/carnitine/choline-binding protein	-1.869526654	2.48E-03
-	PflA506_2445	ABC transporter permease	-1.224405619	7.60E-03
-	PflA506_2448	ABC transporter substrate-binding protein	-1.234401674	4.88E-03
CELL REPLICATION				
recF	PflA506_0003	DNA replication and repair protein RecF	-1.874990985	6.57E-03
mdoH	PflA506_0359	glucans biosynthesis glucosyltransferase H	-1.963883944	7.79E-03
ftsQ	PflA506_0933	cell division protein FtsQ	-2.106889719	1.87E-03
ftsK	PflA506_3183	DNA translocase FtsK	-1.300307408	9.75E-03
TRANSCRIPTIONAL REGULATOR				
-	PflA506_1169	LysR family transcriptional regulator	-1.602136843	8.20E-03
-	PflA506_3292	AraC family transcriptional regulator	-1.572565218	1.35E-03

Gene name[a]	Synonym[b]	Annotated Functions[c]	Fold Change[d] (original values)	EDGE test: blue vs white. tagwise dispersions P-value[e]
-	PflA506_3563	GntR family transcriptional regulator	-1.620803004	2.94E-04
anr	PflA506_3874	transcriptional regulator Anr	-2.094611054	1.42E-03
OTHER				
-	PflA506_0165	hypothetical protein	-245.2594698	2.25E-08
ychF	PflA506_0717	GTP-binding protein YchF	-1.946452337	6.22E-03
-	PflA506_1267	hypothetical protein	-1.914802846	2.10E-03
-	PflA506_1648	hypothetical protein	-	1.22E-04
-	PflA506_1653	ISPpu14-like transposase, Orf1	-	3.31E-05
cmk	PflA506_1700	cytidylate kinase	-3.864295807	1.13E-04
-	PflA506_1759	hypothetical protein	-1.335637252	6.45E-03
-	PflA506_1920	hypothetical protein	-1.454469288	1.26E-03
-	PflA506_2020	hypothetical protein	-	8.97E-04
-	PflA506_2022	hypothetical protein	-	5.97E-03
-	PflA506_2082	hypothetical protein	-56.9058859	1.15E-05
-	PflA506_2084	site-specific recombinase, phage integrase family	-318.3344961	2.00E-11
-	PflA506_2087	peptidase propeptide and YPEB domain-containing protein	-1.375573024	9.21E-03
-	PflA506_2111	hypothetical protein	-1.371835351	5.09E-03
-	PflA506_2702	KaiC domain protein	-1.251335768	9.01E-03
-	PflA506_2866	Translocator protein, LysE family	-1.99836645	3.87E-05
proP	PflA506_2888	osmosensory proline/glycine betaine transporter ProP	-2.13548905	2.86E-03
-	PflA506_3569	addiction module killer protein	-13.08354692	7.45E-10
mscS	PflA506_4234	small-conductance mechanosensitive channel	-2.086199168	5.93E-04
-	PflA506_4261	fumarate hydratase, class I	-1.679578054	4.65E-03
-	PflA506_4404	S-type pyocin/colicin family protein	-128.079872	1.55E-09

imm	PflA506_4405	Colicin-E3 immunity protein	-	7.89E-10
-	PflA506_4638	hypothetical protein	-1.6700303	9.66E-04
-	PflA506_4681	hypothetical protein	-1.514544811	5.15E-03
erpA	PflA506_4852	essential respiratory protein A	-1.515607688	3.04E-03
-	PflA506_4941	hypothetical protein	-1.497811859	6.08E-03
-	PflA506_5173	hypothetical protein	-2.120261937	6.86E-04
hcp1	PflA506_5303	type VI secretion system effector Hcp1	-1.818800594	8.30E-03

[a, b] Gene name and symbol referred to *P. fluorescens* A506 used as reference genome
[c] Annotated function obtained using BioCyc database
[d] Fold change of read number count of blue-pigmenting strains vs non-pigmenting strains; no fold-change value (symbol "-") is reported when no read mapped in one of the two subsets analysed.
[e] Significance of Differential Gene Expression of blue-pigmenting strains vs non-pigmenting strains

3.9. Appendix to Chapter 3

During the investigation of the genomic information of the blue and non-pigmenting strains, *trp* genes were revealed to be potentially involved in the blue-pigment production, as they have been found in a genetic cluster of the accessory genome of the blue strains.

Trp operon has been widely used as a model to investigate the evolutionary relationship between gene and protein, and gene expression.

Multiple copies of *trp* genes have been reported in several bacterial species: for example, for archaea and bacteria, four different groups of TrpB protein were identified, whose combinations have been found in several species (Merkl, 2004). Moreover, in some cases, an additional function for the second copy has been speculated (Xie *et al.,* 2001; Busch *et al.,* 2014). Particularly, the two copies possess substrate specificity, giving different selective advantages to bacteria having both copies, through still unknown mechanisms (Busch *et al.,* 2014).

3.9.1. Screening of the strains for the presence of *trp* genes

To confirm the real correlation between the presence of the aforementioned genes and the ability to produce the blue pigment, 36 strains (eighteen blue- and eighteen non-pigmenting strains) were investigated to identify the presence of these genes. Table 3.8 reports the strains used in the investigation.

Table 3.8 - Strains investigated for the presence of trp genes.

Blue-strains	ps_1, ps_2, ps_5, ps_6, ps_13, ps_22, ps_25, ps_75, ps_76, ps_77, ps_78, ps_79, ps_80, ps_81, ps_82, ps_83, ps_84, ps_85.
Non-pigmenting strains	ps_3, ps_4, ps_7, ps_20, ps_21, ps_27, ps_29, ps_33, ps_37, ps_40, ps_48, ps_51, ps_54, ps_58, ps_59, ps_61, ps_64, ps_74.

The second copy of the *trp* genes (*trpA_2*, *trpB_2*, *trpC_2*, *trpD_2*, *trpF_2*) of the previously completely sequenced genomes of ps_22 and ps_77 were used to design five primer pairs. Primers were designed from the most conserved regions using Primer3

software (http://frodo.wi.mit.edu/primer3/). The complete list of the primers used for PCR amplification and sequencing is reported in Table 3.9.

Table 3.9 - Primers used for amplification and sequencing.

Primer	Sequence (5'-3')	Annealing temperature (°C)
trpA_2_F	TGA CCT TGA CTC TGG CTC TC	59.4
trpA_2_R	CTG TCT CAC CCT GGC GAT AA	59.4
trpB_2_F	AGA GAT ATT CGA GGT GGC GG	59.4
trpB_2_R	GCT ACC CAA TTG ACG GTG TC	59.4
trpC_2_F	GGA CGA GGT TTG TAT TGC GG	59.4
trpC_2_R	GGT AGA ATC CAA GCA AGC CG	59.4
trpD_2_F	CGA CTT ATT GGA GGC CGT TG	59.4
trpD_2_R	TTC GTC CAG TCC CTC TTC AC	59.4
trpF_2_F	TGA TCA AAG TGT GCG GTG TG	57.3
trpF_2_R	CGG TAG TAT CGT TGG CTC CT	59.4

Genomic DNA was extracted as previously described (Paragraph 2.2.4. of this thesis). The PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler in a final volume of 20 μL of amplification mix containing 1U of GoTaq polymerase (Promega, Madison, WI), 1U GoTaq Buffer, 1.5 mM MgCl2, 0.2 mM each deoxynucleotide triphosphate (dNTP), 250 nM each primer and 5 ng of genomic DNA as the template. The reaction mixture was subjected to the following thermal cycle: an initial step at 94 °C for 2 min to activate the polymerase and 35 cycles each of denaturation at 94 °C for 20 s, annealing of the primers at 62 °C for 30 s and extension at 72 °C for 1 min and a final step of extension at 72 °C for 7 min. The amplified products were analysed by electrophoresis on 1.8% agarose-Tris-acetate-EDTA (TAE) gels, stained with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) and visualised on a UV transilluminator (Gel Doc XR, Biorad).

The amplification of the second copy of *trp* genes was possible only for the blue-pigmenting strains. Some aspecific products were visible for some non-pigmenting strains, but they were avoided enhancing the annealing temperature of the reaction. Moreover, the aspecific bands were longer than the specific PCR product. For this reason, they were easily identified and distinguished each other.

The identification of the second copies of *trp* genes only in the blue-pigmenting strains confirmed the possibility to use these genes as a marker for the presence of potential blue

Pseudomonas right in the food plant. The application of this screening might be of easier application than the amplification and sequencing of the seven loci of the MLST scheme, considered in some cases too much time- and money-expensive (Scales *et al.,* 2014).

3.9.2. Is the second copy of *trp* genes the consequence of a duplication or of Horizontal Gene Transfer?

During the discussion of the results of the paper “A genomic and transcriptomic approach to investigate the blue pigment phenotype in *Pseudomonas fluorescens*”, the second copy of each *trp* gene was improperly defined as the results of a duplication. During the revision of the manuscript, the words “duplication” or “duplicated” have been replaced by “second” or “double” to avoid meeting with a mistake.

Gene duplications are considered a fundamental mechanism for gene gain that can facilitate adaptation of bacteria to different environments (Gevers *et al.,* 2004; Vos *et al.,* 2015). The two copies of the same gene are called “Paralogs” if they are a result of a duplication event. On the other hand, a second mechanism can take place: Horizontal Gene Transfer (HGT). HGT is based on the acquisition of external sequences from outside sources. In this case, the two copies of the same gene are called “Xenologs”. To investigate the real nature of the second copy of the aforementioned genes, a Blastp investigation of the second copy of *trp* genes of ps_20 and ps_77 was performed. Briefly, the amino acidic sequence of the proteins encoded by the second copy of the genes were queried against NCBI using Blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). First twenty alignments were selected and sequences were downloaded from NCBI and used to implement the dataset for the phylogenetic reconstruction. The dataset for each of the five Trp proteins was constituted by the protein encoded by the first copy of the gene of *P. fluorescens* A506, ps_20, ps_22, ps_40 and ps_77, the second proteins in ps_22 and ps_77 and the best twenty alignments to the second protein of ps_77. The alignment and the phylogenetic reconstruction was performed using MEGA choosing the Maximum Likelihood algorithm.

The phylogenetic trees are reported in Figure 3.8, 3.9, 3.10, 3.11 and 3.12.

In most of the cases, the so-called first copy, namely the one who has been found also in *P. fluorescens* A506, is quite conserved in the five strains investigated, up to 100% of similarity for TrpD. Also the second copies, namely the one found only in ps_22 and ps_77 are identical in the two blue pigmenting strains.

From the phylogenetic reconstruction it is possible to highlight a clear separation between the proteins encoded by the two copies of *trp* genes. Indeed, the Maximum Likelihood trees showed well supported separated clusters in respect to their "core" counterparts (and also in comparison to other Trp proteins). Similar findings have been reported by Merkl (2007) reporting the phylogenetic reconstruction among Trp Proteins in bacteria and archaea. Indeed, two major groups were identified and specifically in the second one, further subgroups were recognised.

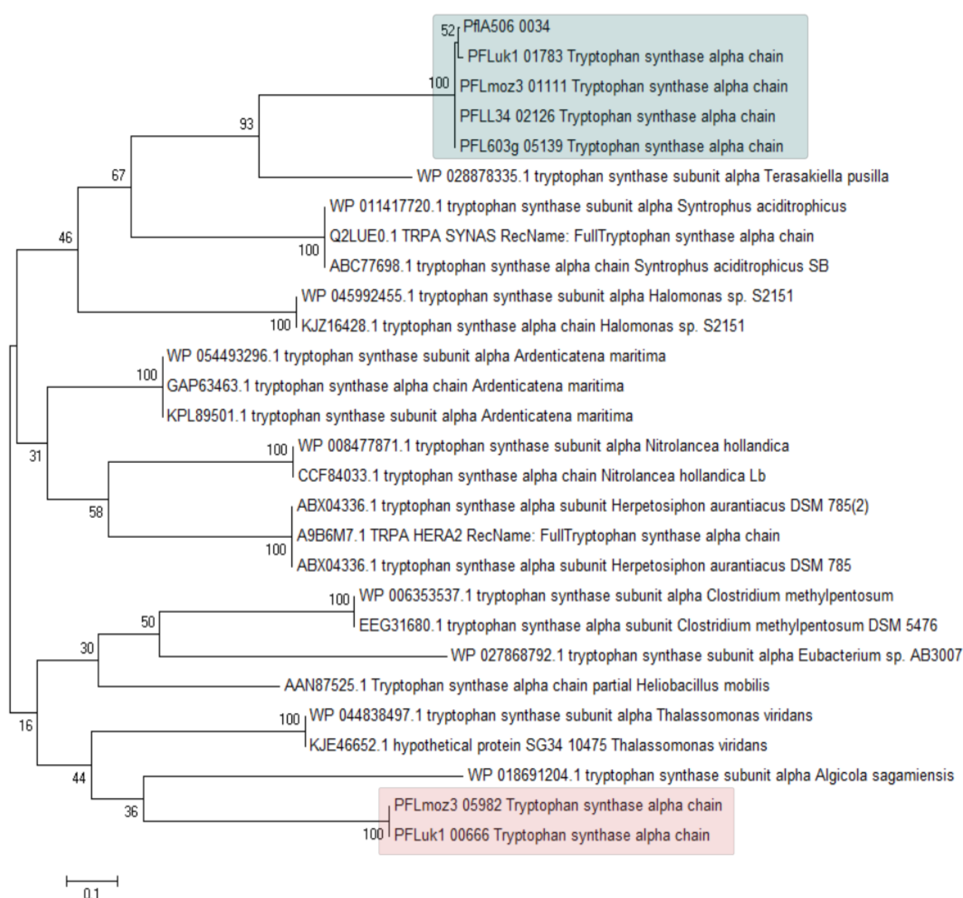


Figure 3.8 - ML phylogenetic tree of TrpA proteins.

Proteins encoded by first copy are highlighted with a light blue box, while the ones by the second copy are highlighted in pink.

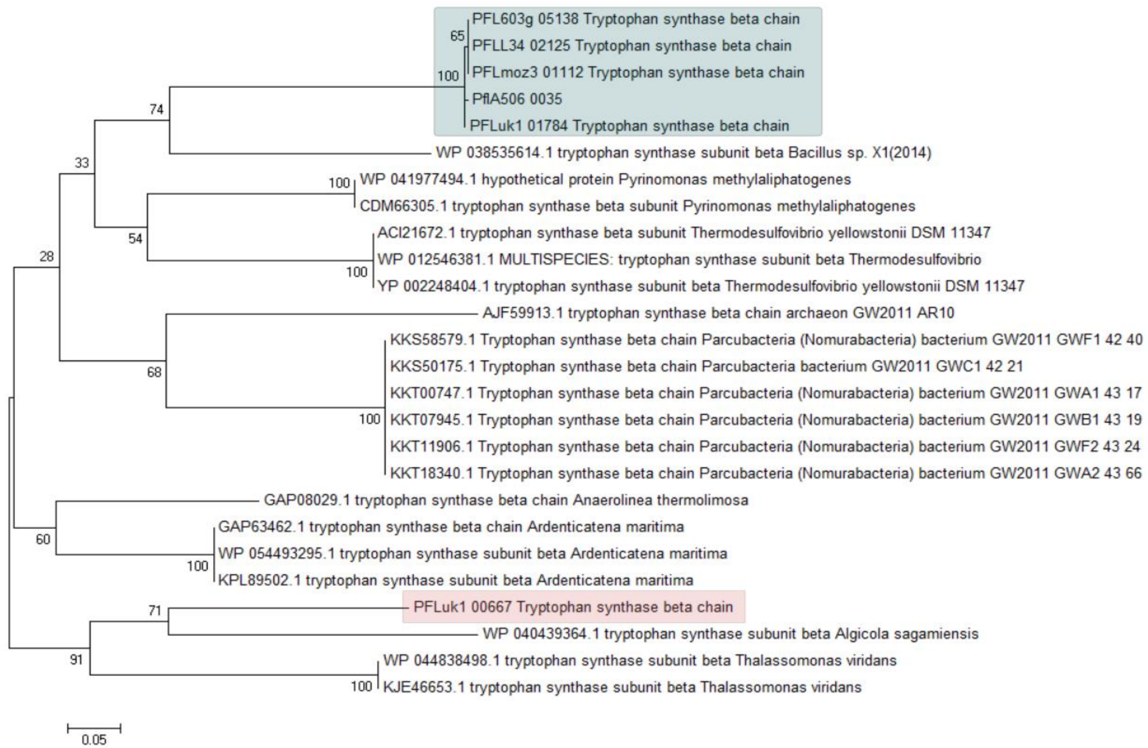


Figure 3.9 - ML phylogenetic tree of TrpB proteins.

Proteins encoded by first copy are highlighted with a light blue box, while the ones by the second copy are highlighted in pink. No protein of TrpB was found in ps_22 due to the lower coverage of the genome.

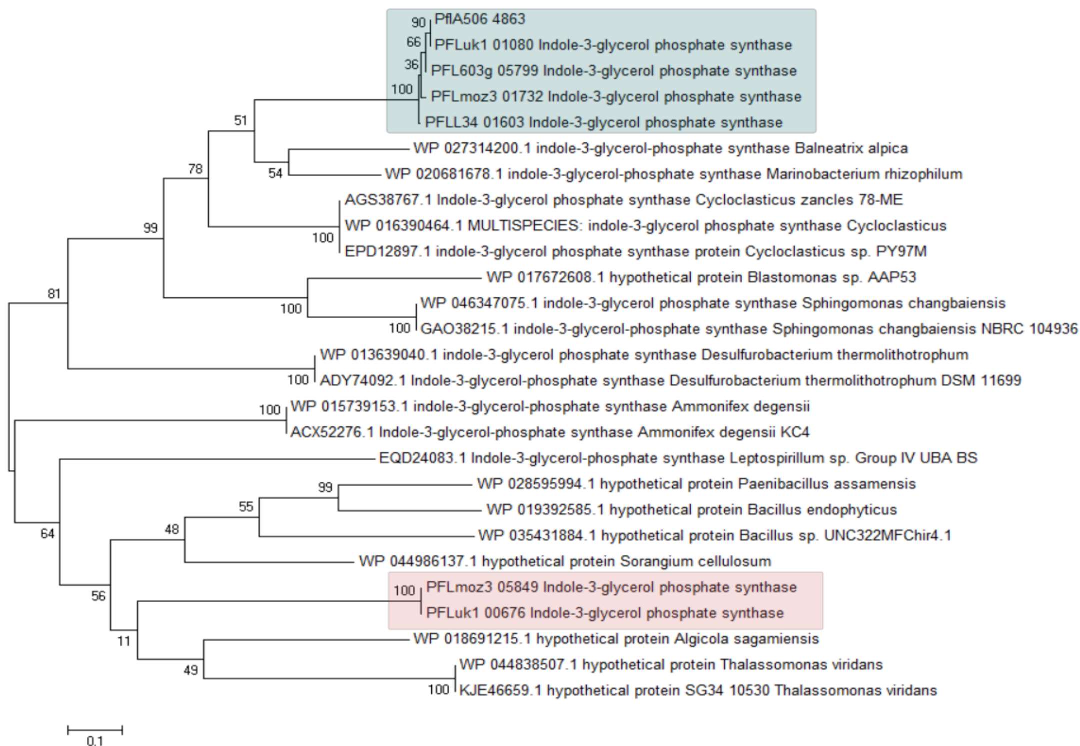


Figure 3.10 - ML phylogenetic tree of TrpC proteins.

Proteins encoded by first copy are highlighted with a light blue box, while the ones by the second copy are highlighted in pink.

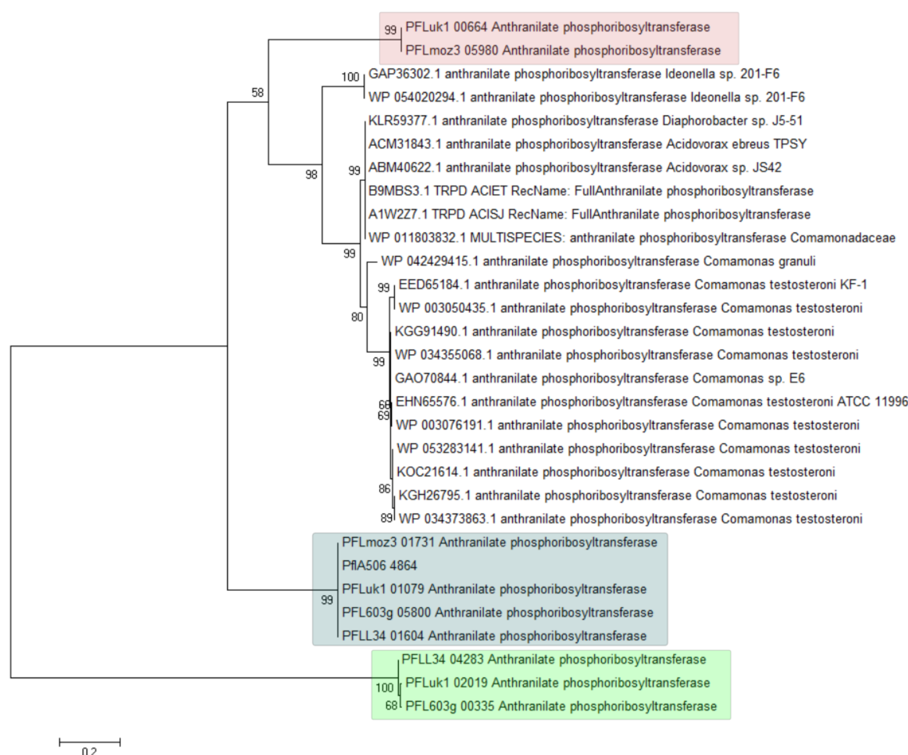


Figure 3.11 - ML phylogenetic tree of TrpD proteins.

Proteins encoded by first copy are highlighted with a light blue box, the ones by the second copy are highlighted in pink, while proteins encoded by a third copy are highlighted in green.

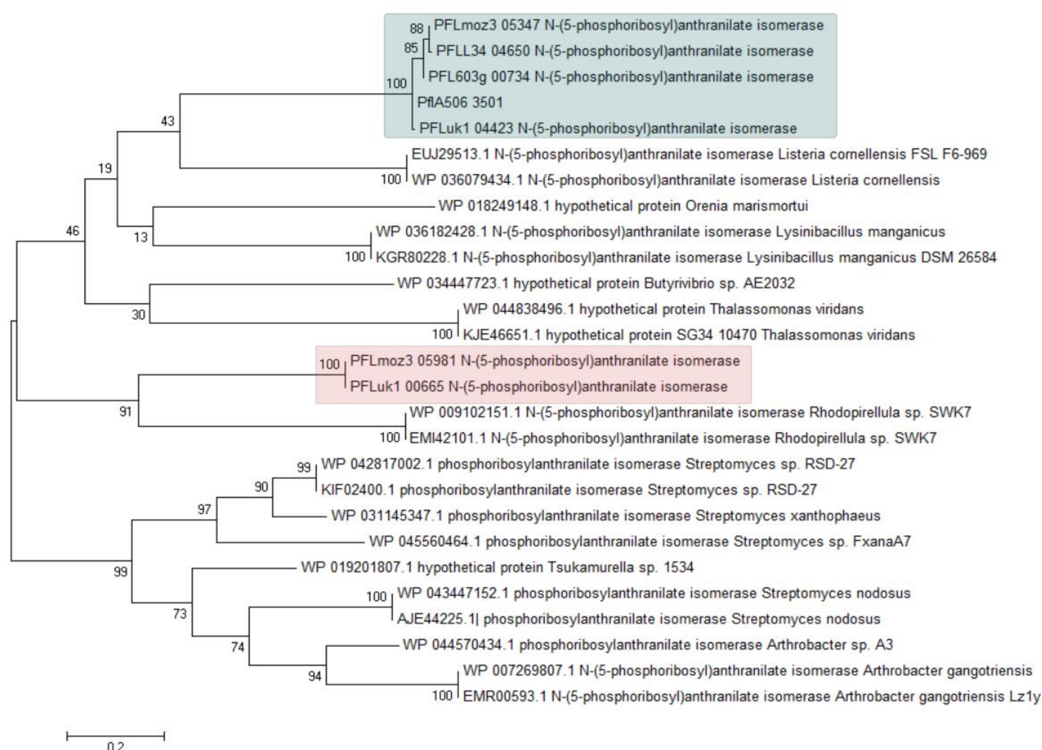


Figure 3.12 - ML phylogenetic tree of TrpF proteins.

Proteins encoded by first copy are highlighted with a light blue box, while the ones by the second copy are highlighted in pink.

To summarize, from the phylogenetic reconstruction it seems to be more likely that the second copy of the investigated genes has been acquired through Horizontal Gene Transfer and not through duplication of the core gene, according to the study of Merkl (2007). This theory is supported by localization of the first and second copy in the two blue-pigmenting *Pseudomonas* strains in different genomic regions.

A more in depth investigation including archaeal Trp protein sequencing might elucidate the evolutionary origin of the genes but this investigation lies outside the goals of the present study.

3.9.3. References

- Busch, F., Rajendran, C., Mayans, O., Löffler, P., Merkl, R., Sterner, R., 2014. TrpB2 enzymes are O-phospho-l-serine dependent tryptophan synthases. Biochemistry, 53, 6078-6083.
- Gevers, D., Vandepoele, K., Simillon, C., Van de Peer, Y., 2004. Gene duplication and biased functional retention of paralogs in bacterial genomes. Trends in Microbiology, 12, 148-154.
- Merkl, R., 2004. SIGI: score-based identification of genomic islands. BMC Bioinformatics, 3, 5-22.
- Merkl, R., 2007. Modelling the evolution of the archeal tryptophan synthase. BMC evolutionary biology, 10, 7-59.
- Scales, B.S., Dickson, R.P., LiPuma, J.J., Huffnagle, G.B., 2014. Microbiology, genomics, and clinical significance of the *Pseudomonas fluorescens* species complex, an unappreciated colonizer of humans. Clinical Microbiological Reviews, 27, 927-948.
- Vos, M., Hesselman, M.C., te Beek, T.A., van Passel, M.W., Eyre-Walker, A., 2015. Rates of Lateral Gene Transfer in Prokaryotes: High but Why?. Trends in Microbiology, 23, 598-605.
- Xie, G., Forst, C., Bonner, C., Jensen, R.A., 2001. Significance of two distinct types of tryptophan synthase beta chain in Bacteria, Archaea and higher plants. Genome Biology, 3, RESEARCH0004.

CHAPTER 4: Transposon-induced pigment deficient mutants of a blue *Pseudomonas fluorescens* strain

---

Andreani Nadia Andrea

In Preparation

Article info:

Paper in preparation

Keywords:

Pseudomonas fluorescens, transposon-induced mutants, phenotypic characterisation, blue-pigment biosynthesis.

4.1. Abstract

Pseudomonas fluorescens ps_77 is a blue-pigmenting strain belonging to the *P. fluorescens* subgroup. The biosynthetic pathway of the pigment seems to be related to tryptophan metabolism, as blue pigmenting strains have a second copy of *trpABCDF* genes, encoding for enzymes involved in tryptophan production. In this paper, random mutagenesis of *P. fluorescens* ps_77 was performed with the aim to identify the genes involved in the blue-pigment production. Genetic analyses based on the mapping of the insertions and gene expression studies were performed. The phenotypic characterisation of the white mutants was conducted to highlight the biological function of the pigment. The mapping of transposon insertions and the evaluation of gene expression in blue- and non-pigmenting strains revealed the complexity of pigment biosynthesis. The *trpB* gene and other genes of the genetic cluster identified solely in blue pigmenting strains are involved in the biosynthetic pathway. Moreover, other genes involved in regulation, biofilm formation, motility, signalling were found to mediate pigment production. The characterisation of the mutants led to the conclusion that the blue molecule does not function as a siderophore, but that iron is necessary to produce the pigment. The biosynthetic pathway, the chemical nature and the function of the blue pigment remain an open question.

4.2. Introduction

Pseudomonas fluorescens is a well-known food spoiler belonging to the genus *Pseudomonas*. The importance of this Gram-negative bacterium is related to public hygiene and food trade, as it is not commonly considered a human pathogen (Scales *et al.,* 2014), even if it can be found in several anatomic locations.

The interest towards this group of bacteria as food spoilers has increased since 2010, when some packages of mozzarella were seized after a blue discolouration was noticed on the external surface of the cheese (Annual Report of RASFF). After the initial uncertainty about the cause of this particular alteration, strains belonging to *Pseudomonas tolaasii* and

Pseudomonas libanensis species were blamed for this unpleasant modification. A subsequent study revealed the strains responsible of the discolouration as belonging to a specific genetic cluster of the *Pseudomonas fluorescens* group (Andreani *et al.,* 2014). This cluster, called the "blue branch", was demonstrated to contain both blue-pigmenting strains and uncoloured bacteria.

Recently, the chemical nature of the blue pigment has been investigated but no clear answer has yet been obtained. According to Caputo and colleagues (2015), the blue pigment observed on mozzarella cheese might be indigoidine; however, the same research group classified producing bacteria as *Pseudomonas fluorescens* biovar IV (namely *Pseudomonas lemonnieri*) in contrast with the conclusions of Andreani and co-workers, whose Multilocus Sequence Typing (MLST) scheme revealed a wide phylogenetic distance between *P. lemonnieri* and the blue pigmenting strains. In this last paper, the blue pigment was supposed to be an indigo-derivative based on a MALDI-TOF Mass spectrometry analysis (Andreani *et al.,* 2015). The blue pigment production seems to be strictly related to the environmental conditions in which blue strains grow. Indeed, the pigment production takes place at lower temperatures (6 and 22 °C) and not at 31 °C and the pigment is formed only on specific solid media (e.g. Potato Dextrose Agar and Minimal Bacterial Medium; Andreani *et al.,* 2014).

A genomic and transcriptomic investigation of two blue- and two non-pigmenting strains belonging to the "blue branch" has been recently published (Andreani *et al.,* 2015). The draft genome analysis and the investigation of accessory genes shared only by the blue-pigmenting strains, revealed the presence of some genes putatively involved in the blue pigment production (Andreani *et al.,* 2015). A cluster of sixteen genes has been identified as present only in the two blue strains investigated. This cluster contains five out of the seven genes involved in the biosynthesis of tryptophan, an amino acid, whose production is common of all *Pseudomonas* species (Bentley, 1990). The analysis of the pangenome of four *P. fluorescens* belonging to the blue cluster revealed that the *trp* genes are present in two copies in blue-pigmenting strains. A screening of eighteen blue-pigmenting strains and eighteen non-pigmenting strains belonging to the *Pseudomonas fluorescens* group revealed the presence of

the second copies in all the analysed blue-pigmenting strains only (Andreani, Appendix to Chapter 3 of the present Manuscript). The complete biosynthetic pathway and the type of enzymes involved in the production of the blue pigment, the chemical nature of the coloured molecule, as well as, the biological function of pigment production remain an open question.

Several fluorescent *Pseudomonas* produce secondary metabolites that can play a role in bacterial survival, fitness and advantage. A good example is *Pseudomonas fluorescens* CHA0 (now *Pseudomonas protegens*), whose production of several secondary metabolites allows the defense response against nematodes, influencing the physiology of predators and enhancing the fitness in the environment (Neidig *et al.,* 2011). Another example is represented by the chromosome of *Pseudomonas fluorescens* Pf-5, a biocontrol strain, whose 6% of the genetic information is dedicated to the biosynthesis of secondary metabolites (including antibiotics, toxins and siderophores; Looper and Gross, 2007). In particular, strains belonging to the genus *Pseudomonas* are well-known siderophore producers, or rather small molecule chelators able to bind extracellular ferric iron, making it bioavailable to microbes (Cornelis, 2010). Previous studies based on the analysis of the transcriptomes revealed a state of iron depletion of the blue-pigmenting strains in comparison to non-pigmenting ones: for this reason, one hypothesis was that the blue-pigment might have a siderophore function (Andreani *et al.,* 2015). Some secondary metabolites might provide oxidative protection against antimicrobial agents (e.g. prodigiosins produced by different bacteria and, specifically, some *Pseudomonas* species; Stankovic *et al.,* 2014; Sumathi *et al.,* 2014).

Transposon mutagenesis has been widely used as a powerful tool to investigate gene function in bacteria. The main basic concept of the method is to randomly disrupt genes using traceable mobile elements. Each random insertion allows the creation of a mutant having a peculiar phenotype, that is easily selected, isolated and screened (Chen *et al.,* 2000b). Mapping of the insertion, namely the identification of the locus the transposon is inserted in, allows the identification of the genes involved in each specific phenotype. Transposon mutagenesis has been used for a wide range of purposes in bacteria, such as the investigation of biosynthetic genes of several metabolites. For example, through transposon mutagenesis

the biosynthetic pathways of secondary metabolites in *P. chlororaphis* have been investigated (Kang *et al.,* 2008), the biosynthetic genes of Phl (3,4-diacetylphloroglucinol) or even the biosynthesis of a red pigmented derivative in *P. fluorescens* Q2-87 (Bangera and Thomashow, 1996).

In this study, a previously characterised strain of the *P. fluorescens* species complex was investigated. Ps_77 was isolated from a blue slice of beef in the UK. The strain produces a dark-blue, extracellular pigment on Potato Dextrose Agar and Minimal Bacterial Medium (Andreani *et al.,* 2015) at 6 °C and 22 °C, as well as on mozzarella cheese. Ps_77 belongs to the *Pseudomonas fluorescens* subgroup and the draft genome sequence is now available (LCYB00000000).

In the present work, more than 10^5 mutants of ps_77 were obtained using transposon mutagenesis, with the aim to collect non-pigmenting strains from the blue wild-type one. Transposon insertions were mapped in 24 non-pigmenting strains and one hyper pigmented mutant with the aim to identify the localization of the transposon in the chromosome. A phenotypic characterisation of the wild-type and the mutant strains was conducted to investigate the biological function of the pigment.

4.3. Materials and methods

4.3.1. Bacterial strains

The *Pseudomonas fluorescens* strain ps_77 was cultured and maintained in Luria Bertani (LB) Broth. The choice of the strain was conducted with the aim of selecting a blue strain for which detailed genomic information was available. Ps_77 produces a dark-blue pigment on Potato Dextrose Agar (PDA; Oxoid Microbiology Products, Thermo Scientific), Minimal Bacterial Medium Agar (0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% tri-sodium citrate, 0.01% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.2% glucose and 1.5% agar; Boles *et al.,* 2004) and mozzarella cheese. The wild-type strain and transposon-induced mutants were stored at -80 °C in LB Broth with 50% v/v glycerol (Sigma-Aldrich). Where appropriate, 50 ng/µL kanamycin (Sigma-Aldrich) was supplemented to the medium.

4.3.2. Transposon mutagenesis and screening of the mutants

Transposon mutagenesis of ps_77 was carried out with EZ-Tn5™ Tnp Transposome™ (Epicentre). Electrocompetent cells were prepared as reported by Choi and colleagues (2006). Briefly, 36 mL of overnight LB broth culture of ps_77 were centrifuged at 16,000 xg for 10 minutes. The pellet was washed trice with 300 mM sterile sucrose and then suspended in 100 µL of sterile sucrose. Electrocompetent cells were mixed with 1 µL of EZ-Tn5 <KAN-2> Tnp Transposome. Cells were electroporated in an electroporation cuvette (ThermoScientific) with an Eporator (Eppendorf; 25 Uf, 200 Ω, 2.5 kV). One mL of LB broth was added to the electroporated cells immediately after the electroporation. Cells were transferred into a tube and incubated at 28 °C for 60 minutes to facilitate cell outgrowth. Screening of kanamycin resistant (Kan^R) mutants was performed on MBM Agar with 50 ng/µL kanamycin (Sigma). Kan^R mutants were picked based on the pigmentation and streaked three times on MBM Agar with 50 ng/µL kanamycin (Sigma) at 28 °C to check the phenotype.

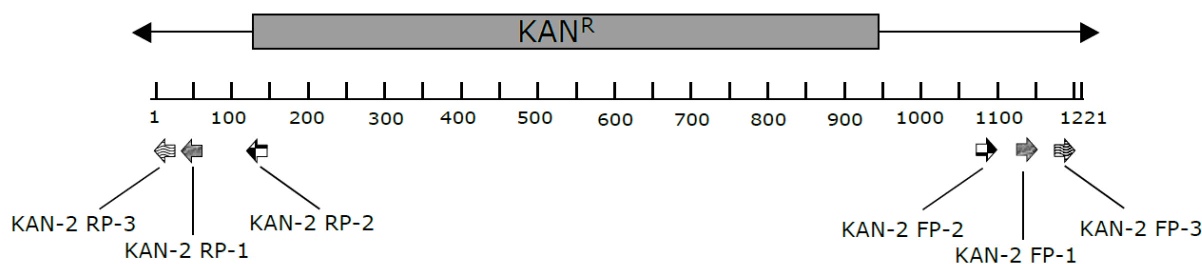
4.3.3. Tn5-flanking sequences of selected mutants

All selected Kan^R mutants were grown in 5 mL of LB Broth with 50 ng/µL kanamycin and incubated overnight at 28 °C. Genomic DNA (gDNA) was extracted using FastDNA™ SPIN Kit (MP BIOMEDICALS) and sequenced as described in Karlyshev *et al.,* 2000. Briefly, gDNA was amplified in a single-primer PCR with the upstream primer KAN-2 FP-2 or the downstream primer KAN-2 RP-2 with the following amplification scheme: 1 minute at 94 °C, 20 cycles of 94 °C for 30s, 50 °C for 30s and 72 °C for 3 min; 30 cycles of 94 °C for 30s, 30 °C for 30s and 72 °C for 2 min; 30 cycles of 94 °C for 30s, 50 °C for 30s and 72 °C for 2 min and the extension for 7 min at 72 °C. The complete list of the primers used for PCR amplification and sequencing and a schematic representation of their position in the transposon is reported in Table 4.1.

PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler in a final volume of 25 µL of amplification mix containing 12.5 µL of 2X GoTaq® Hot Start Green Master Mix Buffer, 2.5 µL of 10 µM upstream KAN-2 FP-1 or downstream KAN-2 RP-1

Table 4.1 - Primers used for the mapping procedures.

Primer	Sequence (5'-3')	Annealing temp (°C)	Reference
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC	61.3	EZ-Tn5™ Tnp Transposome™ (Epicentre)
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG	58.1	EZ-Tn5™ Tnp Transposome™ (Epicentre)
KAN-2 FP2	ACCGTTCCGTGGCAAAGC	58.2	This study
KAN-2 RP2	TGGCTCATAACACCCCTT	53.7	This study
KAN-2 FP-3	ATCCTCTAGAGTCGACCTGCA	59.8	This study
KAN-2 RP-3	TCATCGATGATGGTTGAGATGTG	58.9	This study



primer, 7.5 µL of nuclease-free water and about 100 ng of gDNA as the template. The amplified products were analysed by electrophoresis in a 1.8% agarose-Tris-acetate-EDTA (TAE) gel, stained with Ethidium Bromide (Sigma) and visualised on an UV transilluminator (Gel Doc XR™, Biorad). The most intense band of each amplification was extracted from the gel using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). The so obtained template was sequenced using one of the upstream or the downstream primers (KAN-2 RP-1 or KAN-2 FP-1) of EZ-Tn5™ <KAN-2> Tnp Transposome™ Kit at Macrogen Inc. (Amsterdam, the Netherlands) through direct Sanger sequencing.

The mapping of transposon insertion for Kan^R mutants, for which the sequencing as described previously was not possible, was performed as proposed by Walterson and colleagues (2014) for *Pantoea*. Briefly, the gDNA was digested in a reaction containing 2 units of *ErhI* (SybEnzyme), 2 µL of 10XSE-Buffer 2W, 0.2 µL of BSA (10 µg/mL, SybEnzyme), 15.6 µL of nuclease-free water and 1 µL of digested gDNA (450-850 ng/µL). Samples were incubated at 37 °C for 1 hour to allow the enzyme to cut gDNA and 20 minutes at 65 °C to inactivate the enzyme in an Applied Biosystems 2720 Thermal Cycler. Alternatively, the gDNA was digested in a reaction containing 2 units of *BstACI*

(SybEnzyme), 2 µL of 10XSE-Buffer W, 15.8 µL of nuclease-free water and 1 µL of digested gDNA (450-850 ng/µL). Samples were incubated at 37 °C for 1 hour to allow the enzyme to cut gDNA and 20 minutes at 80 °C to inactivate the enzyme in an Applied Biosystems 2720 Thermal Cycler. An unimolecular ligation was performed in a reaction containing 5,000 units of T4 DNA Ligase (SybEnzyme), 10 µL of 10X Buffer SE-T4 DNA Ligase Buffer, 2.5 µL of 10 mM ATP, 52.5 µL of nuclease-free water and 10 µL of digested gDNA in an Applied Biosystems 2720 Thermal Cycler. A first PCR amplification was performed (4 µL of 5X Phusion HF Buffer, 0.4 µL of 10 mM dNTPs, 2.5 µL of 10 pM KAN-2FP-2, 2.5 µL of 10 pM KAN-2RP-2, 0.2 µL of Phusion DNA Polymerase, 5.4 µL of nuclease-free water and 5 µL of ligated gDNA). The reaction mixture was subjected to the following thermal cycle: an initial step at 98 °C for 30 seconds to activate the polymerase and 35 cycles each of denaturation at 98 °C for 10 seconds, annealing of the primers at 54 °C for 30 seconds and extension at 72 °C for 90 seconds and a final step of extension at 72 °C for 10 minutes. The so obtained PCR product was applied in a second nested PCR (4 µL of 5X Phusion HF Buffer, 0.4 µL of 10 mM dNTPs, 2.5 µL of 10 pM KAN-2FP-1, 2.5 µL of 10 pM KAN-2RP-1, 0.2 µL of Phusion DNA Polymerase, 5.4 µL of nuclease-free water and 5 µL of 1:500 PCR product). The PCR products were sent to Macrogen Inc. (Amsterdam, the Netherlands) for direct Sanger sequencing with the upstream primer (KAN-2 FP-3) or the downstream primer (KAN-2 RP-3).

All the sequences were checked for quality and edited with FinchTV 1.4.0 software (Geospiza).

4.3.4. Genetic analyses and evaluation of gene expression in *Pseudomonas fluorescens* strains

Insertion sequences were queried against NCBI using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). When necessary, HMMER was applied with the aim to highlight protein homology of the query against Reference proteomes or SwissProt (Finn *et al.,* 2015). In selected cases, SIM (Local Similarity Program; Huang and Miller, 1991) was applied to align amino acidic sequences.

Additionally, sequences were queried against ps_77 (LCYB00000000) through the Bioedit Blast Tool using the draft genome as database (Tippmann, 2004). Identification of COG (Cluster of Orthologous) was performed using KOALA (KEGG Orthology and Links Annotation; Kanehisa *et al.,* 2015). The list of previously identified genes was analysed to evaluate the expression in two blue- and non-pigmenting strains of *Pseudomonas fluorescens*. Differential expression analysis was performed using transcriptomic data obtained in a previous work (Andreani *et al.,* 2015; SRR1725678; SRR1725679; SRR1725680; SRR1725681; SRR1725682; SRR1725683; SRR1725684; SRR1725723) and the RNAseq tools of CLCBio Genomics Workbench by applying Empirical analysis of DGE (Differential Gene Expression; minimum length fraction=0.9, minimum similarity fraction=0.8, maximum number of hits for a read=10). Differential expression was considered significant with *p*-values lower than 0.01 according to the CLCBio Genomics Workbench user manual. Gene expression was evaluated using both the draft genome of ps_77 (LCYB00000000) and *P. fluorescens* A506 (NC_017911.1) as reference genomes.

4.3.5. Phenotypic characterisation of the mutants

With the aim to investigate the biological function and the chemical nature of the blue pigment in ps_77, the phenotypic characterisation of ps_77 and some of its transposon insertion mutants was performed. Strains were chosen in order to compare the wild-type strain (ps_77), one hyper pigmented mutant (M25) and five non-pigmenting mutants (M2, M3, M19, M20 and M21). Where appropriate, the wild-type strain *P. fluorescens* SBW25 (previously phenotypically characterised as a strong producer of siderophores, and, in particular, pyoverdine) and a non-producing "cheater" of *P. fluorescens* SBW25, namely PBR840 (producing a lower amount of siderophores, due to the deletion of *pvdL* gene; Luján *et al.,* 2015) were used as control strains. A negative control, with no bacterial strain, was included to check for contamination. All strains were previously grown overnight in LB broth at 28 °C in continuous shaking to reach an average bacterial count of 10^8 CFU/mL. Ten-fold dilutions were performed in proper media to reach 10^3 CFU/mL and to remove traces of LB broth for all the following phenotypic tests.

4.3.5.1. Growth curves

Growth curves were investigated in triplicate in King's B broth (KB; 20 g of proteose peptone; Difco, 1,5 g of K_2HPO_4, 1,5 g of $MgSO_4 \cdot 7H_2O$, 10 mL of glycerol in 1 L of distilled water) to highlight a possible difference in the growth rate of blue and non-pigmenting strains. Growth curves were obtained using a plate reader (Multiskan Series Microplate Readers, Thermo Fisher Scientific), incubating the 96-well plate in continuous shaking at 28 °C and reading the optical density (O.D.) at 600 nm every 30 minutes. Growth rate was calculated using DMFit tool of ComBase, an online tool allowing the shape of microbial growth curve, based on the Gompertz and Baranyi modified equation (Baranyi and Tamplin, 2004; Mytilinaios *et al.,* 2012).

4.3.5.2. Growth curves in iron-free condition and evaluation of siderophore production

As iron was suggested to be involved in the blue-pigment production, the growth curves were carried out in KB with 100 µg/mL human apotransferrin (Sigma), a natural iron chelator, and 20 mM sodium bicarbonate, necessary for iron chelation by apotransferrin (Inglis *et al.,* 2012) to obtain an iron-free environment. Growth curves in iron-free condition were obtained as previously described (paragraph 4.3.5.1.).

To investigate whether the blue pigment has a siderophore function, Chrome Azurol S (CAS) liquid assay was performed, as reported by Schwin and Neilands (1987). CAS solution was prepared as proposed by Alexander and Zuberer (1991). Briefly, solution 1 was prepared by mixing 10 mL of 1 mM FeC1(III)$\cdot 6H_2O$ in 10 mM HCl with 50 mL of an aqueous solution of CAS (12.2 M). The resulting solution was added slowly, with constant stirring, to 40 mL of an aqueous solution of HDTMA (1.82 mg/mL). Then, 100 µL of each bacterial culture in KB were withdrawn in a new 96-well plate and 100 µL of CAS solution was added. The plate was stored in the dark at room temperature for 50 minutes to allow the stabilization of the reaction. Siderophore production was evaluated visually, by observing orange colouration. The liquid CAS assay was conducted at 31 hours of growth to allow all the strains to produce detectable amounts of siderophores.

4.3.5.3. Utilization of carbon sources

To assay whether the blue or the non-pigmenting strains were able to use different carbon sources in comparison to the wild-type strain, a Biolog GN2 Micro Plate was used for each strain. Biolog plates are 96-well plates: on the bottom of each well, a unique different carbon source is bound. If the strain is able to use that specific carbon source, it is able to grow and a colorimetric reaction takes place. Strains were grown overnight at 28 °C in LB broth and a 5-fold dilution was pipetted in each of the 96-wells of the Biolog GN2 plate. Biolog GN2 Micro Plates were incubated at 28 °C and bacterial growth was evaluated after 24 hours by measuring the O.D. at 600 nm with a plate reader.

4.3.5.4. Competition experiment

A competition experiment was set up to highlight the presence of differential fitness of the blue- and non-pigmenting strains in KB broth in different environmental conditions. Two strains having the same growth rate (ps_77 and M19) were inoculated in 2 mL of normal KB broth or iron-limited KB broth at the same concentration. Each competition experiment was conducted using six replicates and three different investigations were set up to test the competition at 4, 22 and 28 °C. The ratio between ps_77 and M19 was checked by plating on MBM agar every day for 5 days for the 22 and 28 °C experiments and 9 days for the 4 °C experiment to allow the strains to reach the final amount of 10^8 CFU/mL.

Similarly, the competition experiment was conducted on four different food matrices: bacon (the meat portion), bacon (the fat portion), mozzarella cheese and the governing liquid of mozzarella cheese. The same amount of ps_77 and M19 were mixed and 100 µL of the mix were dispensed on six samples for each food type. The samples were incubated at 4 °C for 12 days, until the blue pigment was visible on food. The ratio between the wild-type and the non-pigmenting mutant was checked as previously reported.

4.4. Results and discussion

4.4.1. Transposon mutagenesis of ps_77

Mutagenesis of ps_77 was performed twice to enhance the number of mutants losing the ability of producing the blue pigment. Using the EZ-Tn5™ Tnp Transposome™, $1.35 \cdot 10^5$ single individual transposon insertion mutants were obtained from the two different electroporation events. The application of MBM Agar allowed an easy discrimination of the phenotype, as suggested by previous studies (Andreani *et al.,* 2014; Andreani *et al.,* 2015). The transposon bank for this study was restricted to the 24 non-pigmenting strains and a hyper pigmented strain. All the strains of this study are reported in Table 4.2. The mutants were selected and plated in triplicate at 28 °C on MBM with 50 ng/μL kanamycin to evaluate the pigment phenotype. All the phenotypes observed during the preliminary screening were confirmed by following tests on MBM agar, pointing out a stable insertion of Tn-5 transposon in the genome of ps_77.

4.4.2. Mapping of genes disrupted by transposon-insertion

To investigate the nature of the association between pigment production and genetic information encoded by loci in the chromosome, the gDNA region flanking the transposon insertion sites was sequenced for each selected strain.

The complete list of gene insertions observed in the investigated mutants is reported in Table 4.2. The table reports locus name of the genes referring to ps_77 (PFLuk1, LCYB00000000) and, if present, the ortholog gene in *P. fluorescens* A506 (NC_017911.1).

Of 25 selected mutants, only thrirteen insertions could be mapped using the single-primer PCR method proposed by Karlyshev and colleagues (2000). The remaining mutants were analysed applying the protocol proposed by Walterson and co-workers (2014).

The obtainment of single and clear sequences confirmed the presence of unique genomic insertions of the transposon.

The localization of transposon-insertions allowed the identification of genes putatively involved in blue-pigment biosynthesis. Among the 24 non-pigmenting mutants in analysis,

Table 4.2 - Transposon-induced mutants.

The table reports the mutants obtained through transposon mutagenesis, the strain name, the mapping method, the mapped gene, the contig of ps_77 in which the gene is located, the eventual presence in pfA506 and expression values of each locus.

Mutant strain	Colony name	Mapping method	Disrupted gene	Uk1 Contig	Ortholog gene in *P fluorescens* A506	Expression values			
						P fluorescens A506		Ps_77	
						Fold Changed (original values)	EDGE test: blue vs white. tagwise dispersions P-value	Fold Changed (original values)	EDGE test: blue vs white. tagwise dispersions P-value
M1	T0A	**	PFLuk1_01741 Chromosome partitioning protein ParA	4	*parA* (PflA506_5428)	-1.02781591	0.49948346	-1.13734378	0.375229721
M2	T0C	*	PFLuk1_00668 hypothetical protein	4	-	-	-	-395.694949	2.81E-28
M3	35.1	*	PFLuk1_00667 Tryptophan synthase beta chain trpB_1	4	-	-	-	-451.099036	6.64E-35
M4	24A	**	PFLuk1_00673 UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase	4	-	-	-	-453.944497	1.05E-26
M5	22A	**	PFLuk1_00503 ATP phosphoribosyltransferase hisG	4	*hisG* (PflA506_0882)	-1.27098546	0.978572006	-1.36952819	0.765177214
M6	33e	***	PFLuk1_00673 UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase	4	-	-	-	-453.944497	1.05E-26
M7	12.2	**	PFLuk1_00668 hypothetical protein	4	-	-	-	-395.694949	2.81E-28
M8	19C_new	*	PFLuk1_05081 Pyruvate dehydrogenase E1 component	61	*aceE* (PflA506_0451)	-2.34173209	0.110912328	-2.43704809	0.099306393
M9	8.3	**	PFLuk1_05081 Pyruvate dehydrogenase E1 component	61	*aceE* (PflA506_0451)	-2.34173209	0.110912328	-2.43704809	0.099306393
M10	30A	**	PFLuk1_05080 Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	61	*aceF* (PflA506_0450)	-1.10335984	0.86996368	-1.20683554	0.893746763
M11	-1ω	**	PFLuk1_04071 Carboxynorspermidine/carboxyspermidine decarboxylase	56	*nspC* (PflA506_3051)	1.260451805	0.009581448	1.580249488	0.009893684
M12	28A	*	PFLuk1_05234 Biosynthetic arginine decarboxylase speA	61	*speA* (PflA506_0605)	1.112522611	0.228165571	-1.0046584	0.171771883
M13	10A	*	PFLuk1_03159 Phosphoadenosine phosphosulfate reductase cysH	43	PflA506_3944	-2.09364572	0.106962123	-1.87548971	0.09864557
M14	28α	**	PFLuk1_00099 Glucose-6-phosphate 1-dehydrogenase	4	*zwf_2* (PflA506_4150)	-1.08812816	0.828822035	-1.03678357	0.523444756

M15	26α	*	PFLuk1_02002 Signal transduction histidine-protein kinase BarA_1	12	*gacS* (PflA506_3159)	-1.55430186	0.465181759	-1.76941434	0.698141715
M16	3α	*	PFLuk1_02131 Bifunctional protein Fold protein fold	12	*folD* (PflA506_3307)	2.382202777	0.016522646	2.135688167	0.014157701
M17	26.1	*	PFLuk1_05547 Hydroperoxy fatty acid reductase gpx2	61	PflA506_4459	1.452975992	0.278606599	1.356637028	0.249856091
M18	30γ	*	PFLuk1_00100 6-phosphogluconolactonase pgl_1	4	*pgl* (flA506_4149)	-1.47172272	0.725280275	-1.61658486	0.574110832
M19	3B	*	PFLuk1_00674 UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase wbpE_2	4	-	-	-	-427.014246	7,4321E-23
M20	3C	**	PFLuk1_05423 hypothetical protein	61	*TfoX_C* (PflA506_4519)	-2.60711594	0.104880588	-3.20211632	0.065849233
M21	19α	*	PFLuk1_00600 Nicotinate-nucleotide pyrophosphorylase nadC	4	*nadC* (PflA506_0786)	-1.20444913	0.634847453	-1.31156967	0.333597575
M22	9B	**	PFLuk1_05080 Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	61	*aceF* (PflA506_0450)	-1.10335984	0.86996368	-1.20683554	0.893746763
M23	11b	**	PFLuk1_04714 putative cardiolipin synthase YwiE	60	PflA506_1891	-1.57152497	0.536103866	-1.39138319	0.544109672
M24	24E	*	PFLuk1_05081 Pyruvate dehydrogenase E1 component aceE	61	*aceE* (PflA506_0451)	-2.34173209	0.110912328	-2.43704809	0.099306393
M25	26a	*	PFLuk1_00753 Bifunctional enzyme CysN/CysC CysNC	4	*cysN* (PflA506_0749)	-1.09470358	0.861377937	-1.06296458	0.554846202

* mapping method proposed by Karlyshev *et al.*, 2000.

** mapping method proposed by Walterson *et al.*, 2014 by using *Erh I*

*** mapping method proposed by Walterson *et al.*, 2014 by using *BstAC I*

insertions were localized in nineteen different genes. BlastKOALA tool allowed the annotation of only fifteen entries, belonging to different pathways (mainly concerning metabolism and environmental information processing). Briefly, four sequences were categorized as belonging to amino acid metabolism (mainly of Gly, Ser, Thr, Arg, Pro, His, Phe, Tyr and Trp), a further four to carbohydrate metabolism, three genes are involved in energy metabolism, as well as single genes involved in metabolism of cofactors and vitamins, processing of genetic information, processing of environmental information and lipid metabolism. Table 4.3 details the pathways to which all genes belong. On the other hand, PFLuk1_00753 (disrupted in the hyper pigmented strain) was identified as belonging to Energy metabolisms by BlastKOALA tool.

4.4.3. Investigation of presumptive gene function in blue pigment production

4.4.3.1. Blue-pigmenting strains exclusive genes

Four genes (PFLuk1_0667, PFLuk1_0668, PFLuk1_0673 and PFLuk1_0674) belonging to a genic cluster unique to the blue pigmenting strains were affected by transposon insertions giving a non-pigmented phenotype, in M2, M3, M4, M6, M7 and M19. As already reported in Andreani *et al.,* 2015, these genes show a low level of similarity to other bacterial species genes, (as reported in paragraph 3.4.4.).

PFLuk1_00667 has been annotated as Tryptophan synthase beta chain trpB_1; a more in depth analysis conducted with Hmmer revealed the presence of two protein domains (B3-4 superfamily and TrpB_II superfamily) that allowed Prokka software to annotate the locus. This result partially confirms previous conclusions that indicated all the second copies of *trp* genes to be involved in the blue pigment biosynthesis. In fact, it is not clear whether the absence of *trpA*, *trpC*, *trpD* and *trpF* second copy mutants among the white ones is due the induction by the disruption of these genes of a lethal phenotype or to the fact that these disruptions maintain the blue pigment production. Moreover, due to the low number of non-pigmented transposon-induced mutants obtained, a further transposon mutagenesis or site-

Table 4.3 - COGs of disrupted loci in the investigated mutants.

locus name	Cluster of ortholog category		
PFLuk1_00099	metabolism	global and overview maps	metabolic pathway biosynthesis of secondary metabolites microbial metabolism in diverse environments biosynthesis of antibiotics carbon metabolism
		carbohydrate metabolism	pentose phosphate pathway
		metabolism of other amino acids	glutathione metabolism
PFLuk1_00100	metabolism	global and overview maps	metabolic pathway biosynthesis of secondary metabolites microbial metabolism in diverse environments biosynthesis of antibiotics carbon metabolism
		carbohydrate metabolism	pentose phosphate pathway
PFLuk1_00503	metabolism	global and overview maps	metabolic pathway biosynthesis of secondary metabolites biosynthesis of amino acids carbon metabolism
		amino acid metabolism	histidine metabolism
PFLuk1_00600	metabolism	global and overview maps metabolism of cofactors and vitamins	metabolic pathway nicotinate and nicotinamide metabolism
PFLuk1_00667	metabolism	global and overview maps	metabolic pathway biosynthesis of secondary metabolites biosynthesis of amino acids
		amino acid metabolism	glycine, serine and threonine metabolism phenylalanine, tyrosine and tryptophan biosynthesis

locus name	Cluster of ortholog category		
PFLuk1_02002	environmental information processing	signal transduction	two-component system
PFLuk1_02131	metabolism	global and overview maps	metabolic pathway carbon metabolism microbial metabolism in diverse environments
		energy metabolism	carbon fixation pathways in prokaryotes
		metabolism of cofactors and vitamins	one carbon pool by folate
PFLuk1_03159	metabolism	global and overview maps	metabolic pathway microbial metabolism in diverse environments
		energy metabolism	sulphur metabolism
PFLuk1_04071	metabolism	global and overview maps	metabolic pathway
		amino acid metabolism	arginine and proline metabolism
PFLuk1_05080	metabolism	global and overview maps	metabolic pathway biosynthesis of secondary metabolites microbial metabolism in diverse environments biosynthesis of antibiotics carbon metabolism
		carbohydrate metabolism	glycolysis/gluconeogenesis citrate cycle (TCA cycle) pyruvate metabolism
PFLuk1_05081	metabolism	global and overview maps	metabolic pathway biosynthesis of secondary metabolites microbial metabolism in diverse environments biosynthesis of antibiotics carbon metabolism
		carbohydrate metabolism	glycolysis/gluconeogenesis citrate cycle (TCA cycle) pyruvate metabolism

PFLuk1_05234	metabolism	global and overview maps	metabolic pathway
		amino acid metabolism	arginine and proline metabolism
PFLuk1_05547	metabolism	lipid metabolism	arachidonic acid metabolism
		metabolism of other amino acids	glutathione metabolism

directed mutagenesis might be useful to discern the possible role of *trpACDF* in the biosynthetic pathway.

Specifically, double copies of *trpB* genes have been reported in several bacterial and archaeal species (Xie *et al.,* 2001; Busch *et al.,* 2014). It has been demonstrated that usually the second copy possesses substrate specificity as it recognises not L-serine, but O-Phospho-L-serine, giving a selective advantage to strains having the second copy through a better regulation of intracellular amount of indole (Busch *et al.,* 2014). Additionally, it has been reported that, as usually TrpB assembles with TrpA, TrpB2 is not able to take part in this interaction. Finally, even if contributing to tryptophan biosynthesis, TrpB2 seems to have other functions, such as indole salvage (Hiyama *et al.,* 2014).

The investigation of PFLuk1_00668 did not reveal any interesting information: the locus was annotated as Hypothetical Protein for the similarity to a hypothetical protein of other species and no characteristic domain was identified through Hmmer investigation.

PFLuk1_00673 and PFLuk1_00674 were annotated as UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase. Poor similarity to other species was revealed by a Blast analysis. Both proteins seem to have a characteristic domain, namely a DegT/DnrJ/EryC1/StrS aminotransferase domain. DegT/DnrJ/EryC1/StrS aminotransferases have been widely reported as having a regulatory and protein kinase (sensor) function (Murphy *et al.,* 1993; Madduri and Hutchinson, 1995). In *Bacillus stearothermophilus*, *degT* has a proper regulatory function, being involved in the transfer of environmental stimuli (Takagi *et al.,* 1990). Recently, aminotransferases of this family have been identified as involved in aminotrasfers that lead to amino sugars involved in the formation of LPS and aminoglycosides in *P. porphyromonas* (Shoji *et al.,* 2002). On the other hand, Chen and colleagues (2000a) hypotheses that the lower blue pigment production in *P. porphyromonas degT*$^{-}$ mutants might be due the impossibility of the mutant strain to secrete the extracellular pigment through the production of vesicles. DegT/DnrJ/EryC1/StrS aminotransferase domain, the two amino acidic sequences are quite different. First, PFLuk1_00673 and PFLuk1_00674 length differs for six amino acids, as revealed by the alignment. SIM analysis revealed also a low similarity between the two proteins with 31.6%

identity in 345 residues overlap and Gap frequency of 2.0%. This result induces to suppose the two loci have different functions, even if sharing the same domain.

PFLuk1_00668, PFLuk1_00673 and PFLuk1_00674 seem to belong to a poorly conserved operon, since a similar genetic organization has been observed in some Actinobacteria (e.g. *Nocardiopsis* spp. and *Kitosatospora* spp.), as highlighted through Hmmer investigation of the genes belonging to the unique gene cluster in contig_0004 of ps_77.

4.4.3.2. Investigation of the role of other genes involved in the blue pigment production

PFLuk1_00099 was disrupted in M14 and it has been annotated as a Glucose-6-phosphate 1-dehydrogenase (G6PDH): this enzyme has been widely reported as involved in the pentose phosphate pathway and in the glutathione metabolism. Site-directed mutagenesis in different species revealed the implication of G6PDH in secondary metabolites biosynthesis: Δ*zwf P. aeruginosa* strains lose the ability to produce alginate, a polysaccharide involved in virulence (Silo-Suh *et al.,* 2005).

In addition, PFLuk1_00100 resulted to be disrupted in one of the white mutants (M18). This locus encodes for 6-Phosphogluconolactonase (6PGDH) and the locus name is *pgl* in *P. fluorescens* A506. It is located right downstream *zwf* and catalyses the second step of the super pathway of glycolysis and Entner-Doudoroff pathway, acting just after *zwf*. Interestingly, the disruption of this gene induces a lower production of medium chain length PHAs in *P. putida* (Poblete-Castro *et al.,* 2013).

Several studies reported G6PDH and 6PGDH to be activated in presence of glucose, gluconate, glycerol, fructose and mannitol and mainly in early-mid logarithmic phase of growth (O'Brien, 1975; Hager *et al.,* 2000; Petruschka *et al.,* 2002; Silo-Suh *et al.,* 2005); conversely, Hugouvieux-Cotte-Pattat and Robert-Baudouy reported G6PDH to be repressed by glucose in *Erwinia chrysantemi* (1990). The involvement of carbohydrates in the induction of these genes and their putative role in the blue pigment production agree to previous results, demonstrating that different typologies and amounts of sugars can affect pigment production in agar plates (Andreani, unpublished data).

It has been widely reported that G6PDH and 6PGDH, as other enzymes producing NAD^+ and $NADP^+$ molecules are also involved in resistance to oxidative stress, promoting a reductive

environment (Ma *et al.,* 1998; Singh *et al.,* 2005; Bériault *et al.,* 2007). Butler and co-workers (2002) notified that G6PDH and 6PGDH are responsible of the majority of NADPH molecules produced in the bacterial cell. Two main hypotheses can be done about the reason why the disruption of *zwf* and *pgl* induces the loss of the blue phenotype in ps_77. The first one is because NADPH is an important cofactor of several reactions; the lower production of this cofactor might reduce the amount of NADPH, necessary for the production of the blue pigment. The second one is related to the biosynthetic pathway of the amino acid serine, involving these enzymes to produce the parental compound 3-phospo-D-glycerate. Serine, together with indole, is the substrate of Tryptophan synthase beta chain (TrpB) to form tryptophan. As the blue pigment seems to be an indigo-derivative, a lower amount of serine might affect the amount of tryptophan and the final production of the blue molecule.

PFLuk1_00503 was mapped during the investigation of M5 and it encodes for an ATP-Phosphoribosyltransferase and its ortholog in *P. fluorescens* A506 is *hisG*. *hisG* is involved in the biosynthesis of amino acid histidine. Histidine biosynthetic pathway is linked to the tryptophan biosynthetic pathway, in particular as tryptophan and histidine share a common parental compound (Phosphoribosyl pyrophosphate; PRPP). In 2005, Henry and co-workers reported as the deletion of *trpC* induced the accumulation of PRPP in *Salmonella typhimurium*, reducing the activity of *hisG* and the downstream genes. Supposing that the opposite can also take place and that tryptophan metabolism is involved in the blue pigment production, it can be deduced that the disruption of *hisG* might involve the accumulation of PRPP and a downregulation of *trp* metabolism, inducing the loss of the blue pigment phenotype.

One of the non-pigmenting mutants (M21) was characterised by the disruption of PFLuk1_00600, namely the nicotinate-nucleotide phosphorylase *nadC*. This enzyme crosses again the tryptophan biosynthetic pathway, as it requires PRPP to produce nicotinamide mononucleotide, a precursor of NAD (Kurnasov *et al.,* 2003). In *Burkholderia* spp. and *Pseudomonas*, *nadC* is involved in the biosynthesis of secondary metabolites, such as fitormones and indole-3-acetic acid (Patten and Glick, 2002; Wang *et al.,* 2006). Again, two hypotheses can be formulated about the involvement of this locus in the blue pigment

production: the first one involves the accumulation of PRPP, as already stated for *hisG* $^-$. However, as *nadC* is involved in the production of NAD, the lower amount of the cofactor might reduce the utilization of secondary metabolites pathway, as a way to save energy for basal metabolism.

PFLuk1_01741 encodes for the chromosome partition protein ParA; this protein belongs to Walker-Type ATPases protein family and it is involved in chromosome partitioning during cell division (Lasocki *et al.,* 2007; Bartosik *et al.,* 2014). In most species, *parA* is not essential, as the phenotype of *parA*$^-$ strains is not lethal (with the exception of *Caulobacter crescentus*; Mohl and Gober, 1997). *parA* mutants show species-dependent phenotypes: alteration in motility has been demonstrated in *P. aeruginosa*, with no swarming and defective swimming motility (Lasocki *et al.,* 2007). The authors hypothesised this could be due to a defect in signalling system between cells or, less likely, to the disturbance of pool of ATP, necessary for the activity of movement apparatus. *parA* knock-out strains of *P. putida* were characterised by a defective phenotype (characterised by an high number of anucleate cells and smaller dimensions of cells) in presence of a minimal medium (such as M9) and at lower temperature, suggesting a putative role of ParA protein in management of the chromosome in stressing conditions (Godfrin-Estevenon *et al.,* 2002)

One non-pigmenting mutant (M15) was characterised by disruption of gene encoded by PFLuk_02002, namely a signal transduction histidine-protein kinase BarA, annotated as *gacS* in *Pseudomonas* species. *gacS* belongs to the GacS/GacA two-component system, involved in the signal/transduction pathway. Briefly, GacS protein transmembrane domain senses different (not already completely understood) stimuli and induces a phosphorylative cascade. GacS encounters an autophosphorylation, than the phosphoric group is moved to the cognate GacA that changes its conformation and regulates transcription of a wide range of genes. GacS/GacA system has been reported to regulate several phenotypes in bacteria. In *E. coli,* BarA system regulates iron acquisition, through the modulation of siderophore biosynthesis (Sahu *et al.,* 2003). In *Pseudomonas fluorescens* FD6 (a biocontrol strain), it has been demonstrated that *gacS* knock-out strains cannot produce a wide range of secondary metabolites. In particular, biofilm formation and siderophore production were

downregulated, as well as the protection activity against *Botrytis cinerea* HY2-1 (Chang *et al.,* 2014). *ΔgacS Pseudomonas chlororaphis* strain PA23 showed no growth defect; however, defects in colonisation and pathogenicity of *Caenorhabditis elegans* were observed as the result of the loss of production of toxic metabolites including pyrrolnitrin, phenazine and hydrogen cyanide (Nandi *et al.,* 2015). Kim and colleagues reported a strict correlation between tryptophan biosynthesis and GacS regulation; in particular, *P. chlororaphis* O6 *gacS*$^{-}$ strains showed a low expression of *trpE* gene, namely the enzyme responsible of the first step of tryptophan production (Kim *et al.,* 2014a). The same research group reported that a mutation in *gacS* gene in the previously studied *P. chlororaphis* strain induced changes in morphology (cells appear elongated) and motility (with an increased number of flagella; Kim *et al.,* 2014b). To summarise, it seems that the metabolic pathways influenced by the GacS/GacA system are dependent on genetic background. However, the role of this protein in the blue pigment production is obvious, either in the induction of genes involved in the blue pigment production or in the sensing of extracellular stimuli inducing the pigment biosynthesis.

PFLuk1_02131 (disrupted in M16) encodes for the Bifunctional protein Fold protein, also annotated as bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase. This enzyme is important for bacterial cell, together with all enzymes for the biosynthesis of folate, because of the ability to produce important cofactors, mainly NADPH (Eadsforth *et al.,* 2012; Sah and Varshney, 2015). It can be hypothesised that the loss of pigment production might be due to a significant stress in which *Pseudomonas* strains might switch toward the maintenance of only basal metabolism.

M13 was characterised by the insertion in locus PFLuk1_03159, encoding a phosphoadenosine phosphosulfate reductase (namely CysH). CysH is involved in sulphur metabolism and in the biosynthetic pathway of two amino acids, cysteine and methionine. This protein, similarly to others containing reduced-sulphur, are involved in resistance to oxidative stress (Senaratne *et al.,* 2006). In *P. aeruginosa, cysH* is involved in biofilm production (Xu *et al.,* 2013). The absence of pigment production after the disruption of PFLuk1_03159 might be an indirect effect: the higher oxidative stress that bacterial cell

encounters because of *cysH* deletion, might reduce the inclination to produce an expensive molecule, such the blue pigment. On the other hand, hypothesizing the importance of biofilm formation in the production of the pigment, *cysH* deletion might nullify the blue-pigment production in ps_77.

PFLuk1_04071 was disrupted in strain M11: this locus encodes for *nspC*, a carboxynorspermideine/carboxyspermidine decarboxylase. In *Vibrio cholerae*, increased activity of NspC induces a higher production of biofilm, reducing, in the meantime, motility (Parker *et al.,* 2012). A similar result was obtained for *Salmonella enterica* (Nesse *et al.,* 2015). Actually, recent investigations revealed exactly the opposite trend, revealing as different levels of norspermidine remarkably prevent biofilm formation in *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli* affecting cellular adhesion and reducing exopolysaccharide production (Si *et al.,* 2015). No evidences are nowadays available about further NspC function in *P. fluorescens*, therefore no real conjectures can be argued. The phenotypic characterisation of M11 mutant might shed light on the function of the protein to further investigate its role in pigment biosynthesis.

PFLuk1_04714 was disrupted in one non-pigmenting ps_77 mutant (M23): genomic annotation performed in a previous study (Andreani *et al.,* 2015) revealed it to be a putative cardiolipin synthase, as identified in a phenazine-producing *P. fluorescens* strain. This enzyme catalysed the production of cardiolipin (CL) in a wide range of prokaryote and eukaryote organisms (Carman, 2012) and is supposed to have a role in energy production. Lin and co-workers revealed a correlation between CL production and cell shape and the ability to produce biofilm in *Rhodobacter sphaeroides* (Lin *et al.,* 2015).

Five non-pigmenting mutants (M8, M9, M10, M22 and M24) had insertions in PFLuk_05080 and PFLuk_05081, namely *aceF* and *aceE* genes, coding for two of the three subunits of pyruvate dehydrogenase complex (Patel *et al.,* 2014). These enzymes catalyse the formation from Pyruvate of Acetyl-Coenzyme A (Acetyl-CoA) and ATP. After the production, Acetyl-CoA enters the TCA cycle or it is used for the synthesis of ATP and acetate, an important carbon source for the cell. The disruption of these loci directly perturbs the energetic situation of the cell that encounters a stressing event as also reported in the study of

Tomar and co-workers (2003). As already highlighted for PFLuk1_00600, supposing that the blue pigment production might require high energetic cost, *Pseudomonas* might be disadvantaged and might principally invest energy in the basal metabolism. This seems to be likely as it involved metabolism of tryptophan, whose biosynthesis is biochemically the most expensive in bacteria (Bentley, 1990).

Another gene involved in polyamines biosynthesis (PFLuk1_05234) was interested in a disruption in one of the non-pigmenting mutants (M12). This locus encodes for the biosynthetic arginine decarboxylase, responsible of the synthesis of agmatine from arginine, a precursor of putrescine (Moore and Boyle, 1990). The deletion of *speA* gene in *Yersinia pestis* corresponds to two main events: the lower production of putrescine and the decrease in biofilm formation attitude (Patel *et al.,* 2006). Putrescine has actually different functions, others than biofilm production: in fact, as other polyamines, it protects bacteria from oxidative stress, as demonstrated in *E. coli* (Chattopadhyay *et al.,* 2003). In *Proteus mirabilis*, putrescine acts as signal molecules to induce swarming motility (Sturgill and Rather, 2004). Thanks to these data, it seems evident the involvement of bacterial signalling and biofilm formation, as a prerogative for the pigment biosynthesis.

M20 was characterised by gene disruption of PFLuk1_05423, annotated as Hypothetical protein by Prokka, showing high level of similarity with *tfoX_C* encoding for a competence protein. No particular phenotype has been reported in literature as a result of gene deletion, if not the loss of competence, namely the ability to uptake DNA from the environment (Smeets *et al.,* 2006). It is not clear how this gene could be involved in the pigment production.

Finally, one non-pigmenting mutant (M17) was characterised by the disruption of PFLuk1_05547, annotated as an hydroperoxy fatty acids reductase *gpx2*, that is a glutathione peroxidase involved in oxidative stress resistance (Leisinger *et al.,* 2001). Interestingly, the activity of this enzyme is induced under oxidative stress and is addressed against large hydroperoxydes, such as nucleic acids, proteins and fatty acids (Avery and Avery, 2001). As previously supposed, the blue pigment biosynthetic pathway might cause oxidative stress

issue to bacterial cell. Indeed, no production is visible when an important enzyme for oxidative stress is silenced.

To summarize, the blue pigment biosynthetic pathway is strictly related to *trp* genes, confirming the importance of the genomic region previously identified in contig_0004 of ps_77. Actually, a more in depth analysis of this genetic cluster is necessary to understand the function of each gene. The production seems to be strictly related to the activity of the two component system GacS/GacA, involved in several cell functions, such as motility, biofilm formation, signalling among cells and secondary metabolite production. It is again not clear whether the pigment acts as a signal molecule or it requires signalling molecules to be synthesized. Similar conclusions can be done for the correlation between biofilm formation and blue pigment production. Anyway, it seem likely to suppose that defects in the energetic apparatus are correlated to a reduction of biochemically expensive metabolite production, such as those involving tryptophan.

4.4.4. Evaluation of gene expression

Evaluation of the gene expression aimed the identification of differential expression among two blue (ps_22 and ps_77) and two non-pigmenting strains (ps_20 and ps_40) as a way to confirm the real involvement of genes previously identified through the mapping in the mutants. Results are reported in Table 4.2. Both expression values evaluated using *P. fluorescens* A506 and ps_77 as reference genome are reported, based on a previous transcriptomic experiment (Andreani *et al.,* 2015). Differential gene expression was considered with p-values lower than 0.01.

No differential gene expression was highlighted using *P. fluorescens* A506 as reference genome, made exception for expression of *nspC,* weakly significantly over expressed in white strains (p-value of 0.0099). Similar results were obtained using ps_77 as reference genome, except for the genes belonging to the blue strain unique cluster. For these genes, significance of differential expression is due to the absence of those genes in the white strains. To verify the actual expression at high levels of those genes in the blue-pigmenting strains, as a confirmation of the real involvement of these genes in the blue pigment production, number of reads that mapped to PFLuk1_0667, PFLuk1_0668, PFLuk1_0673 and PFLuk1_0674 was

checked. Number of reads of ps_22 and ps_77 mapping against ps_77 used as reference genome was substantially high, especially for *trpB_1* and *wbpE_1,* showing more than 18,000 and 9,000 reads respectively (see Table 4.4 for details). Indeed, number of reads for the aforementioned loci was comparable or substantially higher to number of reads mapping to *gyrB*, a housekeeping gene. These results further support putative role of these genes in the blue pigment production.

Table 4.4 - Number of transcriptome reads mapping to ps_22 and ps_77 genomes.

Locus name	Locus number	Number of reads	
		Ps_22	**Ps_77**
trpB_1	PFLuk1_00667	14,290	18,410
-	PFLuk1_00668	2,122	1,621
wbpE_1	PFLuk1_00673	2,652	1,939
wbpE_2	PFLuk1_00674	15,778	9,751
gyrB	PFLuk1_01753	2,688	3,800

Expression results highlight that disrupted genes, other than those belonging to the blue-strain unique cluster, might have multiple functions in *P. fluorescens* and in some cases might only be indirectly responsible in the pigment production. Specifically, the knock-out of these genes creates the conditions and the assumptions that make the blue pigment production impossible or disadvantaged.

4.4.5. Phenotypic characterisation of the mutants

4.4.5.1. Growth curves

Growth rate was investigated for the wild-type strain (ps_77), the hyper pigmented strain (M25) and five non-pigmenting mutants (M2, M3, M19, M20 and M21) in KB broth at 28 °C. Furthermore, also *P. fluorescens* SBW25 and PBR840 were included in the analysis and used as control strains. Growth curves in normal KB are reported in Figure 4.1, panel A.

Growth rate of each investigated strain was calculated using DMFit of ComBase. Even if the model was initially created for the investigation of growth data in log CFU format, recent investigations revealed its applicability also to optical density-based growth curve (Mytilinaios *et al.,* 2012; Rickett *et al.,* 2015). Growth rates are reported in Table 4.5.

As expected the wild-type strain of *P. fluorescens* SBW25 had a higher growth rate (0.0911 ± 0.019) in comparison to the non-producer cheater (0.0627 ± 0.00451; Figure 4.1).

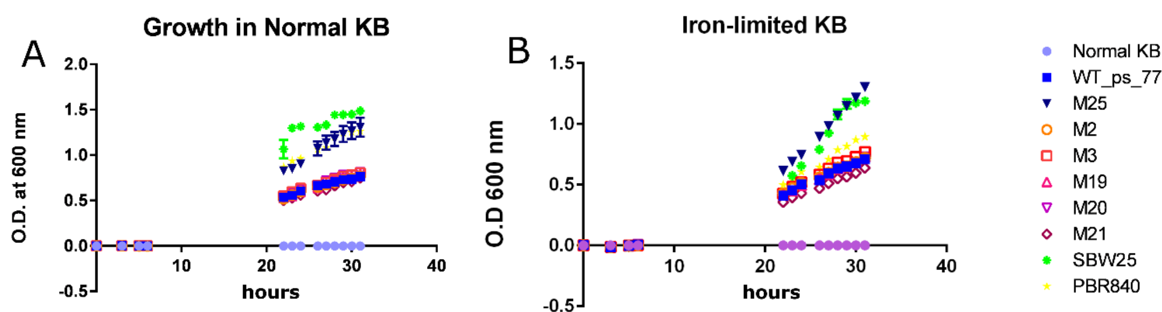


Figure 4.1 - Growth curves in KB and in iron-free KB.

Growth curves in KB (Panel A) and in iron-free KB (Panel B) by measuring the O.D. at 600 nm. SD is reported in the graph.

Table 4.5 - Growth rates calculated through Combase.

	Growth rate in KB	Growth rate in iron-free KB	Note
Ps_77	0.0398 ± 0.00275	0.0358 ± 0.00116	wild-type strain
M25	0.0672 ± 0.00375	0.0757 ± 0.00104	blue (hyper pigmented)
M2	0.0376 ± 0.00219	0.0354 ± 0.00106	white
M3	0.0403 ± 0.00251	0.0382 ± 0.000853	white
M19	0.0391 ± 0.00215	0.036 ± 0.00119	white
M20	0.0414 ± 0.00198	0.0374 ± 0.00103	white
M21	0.035 ± 0.00237	0.0309 ± 0.00087	white
SBW25	0.0911 ± 0.019	0.101 ± 0.00766	Control strain
PBR840	0.0627 ± 0.00451	0.0475 ± 0.00303	Control strain

Interestingly, ps_77 and the analysed non-pigmenting mutants had a similar growth rate. One exception was observed: M25, the blue pigmenting strain characterised by a massive production of the pigment (Figure 4.2), grew with a higher rate (0.0672 ± 0.00375), showing a better fitness in comparison to other mutants.

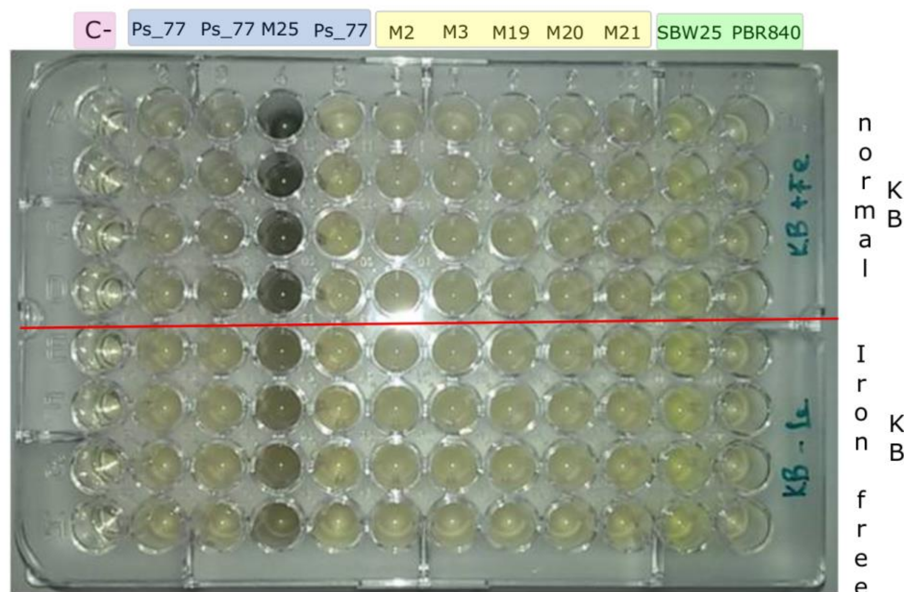


Figure 4.2 - 96-well plate for the evaluation of growth and pigment production of the strains.

4.4.5.2. Growth curves in iron-free condition and evaluation of siderophore production

Growth rate in absence of free-iron was investigated in KB with 100 µg/mL human apotransferrin and 20 mM sodium bicarbonate (Figure 4.1, Panel B).

As already reported (Moon *et al.,* 2007; Luján *et al.,* 2015), *P. fluorescens* SBW25 growth rate was superimposable in both normal and iron-limited KB (0.0911 ± 0.019 and 0.101 ± 0.00766, respectively) as expected due to the production of pyoverdine. A lower growth rate was observed for the cheater strain, unable to synthesize the siderophore (Moon *et al.,* 2007). Similar growth rates were observed for ps_77 and mutant strains in KB broth and KB broth with apotransferrin. Indeed, ps_77 strain is a siderophore producer: in addition to pyoverdine, also pseudomonine is produced (Andreani, personal communication). These results lead to the conclusion that the blue molecule has not a siderophore function: if this hypothesis was verified, a reduced growth rate had to be observed in non-pigmenting mutants in iron-depletion condition. Actually, the WT strain and the mutants, with the exception of M25, have a similar growth rate.

M25 was able to grow better in absence of iron (with growth rates of 0.0672 ± 0.00375 and 0.0757 ± 0.00104, respectively in normal KB and iron-free KB), having a similar growth rate to *P. fluorescens* SBW25 that is a well-known producer of siderophores. After 31 hours of

growth, M25 produced a high amount of pigment compared to the wild-type strain (as reported in Figure 4.2). In particular, the blue-pigment production was more pronounced in normal KB than in presence of chelators. These results suggested that M25 is a strong siderophore producer.

To confirm previous findings, the liquid CAS assay was conducted at 31 hours of growth, when a dark halo was visible in the wells containing M25 and only a grey shadow was visible for ps_77. Interestingly, already at 31 hours of growth an intense orange pigment was visible for M25 because of the activity of siderophores in solution, able to force out iron from preformed HDTMA-Fe complexes (Alexander and Zuberer, 1991). Moreover, the colorimetric reaction was more marked in iron-free environment, according to expectation, as siderophores are usually produced in condition of poor availability of iron (Cornelis, 2010). No colorimetric reaction was visible for ps_77, other mutants, *P. fluorescens* SBW25 and PBR840. Results are reported in Figure 4.3.

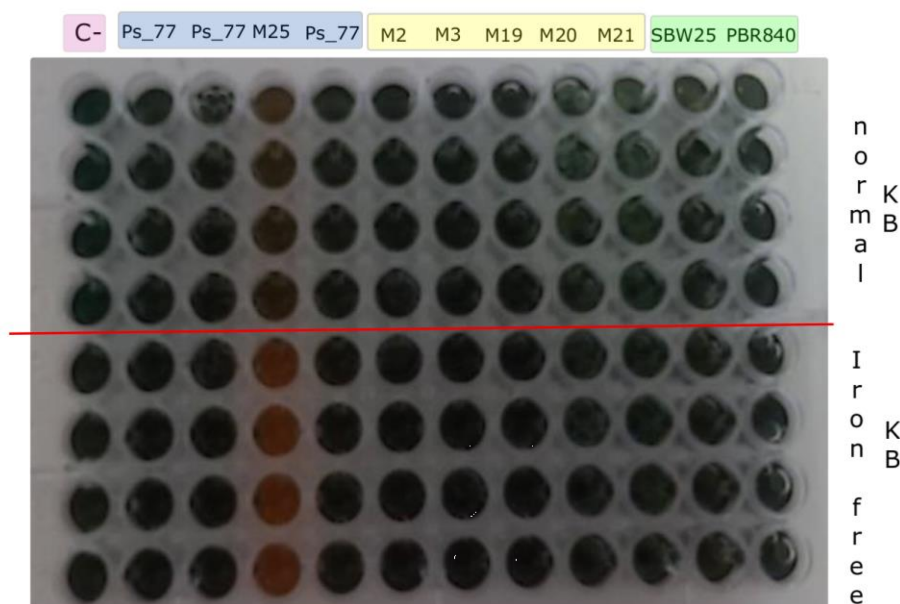


Figure 4.3 - 96-well plate for the execution of the CAS assay.

It seems most likely that the blue pigment is not a siderophore, as growth rate in normal and iron-limited KB broth were similar for the blue- and non-pigmenting strains. Moreover, it could be demonstrated that M25 is able to produce a great amount of the pigment, as well as of siderophores. These results are consistent with a scenario where iron-uptake through the production of siderophores is involved in the production of the blue molecule.

4.4.5.3. Utilization of carbon sources

In bibliography, Biolog GN2 Micro Plates were used to investigate the utilization of different carbon sources. The major aim was to identify a common patter of the blue-pigmenting mutant (M25) and the wild-type strain, not shared by the non-pigmenting strains. No correlation was observed between the ability to produce the pigment and the utilization of the different carbon sources (Figure 4.4)

The comparison of the obtained results with Biolog GN2 results reported by Arnaut-Rollier and colleagues (1999) revealed a really similar utilisation pattern of ps_77 and its transposon-induced mutants with *Pseudomonas fluorescens* strains isolated from poultry meat. Few exceptions were highlighted, such as absent or reduced growth in presence of N-acetyl-D-Galactosamine, Maltose, D-Psicose, α-Keto-Butyric Acid, α-Keto-Valeric Acid, Succinamic Acid, L-Alaninamide, L-Leucine and D-Serine and an higher growth on Sucrose than previously reported. These results demonstrated the extreme heterogeneity of the strains belonging to the *P. fluorescens* species complex strains (Silby *et al.,* 2011).

4.4.5.4. Competition experiment

With the aim to highlight whether the pigment production has the ability to confer higher fitness to *P. fluorescens* strains, competition experiments were set up. The growth rate of the two chosen strains (ps_77 and M19) was superimposable in KB and iron-limited KB, as already reported in the paragraphs 4.4.5.1. and 4.4.5.2.

The variation of the ratio of the two strains at three temperatures is summarised in Figure 4.5. Ratio values have been previously normalized using the real ratio measured at the beginning of the competition test, so that 0 value corresponds to no deviation from the starting ratio.

It is possible to notice that the variation measured considering the six replicates for each tested condition is considerably high. This result clearly indicates that there is a heterogeneous behaviour of the two strains. In particular, at 4 °C the ratio between the two strains is fluctuating close to zero.

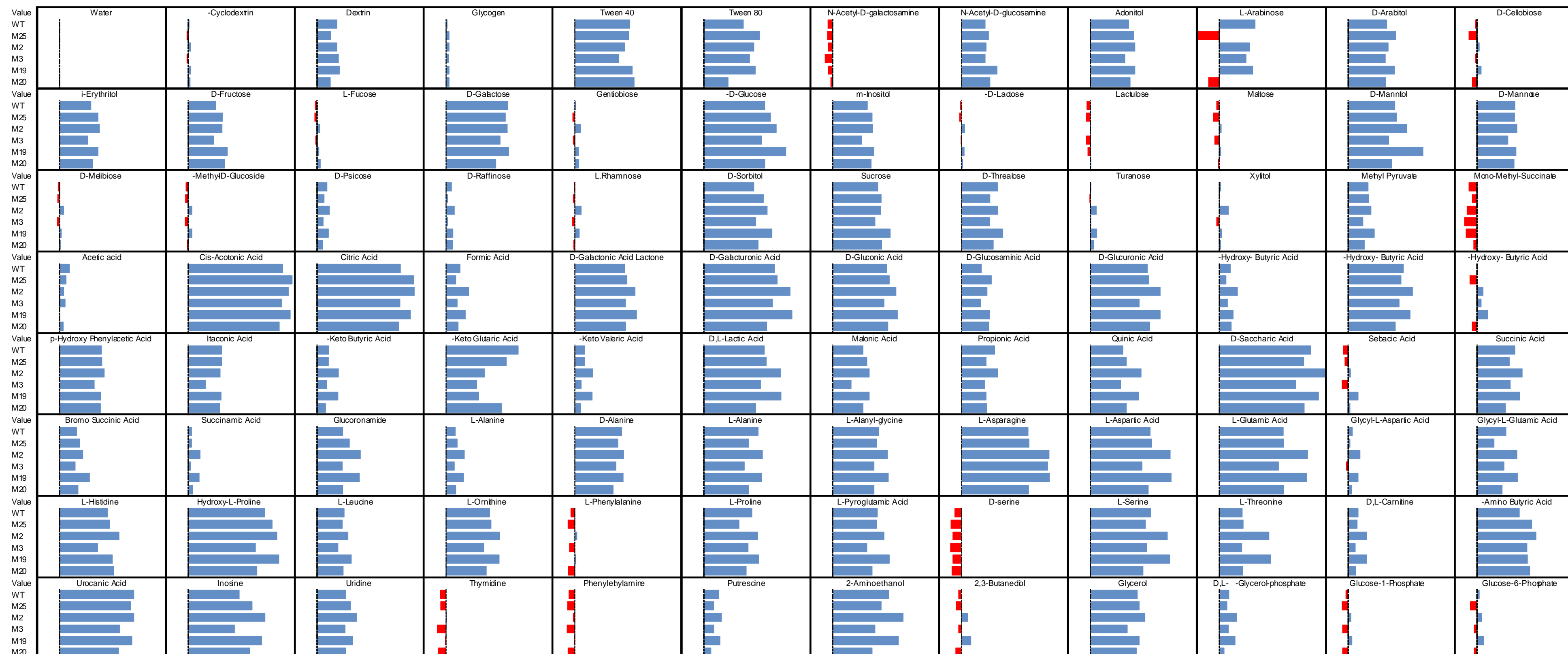


Figure 4.4 - Biolog GN2 test.

Blue bars represent growth measured through a Plate reader as reported in the Material and methods. Red bars represent no growth in presence of the reported carbon source.

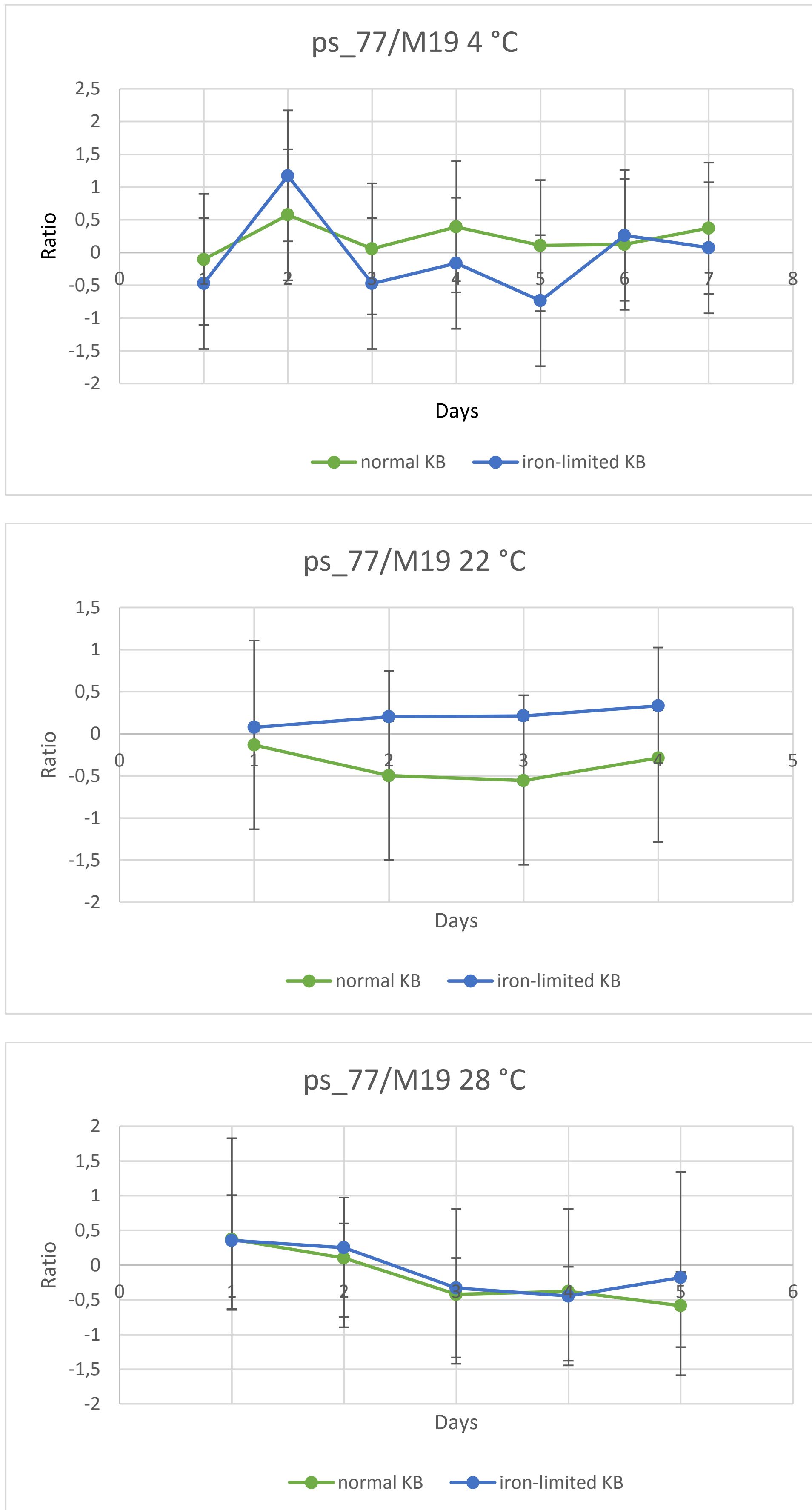


Figure 4.5 - Ratio of tested strains during the competition experiment in normal KB and iron-limited KB.

At 22 °C, ps_77 seems to have a better fitness in iron-free conditions, in comparison to the growth in normal KB. Finally, the white mutant showed a better fitness at 28 °C, without overcoming on the other strain.

The competition experiment was performed also on four food matrices already tested in previous challenge tests (Andreani, personal communication). The same amount of the two overnight cultures was mixed and spotted on about 1 cm^3 of food or in 1 mL of governing liquid of mozzarella cheese. Ratio between the two strains was measured in the mixture spotted at the beginning and when the blue pigment was visible at least in one of the samples. In Figure 4.6, pigment production in the samples is reported.

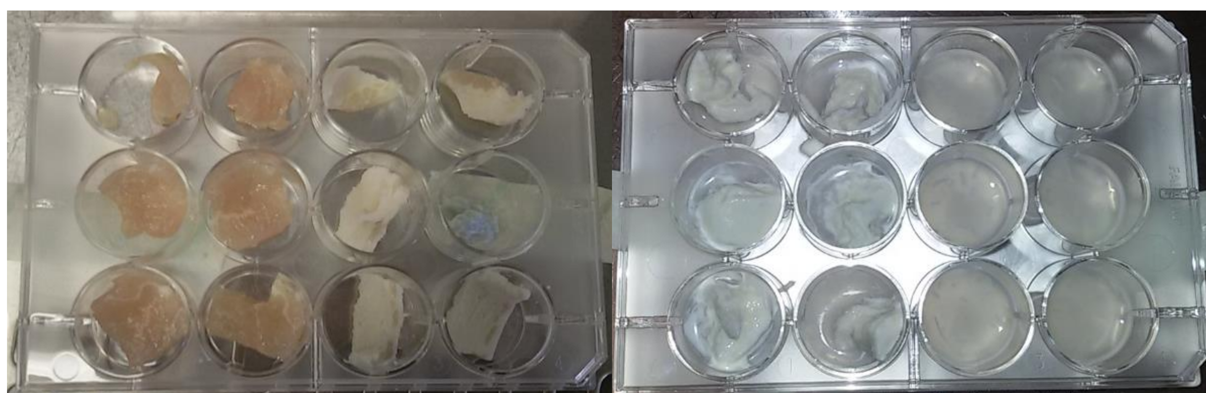


Figure 4.6 - Competition experiment in food matrices.

Ratio between the two strains is reported in Figure 4.7.

Again, the ratio between the two strains fluctuates in the six replicates. These results agree with the ones obtained by Luján and co-workers when observing the competition between the WT strain of *P. fluorescens* SBW25 and its cheater strain PBR840 (Luján *et al.,* 2015). In this study the ratio between the two strains was close to 1, underlying that several *Pseudomonas* are able to take up siderophores produced and released in the environment by other bacteria (Cornelis, 2010). Excluding the molecule to be a siderophore, it is likely that the blue pigment could have a favourable effect also to non-pigmenting strains sharing the environment with the producers, whatever its function was.

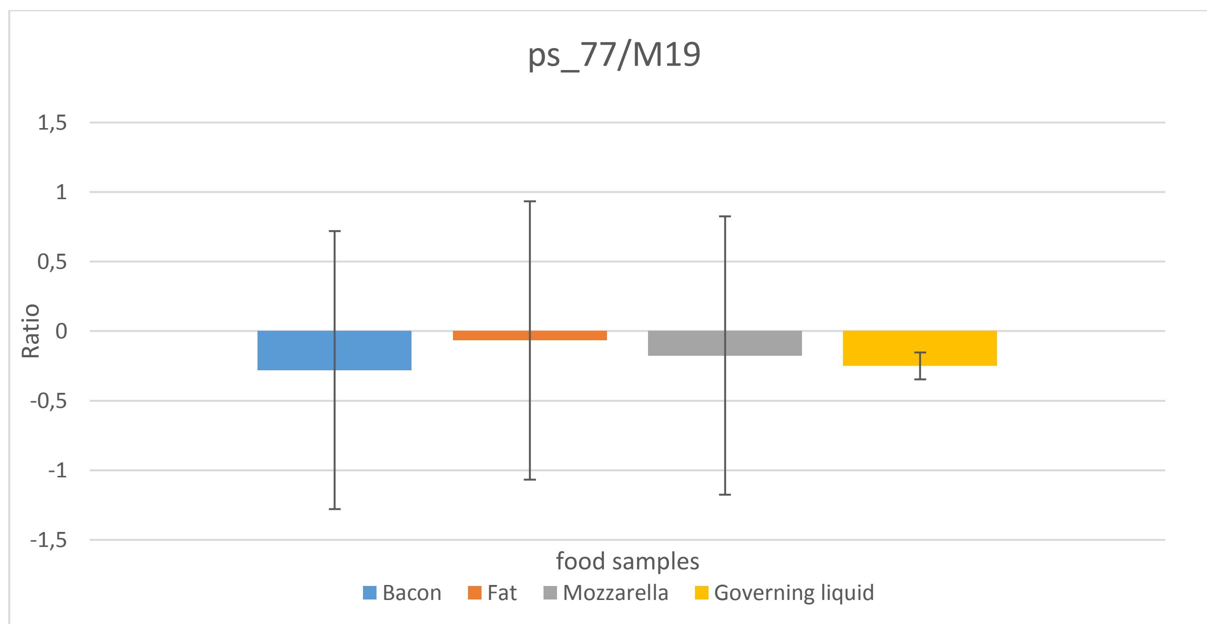


Figure 4.7 - Ratio of the strains during the competition experiment on food matrices.

4.5. Conclusion

In the present work, the blue pigment production of ps_77, a *P. fluorescens* strain was investigated. The strategy was to isolate mutants presenting insertions in genes involved directly or having an indirect effect on pigment production with the aim to find genes responsible for pigment production.

As expected, sequencing of the flanking regions to the transposons confirmed the importance of a unique gene cluster in the blue pigment production, containing *trp* genes. Additionally, genes previously not implicated and other loci whose role in the biosynthesis and excretion has to be disclosed have been reported in this study.

Several mechanisms are involved in the blue molecule production, such as oxidative stress response, biofilm formation and signalling among cells, such as production of quorum sensing molecules. However, the biosynthetic process seems to be centered on tryptophan metabolism, as several genes investigated lead to tryptophan biosynthetic pathway.

Iron plays a key role in the biosynthetic pathway as its enhanced uptake induces a higher pigment production. No visible enhanced fitness has been reported through competition experiments, underlying the possibility of the non-producing strains to benefit of the pigment produced by the blue-pigmenting strains growing in co-culture.

To conclude, further studies are required to shed light on the complex process of the biosynthesis. However, this leading study helps in clarifying next goals for a complete understanding of the pigment and its function.

4.6. Acknowledgements

The study was supported by the University of Padova (Progetto di Ricerca di Ateneo 2011 to L.F.) and by the PhD school of Veterinary Science of the University of Padova which has supported the education of N.A.A. The authors are grateful to Adela Luján for providing *P. fluorescens* SBW25 and PBR840 strains. Finally, N.A.A. is grateful to Fondazione Ing. Aldo Gini that supported her internship to Penryn (Cornwall, the U.K).

4.7. References

- Alexander, D.B., Zuberer, D.A., 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biology and Fertility of Soils, 12, 39-45.
- Andreani, N.A., Martino, M.E., Fasolato, L., Carraro, L., Montemurro, F., Mioni, R., Bordin, P., Cardazzo, B., 2014. Tracking the blue: a MLST approach to characterise the *Pseudomonas fluorescens* group. Food Microbiology, 39, 116-126.
- Andreani, N.A., Carraro, L., Martino, M.E., Fondi, M., Fasolato, L., Miotto, G., Magro, M., Vianello, F., Cardazzo, B., 2015. A genomic and transcriptomic approach to investigate the blue pigment phenotype in *Pseudomonas fluorescens*. International Journal of Food Microbiology, 213, 88-98.
- Arnaut-Rollier, I., Vauterin, L., De Vos, P., Massart, D.L., Devriese, L.A., De Zutter, L., Van Hoof, J., 1999. A numerical taxonomic study of the *Pseudomonas* flora isolated from poultry meat. Journal of Applied Microbiology, 87, 15-28.
- Avery, A.M., Avery, S.V., 2001. *Saccharomyces cerevisiae* expresses three phospholipid hydroperoxide glutathione peroxidases. Journal of Biological Chemistry, 276, 33730-33735.

- Bangera, M.G., Thomashow, L.S., 1996. Characterization of a genomic locus required for synthesis of the antibiotic 2,4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. Molecular Plant-Microbe Interactions, 9, 83-90.
- Baranyi, J., Tamplin, M.L., 2004. ComBase: a common database on microbial responses to food environments. Journal of Food Protection, 67, 1967-1971.
- Bartosik, A.A., Glabski, K., Jecz, P., Lasocki, K., Mikosa, M., Plochocka, D., Thomas, C.M., Jagura-Burdzy, G., 2014. Dissection of the region of *Pseudomonas aeruginosa* ParA that is important for dimerization and interactions with its partner ParB. Microbiology, 160, 2406-2420.
- Bentley, R., 1990. The shikimate pathway-A metabolic tree with many branches. CRC Critical Reviews in Biochemistry and Molecular Biology, 25, 307-384.
- Bériault, R., Hamel, R., Chenier, D., Mailloux, R.J., Joly, H., Appanna, V.D., 2007. The overexpression of NADPH-producing enzymes counters the oxidative stress evoked by gallium, an iron mimetic. Biometals, 20, 165-176.
- Boles, B.R., Thoendel, M., Singh, P.K., 2004. Self-generated diversity produces "insurance effects" in biofilm communities. Proceeding of the National Academy of Sciences, 101, 16630-16635.
- Busch, F., Rajendran, C., Mayans, O., Löffler, P., Merkl, R., Sterner, R., 2014. TrpB2 enzymes are O-phospho-l-serine dependent tryptophan synthases. Biochemistry, 53, 6078-6083.
- Butler, M.J., Bruheim, P., Jovetic, S., Marinelli, F., Postma, P.W., Bibb, M.J., 2002. Engineering of primary carbon metabolism for improved antibiotic production in *Streptomyces lividans*. Applied and Environmental Microbiology, 68, 4731-4739.
- Caputo, L., Quintieri, L., Bianchi, D.M., Decastelli, L., Monaci, L., Visconti, A., Baruzzi, F., 2015. Pepsin-digested bovine lactoferrin prevents Mozzarella cheese blue discoloration caused by *Pseudomonas fluorescens*. Food Microbiology, 46, 15-24.

- Carman, G.M., 2012. An unusual phosphatidylethanolamine-utilizing cardiolipin synthase is discovered in bacteria. Proceedings of the National Academy of Sciences of the United States of America, 109, 16402-16403.
- Chang, L., Xiao, Q., Tong, Y.-H., Xu J.-Y., Zhang Q.-X., 2014. Functional Analysis of the *gacS* Gene in a Tomato Grey Mould Suppressive Bacterium *Pseudomonas fluorescens* FD6. Acta Horticulturae Sinica, 41, 681-686.
- Chattopadhyay, M.K., Tabor, C.W., Tabor, H., 2003. Polyamines protect *Escherichia coli* cells from the toxic effect of oxygen. Proceedings of the National Academy of Sciences of the United States of America, 100, 2261-2265.
- Chen, T., Dong, H., Yong, R., Duncan, M.J., 2000a. Pleiotropic pigmentation mutants of *Porphyromonas gingivalis*. Microbial Pathogenesis, 28, 235-247.
- Chen, T., Dong, H., Tang, Y.P., Dallas, M.M., Malamy, M.H., Duncan, M.J., 2000b. Identification and cloning of genes from *Porphyromonas gingivalis* after mutagenesis with a modified Tn4400 transposon from *Bacteroides fragilis*. Infection and Immunity, 68, 420-423.
- Choi, K., Kumar, A., Schweizer, H.P., 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. Journal of Microbiological Methods, 64, 391-397.
- Cornelis, P., 2010. Iron uptake and metabolism in pseudomonads. Applied Microbiology and Biotechnology, 86, 1637-1645.
- Eadsforth, T.C., Maluf, F.V., Hunter, W.N., 2012. *Acinetobacter baumannii* FolD ligand complexes --potent inhibitors of folate metabolism and a re-evaluation of the structure of LY374571. FEBS Journal, 279, 4350-4360.
- Finn, R.D., Clements, J., Arndt, W., Miller, B.L., Wheeler, T.J., Schreiber, F., Bateman, A., Eddy, S.R., 2015. HMMER web server: 2015 update. Nucleic Acids Research, 43, W30-W38.

- Godfrin-Estevenon, A.M., Pasta, F., Lane, D., 2002. The *parAB* gene products of *Pseudomonas putida* exhibit partition activity in both *P. putida* and *Escherichia coli*. Molecular Microbiology, 43, 39-49.
- Hager, P.W., Calfee, M.W., Phibbs, P.V., 2000. The *Pseudomonas aeruginosa devB/SOL* homolog, *pgl*, is a member of the hex regulon and encodes 6-phosphogluconolactonase. Journal of Bacteriology, 182, 3934-3941.
- Henry, T., García-Del Portillo, F., Gorvel, J.P., 2005. Identification of *Salmonella* functions critical for bacterial cell division within eukaryotic cells. Molecular Microbiology, 56, 252-267.
- Hiyama, T., Sato, T., Imanaka, T., Atomi, H., 2014. The tryptophan synthase β-subunit paralogs TrpB1 and TrpB2 in *Thermococcus kodakarensis* are both involved in tryptophan biosynthesis and indole salvage. FEBS Journal, 281, 3113-3125.
- Huang, X., Miller, W., 1991. A time-efficient, linear-space local similarity algorithm. Advances in Applied Mathematics, 12, 337-357.
- Hugouvieux-Cotte-Pattat, N., Robert-Baudouy, J., 1990. Molecular analysis of the *Erwinia chrysanthemi* region containing the *kdgA* and *zwf* genes. Molecular Microbiology, 11, 67-75.
- Inglis, R.F., Brown, S.P., Buckling, A., 2012. Spite versus cheats: competition among social strategies shapes virulence in *Pseudomonas aeruginosa*. Evolution, 66, 3472-3484.
- Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2015. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Research. pii: gkv1070.
- Kang, B.R., Han, S.H., Cho, S.M., Anderson, A.J., Kim, I.S., Park, S.K., Kim, Y.C., 2008. Characterization of a Homogentisate Dioxygenase Mutant in *Pseudomonas chlororaphis* O6. Current Microbiology, 56, 145-149.
- Karlyshev, A.V., Pallen, M.J., Wren, B.W., 2000. Single-Primer PCR Procedure for Rapid Identification of Transposon Insertion Sites. BioTechniques, 28, 1078-1082.

- Kim, C.H., Kim, Y.H., Anderson, A.J., Kim, Y.C., 2014a. Proteomic Analysis of a Global Regulator GacS Sensor Kinase in the Rhizobacterium, *Pseudomonas chlororaphis* O6. The Plant Pathology Journal, 30, 220-227.
- Kim, J.S., Kim, Y.H., Anderson, A.J., Kim, Y.C., 2014b. The Sensor Kinase GacS Negatively Regulates Flagellar Formation and Motility in a Biocontrol Bacterium, *Pseudomonas chlororaphis* O6. The Plant Pathology Journal, 30, 215-219.
- Kurnasov, O., Goral, V., Colabroy, K., Gerdes, S., Anantha, S., Osterman, A., Begley, T.P., 2003. NAD biosynthesis: identification of the tryptophan to quinolinate pathway in bacteria. Chemistry and Biology, 10, 1195-1204.
- Lasocki, K., Bartosik, A.A., Mierzejewska, J., Thomas, C.M., Jagura-Burdzy, G., 2007. Deletion of the *parA* (*soj*) Homologue in *Pseudomonas aeruginosa* Causes ParB Instability and Affects Growth Rate, Chromosome Segregation, and Motility. Journal of Bacteriology, 189, 5762-5772.
- Leisinger, U., Rüfenacht, K., Fischer, B., Pesaro, M., Spengler, A., Zehnder, A.J., Eggen, R.I., 2001. The glutathione peroxidase homologous gene from *Chlamydomonas reinhardtii* is transcriptionally up-regulated by singlet oxygen. Plant Molecular Biology, 46, 395-408.
- Lin, T.Y., Santos, T.M., Kontur, W.S., Donohue, T.J., Weibel, D.B., 2015. A Cardiolipin-deficient Mutant of *Rhodobacter sphaeroides* has an Altered Cell Shape and Is Impaired in Biofilm Formation. Journal of Bacteriology, 197, 3446-3455.
- Looper, J.E., Gross, H., 2007. Genomic analysis of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5. European Journal of Plant Pathology, 119, 265-278.
- Luján, A.M., Gómez, P., Buckling A., 2015. Siderophore cooperation of the bacterium *Pseudomonas fluorescens* in soil. Biology Letters, 11, 20140934.
- Ma, J.-F., Hager, P.W., Howell, M.L., Phibbs, P.V., Hassett, D.J., 1998. Cloning and characterization of the *Pseudomonas aeruginosa zwf* Gene Encoding Glucose-6-Phosphate Dehydrogenase, an Enzyme Important in Resistance to Methyl Viologen (Paraquat). Journal of Bacteriology, 180, 1741-1749.

- Madduri, K., Hutchinson, C.R., 1995. Functional characterization and transcriptional analysis of a gene cluster governing early and late steps in daunorubicin biosynthesis in *Streptomyces peucetius*. Journal of Bacteriology, 177, 3879-3884.
- Mohl, D.A., Gober, J.W., 1997. Cell cycle-dependent polar localization of chromosome partitioning proteins in *Caulobacter crescentus*. Cell, 88, 675-684.
- Moon, C.D., Zhang, X.X., Matthijs, S., Schäfer, M., Budzikiewicz, H., Rainey, P.B., 2008. Genomic, genetic and structural analysis of pyoverdine-mediated iron acquisition in the plant growth-promoting bacterium *Pseudomonas fluorescens* SBW25. BMC Microbiology, 8, 7.
- Moore, R.C., Boyle, S.M., 1990. Nucleotide sequence and analysis of the *speA* gene encoding biosynthetic arginine decarboxylase in *Escherichia coli*. Journal of Bacteriology, 172, 4631-4640.
- Murphy, P.J., Trenz, S.P., Grzemski, W., De Bruijn, F.J., Schell, J., 1993. The *Rhizobium meliloti* rhizopine *mos* locus is a mosaic structure facilitating its symbiotic regulation. Journal of Bacteriology, 175, 5193-5204.
- Mytilinaios, I., Salih, M., Schofield, H.K., Lambert, R.J., 2012. Growth curve prediction from optical density data. International Journal of Food Microbiology, 154, 169-176.
- Nandi, M., Selin, C., Brassinga, A.K., Belmonte, M.F., Fernando, W.G., Loewen, P.C., de Kievit, T.R., 2015. Pyrrolnitrin and Hydrogen Cyanide Production by *Pseudomonas chlororaphis* Strain PA23 Exhibits Nematicidal and Repellent Activity against *Caenorhabditis elegans*. PLoS One, 10, e0123184.
- Neidig, N., Paul, R.J., Scheu, S., Jousset, A., 2011. Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. Microbial Ecology, 61, 853-859.
- Nesse, L.L., Berg, K., Vestby, L.K., 2015. Effects of norspermidine and spermidine on biofilm formation by potentially pathogenic *Escherichia coli* and *Salmonella enterica* wild-type strains. Applied and Environmental Microbiology, 81, 2226-2232.

- O'Brien, R.W., 1975. Enzymatic analysis of the pathways of glucose catabolism and gluconeogenesis in *Pseudomonas citronellolis*. Archives of Microbiology, 103, 71-76.
- Parker, Z.M., Pendergraft, S.S., Sobieraj, J., McGinnis, M.M., Karatan, E., 2012. Elevated levels of the norspermidine synthesis enzyme NspC enhance *Vibrio cholerae* biofilm formation without affecting intracellular norspermidine concentrations. FEMS Microbiology Letters, 329, 18-27.
- Patel, C.N., Wortham, B.W., Lines, J.L., Fetherston, J.D., Perry, R.D., Oliveira, M.A., 2006. Polyamines are essential for the formation of plague biofilm. Journal of Bacteriology, 188, 2355-2363.
- Patel, M.S., Nemeria, N.S., Furey, W., Jordan, F., 2014. The pyruvate dehydrogenase complexes: structure-based function and regulation. The Journal of Biological Chemistry, 289, 16615-16623.
- Patten, C.L., Glick, B.R., 2002. Regulation of indole acetic acid production in *Pseudomonas putida* GR12-2 by tryptophan and the stationary phase sigma factor RpoS. Canadian Journal of Microbiology, 48, 635-642.
- Petruschka, L., Adolf, K., Burchhardt, G., Dernedde, J., Jürgensen, J., Herrmann, H., 2002. Analysis of the *zwf-pgl-eda*-operon in *Pseudomonas putida* strains H and KT2440. FEMS Microbiology Letters, 215, 89-95.
- Poblete-Castro, I., Binger, D., Rodrigues, A., Becker, J., Martins Dos Santos, V.A., Wittmann, C., 2013. In-silico-driven metabolic engineering of *Pseudomonas putida* for enhanced production of poly-hydroxyalkanoates. Metabolic Engeneering, 15, 113-123.
- Rapid Alert System for Food and Feed (RASFF). Annual Report, 2010.
- Rickett, L.M., Pullen, N., Hartley, M., Zipfel, C., Kamoun, S., Baranyi, J., Morris, R.J., 2015. Incorporating prior knowledge improves detection of differences in bacterial growth rate. BMC System Biology, 9, 60.
- Sah, S., Varshney, U., 2015. Impact of Mutating the Key Residues of a Bifunctional 5,10-Methylenetetrahydrofolate Dehydrogenase-Cyclohydrolase from *Escherichia coli* on Its Activities. Biochemistry, 54, 3504-3513.

- Sahu, S.N., Acharya, S., Tuminaro, H., Patel, I., Dudley, K., LeClerc, J.E., Cebula, T.A., Mukhopadhyay, S., 2003. The bacterial adaptive response gene, *barA*, encodes a novel conserved histidine kinase regulatory switch for adaptation and modulation of metabolism in *Escherichia coli*. Molecular and Cellular Biochemistry, 253, 167-177.
- Scales, B.S., Dickson, R.P., LiPuma, J.J., Huffnagle G.B., 2014. Microbiology, genomics, and clinical significance of the *Pseudomonas fluorescens* species complex, an unappreciated colonizer of humans. Clinical Microbiological Reviews, 27, 927-948.
- Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry, 160, 47-56.
- Senaratne, R.H., De Silva, A.D., Williams, S.J., Mougous, J.D., Reader, J.R., Zhang, T., Chan, S., Sidders, B., Lee, D.H., Chan, J., Bertozzi, C.R., Riley, L.W., 2006. 5'-Adenosinephosphosulphate reductase (CysH) protects Mycobacterium tuberculosis against free radicals during chronic infection phase in mice. Molecular Microbiology, 59, 1744-1753.
- Shoji, M., Ratnayake, D.B., Shi, Y., Kadowaki, T., Yamamoto, K., Yoshimura, F., Akamine, A., Curtis, M.A., Nakayama, K., 2002. Construction and characterization of a non-pigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. Microbiology, 148, 1183-1191.
- Si, X., Quan, X., Wu, Y., 2015. A small-molecule norspermidine and norspermidine-hosting polyelectrolyte coatings inhibit biofilm formation by multi-species wastewater culture. Applied microbiology and biotechnology, 99, 10861-10870.
- Silby, M.W., Winstanley, C., Godfrey, S.A.C., Levy, S.B., Jackson, R.W., 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiology Review, 35, 652-680.
- Silo-Suh, L., Suh, S.J., Phibbs, P.V., Ohman, D.E., 2005. Adaptations of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment can include deregulation of *zwf*, encoding glucose-6-phosphate dehydrogenase. Journal of Bacteriology, 187, 7561-7568.

- Singh, R., Bériault, R., Middaugh, J., Hamel, R., Chenier, D., Appanna, V.D., Kalyuzhnyi, S., 2005. Aluminum-tolerant *Pseudomonas fluorescens*: ROS toxicity and enhanced NADPH production. Extremophiles, 9, 367-373.
- Smeets, L.C., Becker, S.C., Barcak, G.J., Vandenbroucke-Grauls, C.M., Bitter, W., Goosen, N., 2006. Functional characterization of the competence protein DprA/Smf in *Escherichia coli*. FEMS Microbiology Letters, 263, 223-228.
- Stankovic, N., Senerovic, L., Ilic-Tomic, T., Vasiljevic, B., Nikodinovic-Runic, J**.,** 2014. Properties and applications of undecylprodigiosin and other bacterial prodigiosins. Applied Microbiology and Biotechnology, 9, 3841-3858.
- Sturgill, G., Rather, P.N., 2004. Evidence that putrescine acts as an extracellular signal required for swarming in *Proteus mirabilis*. Molecular Microbiology, 51, 437-446.
- Sumathi, C., MohanaPriya, D., Swarnalatha, S., Dinesh, M.G., Sekaran, G., 2014. Production of prodigiosin using tannery fleshing and evaluating its pharmacological effects. Scientific World Journal, 2014, 290327.
- Takagi, M., Takada, H., Imanaka, T., 1990. Nucleotide sequence and cloning in *Bacillus subtilis* of the *Bacillus stearothermophilus* pleiotropic regulatory gene *degT*. Journal of Bacteriology, 172, 411-418.
- Tippmann, H.F., 2004. Analysis for free: comparing programs for sequence analysis. Briefings in Bioinformatics, 5, 82-87.
- Tomar, A., Eiteman, M.A., Altman, E., 2003. The effect of acetate pathway mutations on the production of pyruvate in *Escherichia coli*. Applied Microbiology and Biotechnology, 62, 76-82.
- Walterson, A.M., Smith, D.D.N., Stavrinides, J., 2014. Identification of a *Pantoea* Biosynthetic Cluster That Directs the Synthesis of an Antimicrobial Natural Product. PLoS ONE, 9, e96208.
- Wang, K., Conn, K., Lazarovits, G., 2006. Involvement of quinolinate phosphoribosyl transferase in promotion of potato growth by a *Burkholderia strain*. Applied and Environmental Microbiology, 72, 760-768.

- Xie, G., Forst, C., Bonner, C., Jensen, R.A., 2001. Significance of two distinct types of tryptophan synthase beta chain in Bacteria, Archaea and higher plants. Genome Biology, 3, RESEARCH0004.
- Xu, Z., Fang, X., Wood, T.K., Huang, Z.J., 2013. A systems-level approach for investigating *Pseudomonas aeruginosa* biofilm formation. PLoS One, 8, e57050.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

In the present thesis, a comprehensive study of the spoilage activity of *P. fluorescens* strains isolated from food products has been conducted, paying particular attention to strains able to produce a blue pigment.

This study represents the first investigation of a combined evaluation of phenotypic and genetic information that was able to correlate the blue-pigment production to a specific phylogenetic group through the creation and application of a MLST approach.

The blue pigment production takes place only in particular environmental conditions (i.e. at 6 °C and 22 °C, but not at 31 °C, on MBM agar and PDA, in presence of oxygen, when high cell density is present). This information helps in the understanding of the spoilage mechanisms of the strains and in preventing pigment production.

The genomic analyses, and specially the pangenome analysis, revealed a first panel of five genes (namely *trpABCDF*) involved in the blue pigment production. Moreover, a key role of a genetic cluster found exclusively in the blue pigmenting strains investigated has been reported.

Mutagenesis revealed the involvement of TrpB and further three loci of the blue-unique cluster in the blue molecule production. However, the involvement of the other *trp* genes can be finally excluded only through the application of site-directed mutagenesis.

The chemical nature of the pigment remains an unsolved issue, at the moment: chemical investigation conducted through LC-MS/MS reveals that the pigment is an indigo-derivative, even if the molecular composition remains unclear. However, the blue pigment is not indigoidine and the producing strains are not *P. lemonnieri* strains (*P. fluorescens* biovar IV), in contrasts with findings of Caputo and colleagues (2015).

A deeper phenotypic characterisation of the transposon-induced mutants is necessary to highlight the role of the pigment. For instance, antimicrobial activity of the pigment might be tested against different bacterial species, as well as moulds and yeasts. Moreover, the

eventual protection against oxidative stress might be investigated, or rather the ability to produce biofilm and signal molecules of the mutants. Additionally, the application of further bioinformatics tools might shed light on the complete biosynthetic pathway.

Besides the mere research goals, the application of the MLST scheme, combined with simple phenotypic tests are useful in food industry to identify potential spoilers in raw materials, in processing plants and in final products, allowing to highlight also the routes and sources of contamination. However, the relatively high cost of MLST approach, based on the amplification and sequencing of seven loci, might reduce the applicability of the methods in food industry. A quicker screening might be provided by the identification of *trp* genes, which presence can be easily investigated through a simple PCR amplification and amplicon visualisation through gel electrophoresis.

Rigorous hygiene standards must be applied to reduce the event of exogenous contamination that involve food product after food processing. A particular attention must be paid to the cold chain, when required.

APPENDIX

TODAY'S RESEARCH

The strange case of blue mozzarella

by Nadia Andreani, PhD student in Veterinary science

Close your eyes and think... mummy has prepared a pizza for dinner! The dough is ready, the tomato is smoothed, you take the mozzarella from the fridge and... what strange magic is this? The mozzarella is blue! You rub your eyes: "Maybe...I am dreaming!", you think. It is a normal reaction, if you open a package of mozzarella and you find it covered with blue spots!

Who is responsible of this? Let's ask Barbara Cardazzo, researcher at the University of Padua who has been dealing with the strange case of blue mozzarella for years.

«The responsible is a tiny organism belonging to the world of Bacteria. Its name is Pseudomonas fluorescens», the expert says. «As the name suggest, if we en light them with a special lamp, they become fluorescent».

A blue mozzarella, produced by Barbara for her tests. To obtain it, Barbara put together a white mozzarella and the blue bacteria.

FLUORESCENT SUBSTANCES?

The Pseudomonas Fluorescens releases a blue light because it contains a substance called pyoverdine, which is fluorescent. Fluorescent substances store up a light of a specific colour – usually an ultraviolet light – and they release a light of a different colour – usually visible. The words fluorescent and phosphorescent have not the same meaning: fluorescent substances store up and release the light immediately, phosphorescent substances release the light gradually after a few minutes, sometimes after hours.

Would you eat a blue mozzarella? And if I get sick? But Barbara is sure: «If you eat a blue mozzarella, nothing bad will happen! Pseudomonas fluorescens can change the aspect, or the colour of food, but it is harmless for us».

You are probably wandering how this bacterium fall into the mozzarella. You can often find Pseudomonas fluorescens inside the water of foods, or the water used for their production. For this reason it is not unusual to find this bacteria inside them, especially milk derivatives (like mozzarella and ricotta) or meat. But only in some cases this bacterium produces those blue spots. It means that we always eat Pseudomonas fluorescens, but we do not realise it.

So what are you going to do with a blue mozzarella? Would you throw it away? No way! From now on, always have a look to the mozzarella you have in your fridge, you could find other samples and help Barbara with her research. She is now trying to understand why the colour of the spots is blue. What is there behind this? We hope to find the answer soon!

Barbara Cardazzo

Barbara Cardazzo is a researcher at the Department of Comparative Biomedicine and Food Science at the University of Padua. She has a husband, Luca, and two children, Matteo e Gaia. She is very good at cooking and everybody knows her for her delicious bread and pizza (and she uses white mozzarella, of course!). She studies the microbiology of food, the science which deals with the microorganisms living in our food, like bacteria, virus, fungus and yeasts. Consider that they are so small that you cannot see them to the naked eye.

RICERCA

Lo strano caso della mozzarella blu

di Nadia Andreani, dottoranda in Scienze veterinarie

Chiudete un attimo gli occhi e immaginate: mamma stasera ha preparata la pizza! La pasta è stata tirata, il pomodoro è già stato steso, aprite una confezione di mozzarella e... ma che strana magia è questa? La mozzarella è blu! Vi stropicciate gli occhi: "Forse sto sognando!", pensate. È la reazione che hanno avuto alcune persone quando, aprendo una confezione di mozzarella, hanno visto comparire strane macchie azzurre.

Le mozzarelle incriminate sono state subito analizzate: chi è stato il responsabile di questo strano caso? Lo abbiamo chiesto a **Barbara Cardazzo**, ricercatrice dell'Università di Padova che da anni si sta occupando dello strano caso delle mozzarelle blu.

«Il responsabile del misfatto è un minuscolo organismo che appartiene al regno dei Batteri e che si chiama **Pseudomonas fluorescens** - ci dice l'esperta -. Il nome di questo batterio parla chiaro: molt di loro se illuminati da una lampada speciale diventano **fluorescenti**».

Una mozzarella blu ottenuta in laboratorio mettendo insieme una mozzarella e Pseudomonas Fluorescens

SOSTANZE FLUORESCENTI

Pseudomonas Fluorescens emette luce blu perché contiene una sostanza chiamata pioverdina, che è fluorescente. Le sostanze fluorescenti assorbono luce di un certo colore (di solito ultravioletta), ed emettono luce di un colore diverso (di solito visibile). Le sostanze fluorescenti non vanno confuse con quelle fosforescenti. Le prime emettono la luce subito dopo averla assorbita, le seconde la emettono dopo alcuni minuti o addirittura ore.

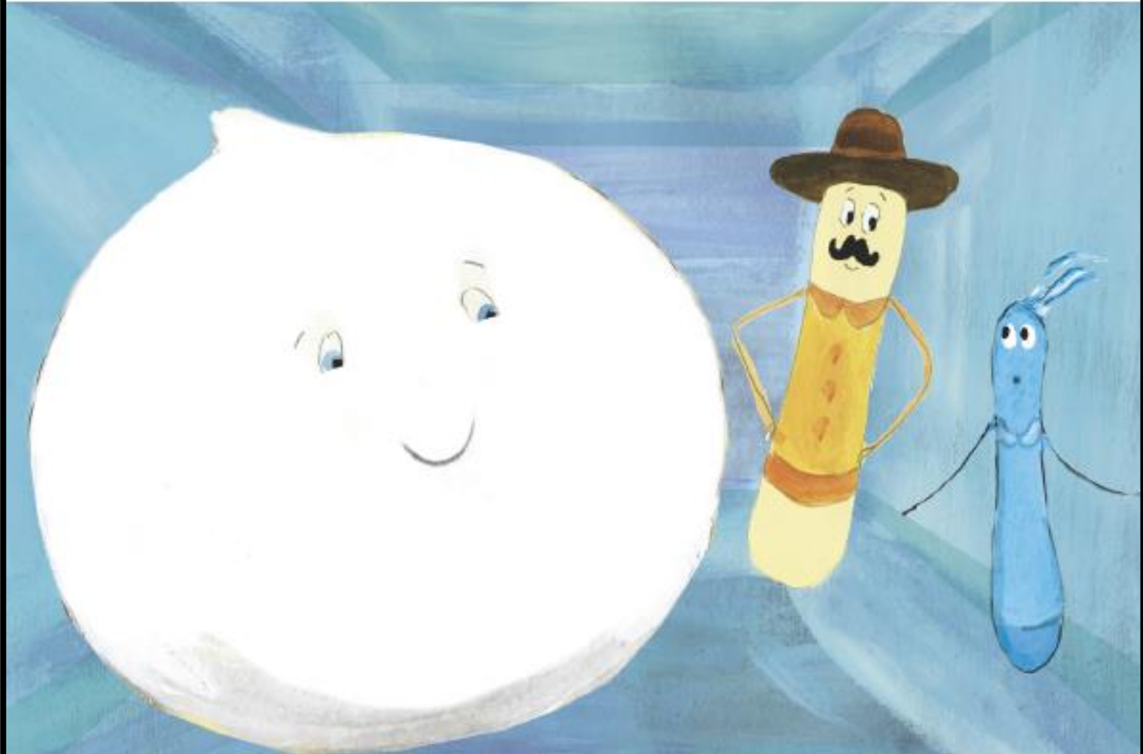
Voi la mangereste una mozzarella blu? E se poi ci fa ammalare? Barbara ci rassicura: «Se mangiate una mozzarella blu non succede niente! Pseudomonas fluorescens è un batterio che può causare dei cambiamenti un po' spiacevoli nel cibo, come il colore blu, ma non è capace di fare ammalare l'uomo».

Forse vi starete chiedendo come ha fatto questo batterio a finire nella mozzarella. Pseudomonas fluorescens si trova spesso nell'acqua contenuta negli alimenti o usata per la loro produzione. Per questo è normale che finisca nei cibi, soprattutto negli alimenti derivati dal latte (come la mozzarella o la ricotta) o nella carne, ma solo in alcuni casi il batterio produce il colore blu. Insomma, noi Pseudomonas fluorescens ce lo mangiamo e beviamo sempre, solo che non ce ne accorgiamo.

E adesso che farete? Smetterete di mangiare mozzarelle per paura di trovarne di blu? Macché! Da oggi inizia la ricerca e magari troverete nuovi campioni che aiuteranno Barbara a rispondere ad alcune domande ancora irrisolte: perché Pseudomonas fluorescens produce questo colore blu? E cosa è davvero la sostanza blu? Speriamo di scoprirlo presto!

Barbara Cardazzo

Barbara Cardazzo è una ricercatrice del Dipartimento di Biomedicina Comparata e dell'Alimentazione dell'Università di Padova. È mamma di Matteo e Gaia ed è sposata con Luca. È un'ottima cuoca ed è famosa tra i colleghi per il suo pane, le sue focacce e la sua straordinaria pizza (con mozzarella non blu, ovviamente!). Si occupa di microbiologia degli alimenti, la scienza che studia i microrganismi (esseri viventi non visibili ad occhio nudo, come batteri, virus, funghi e lieviti) che si trovano nel cibo.


The Adventures of Flo,
the special Bacterium
UNIVERSITY OF EXETER
A book of Professor Penny Stories
Nadia Andrea Andreani
illustrations by Kate Nelms

Text © 2015
Nadia Andrea Andreani

Illustrations © 2015
Kate Nelms

All rights reserved.
This book and the illustrations are licensed for your personal enjoyment only and only and may not be reproduced without prior permission from the author.

10 9 8 7 6 5 4 3 2

ISBN

www.exeter.ac.uk

to my family and to Simo
NAA

A special thank to
Ing. Aldo Gini Foundation
for the economic support of my internship
to Penryn (Cornwall)

The Adventures of Flo, the special Bacterium

How to read this book?

This is a story about a teeny tiny bacterium,

an adventure and an amazing friendship.

Don't you understand some words?

Check on the Glossary at the end of the story!

Difficult words are highlighted

in **bold**.

Flo is a baby, a teeny tiny baby.

No, he is not a puppy dog, he is smaller than a puppy dog!

No, neither is he a kitten, he is smaller than a kitten!

He is smaller than a mouse, a fly or a flea.

In fact he is even tinier than the naked eye can see.

Flo is a teeny tiny **bacterium** *and this is his story.*

"Mum, what's wrong in me? Why does everybody look at me like
I was an alien?" asked Flo.

"Nothing is wrong in you, Flo. Everybody looks at you because you
are special" said Flo's mum.

*Flo is different to the other bacteria in his colony
and he doesn't know yet.*

"Mum, why are all my friends leaving the colony?"
I want to leave, too." complained Flo.

""You can't leave, Flo! I can only protect you if you stay here
in the colony" exclaimed Flo's Mum.

*But Flo is an adventurous bacterium,
and so one day he decides to leave the safe colony to explore
the rest of the world.*

"Mum is finally sleeping" thought Flo.

And taking a deep breath, he said goodbye to his colony.

"I am 8-hours old now and all my friends already left the colony.
I should go!"

Flo is a brave bacterium, but he doesn't know what a big adventure is awaiting him.

Then, all of a sudden he fell into a fast moving river!

Flo realized his colony had been hanging above a water pipe,

and he was in great danger because he couldn't swim!

"If only I had listened to Mum!" said Flo "I feel so alone!"

and while he was despairing, the water carried him down through the pipe, out through a tap and into a washing up bowl full of water with a cleaning cloth.

"Welcome! I am Regine and who are you?" said the cleaning cloth.

"I am Flo and I am a bacterium" answered Flo.

"A bacterium? Come on Flo, you are in serious danger:
the man downthere is going to add the soap to this washing up bowl!
Hold onto me and I'll save you..!" screamed Regine.
And just then, a human hand grabbed both Regine and Flo
and placed them on a clean surface.

Flo sees a man for the first time and he looks big and frightening.

"What are you doing here, Flo?" asked Regine.
"I am asking myself the same question, Regine" answered Flo.
"I just wanted to see the world outside my colony,
but now I feel so sad! Where are we?"
"We are in a dairy plant Flo! Do you know what it means?" said Regine.
"Actually, I don't!" muttered Flo.
"A **dairy plant** is a place where humans make cheese!
This place is full of bacteria like you!" explained Regine...

...and in a moment Flo stops having fear and feeling alone!

"Why are there so many the bacteria in a dairy plant, Regine?"

asked Flo curiously.

"Because some bacteria are really helpful in making food"

replied Regine.

"For example, some bacteria are used to make wine or vinegar,

others for preparing sausages and salami.

But the most important bacteria that you will meet here

are milk bacteria. They help humans to ripen the cheese.

They are all there" said Regine, pointing at a big tank

a little further on.

"I would like to meet them!" enthused Flo .

...And finally Flo feels excited rather than scared, certain his adventure is truly beginning.

Then, Flo caught sight of his reflection in the water of the tank.
"Now I can understand why everyone was looking at me oddly!"
he exclaimed with dismay. "My skin seems to shine! Look there...!"
said Flo, trying to catch the attention of Regine.
"Your skin is rather different from the other bacteria I have
met in my life", admitted Regine. "But it has a beautiful color,
like the blue of a cornflower petals or a summer sky.
I have never seen such a lovely color in my life" Regine enthused.

But Flo now is really confused: why does he have blue skin?
Is he an alien?

And whilst Flo was trying to think at his situation,
a smart bacterium wearing a fluorescent suit
stopped his reflection and introduced himself.

"Hey stranger! My name is Fra! Don't be shy! Jump in and join us in our beautiful swimming pool!" said his new friend.

"But I am not able to swim!" whispered Flo.

"Don't be afraid, you just have to jump. The water is full of salt, it will help you to float" assured Fra.

And waving goodbye to Regine, Flo dived in the tank…

"What's your name?" asked Fra.

"I am Flo. I lived in the water pipe here above with my family" replied sadly Flo.

"Don't be sad, young man! We're a big family here! You won't feel alone here with us" said Fra.

And, taking him by the arm, Fra started a tour of the tank to show everything to Flo.

"What are those floating balls over there?" asked Flo, pointing a little further away.

"They are mozzarellas. Mozzarella is an Italian

pasta filata cheese.

They are amazing: soft and white as the snow" explained Fra.

Flo is now really confused: what does a family of bacteria do in a tank full of mozzarellas?

"You seem thoughtful, my dear" said Fra, reading Flo's mind

"Don't you think this is one of the best place to live in?

Mozzarellas are really funny girls! They give us something to eat

and, in exchange, we keep company with them.

There is only a pact among us:

no human has to know of our presence here or they will try

to eliminate us!

Humans are cruel: they all think all bacteria are naughty.

It is not true! Look at me: I don't cause illness and

I keep the tank clean" complained Fra.

"Luckily, there is one human
who is different. Look over there.
Do you see the girl with wild red hair?
Professor Penny is her name and she is
pretty, isn't she?
"Yes, she is" said shyly Flo,
flushing bright pink.

"She is a scientist. She comes every week,
she takes some water from the tank
and that is the best moment of my week"
said Fra.
And while they were speaking Professor Penny
withdraws some water in a tube for her test.

Flo wonders if he will see her again.

"Isn't she lovely? One day, someone told me that she was

interested in bacteria!

Let me introduce you to another friend of mine!"

and swimming toward the tank, Fra approached a shy mozzarella.

"Flo, this is Bianca! She is really young,

she arrived in the tank only few hours ago!" said Fra.

"Bianca, here is Flo! He is new, as you are! You should be friends!"

"Welcome, Flo. Nice to meet you" mumbled Bianca.

"Why are you sad, Bianca?" asked Flo with the curiosity of a young boy.

"I am just worried about the future, Flo.

In few hours, I will be taken away and a new life will start for me.

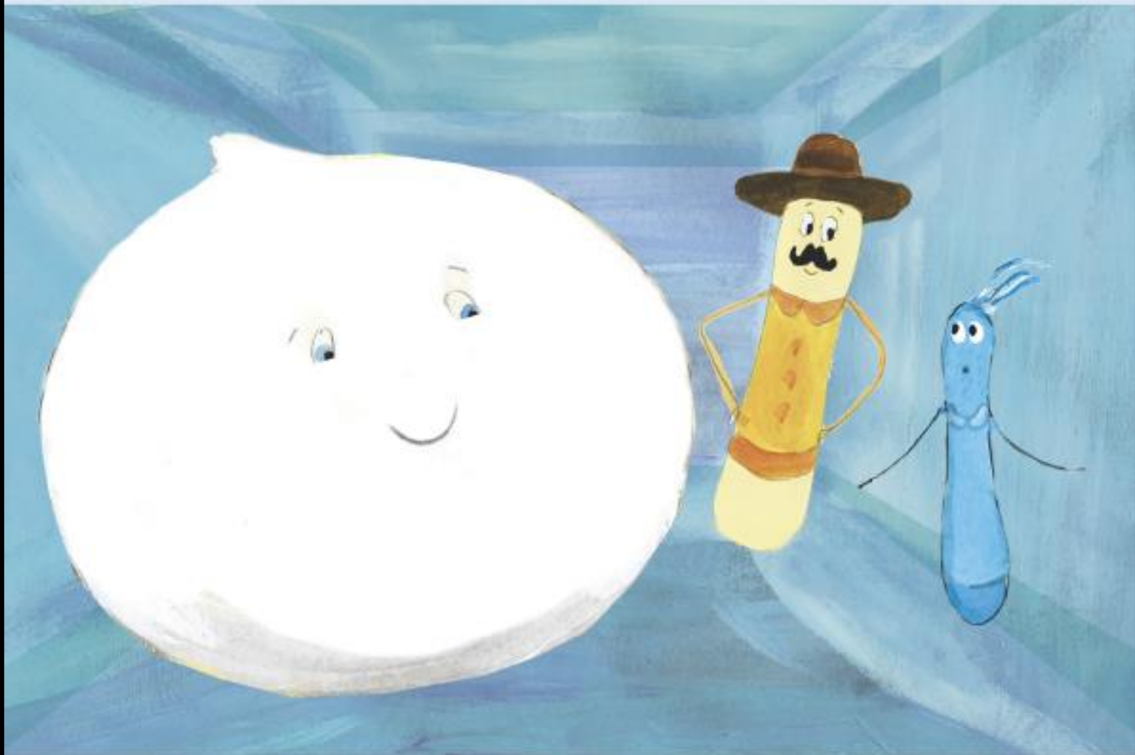
I will reach a supermarket and a family will take me home.

That's my destiny! And you? What's your destiny, Flo?"

asked Bianca.

"Actually, I never asked Mum about my destiny.

I don't know anything about myself or my future" replied Flo.



Suddenly, someone reaches in, scoops up Bianca and places her in a plastic bag full of water.

In a moment, Flo decides to follow Bianca wherever she is going.

"Hold on to me, Flo!" Bianca shouts.

"Don't be scared, everything will be ok!"

"Bianca, never let me go, please!" whined Flo.

Bianca is so impatient to discover what her destiny will be.

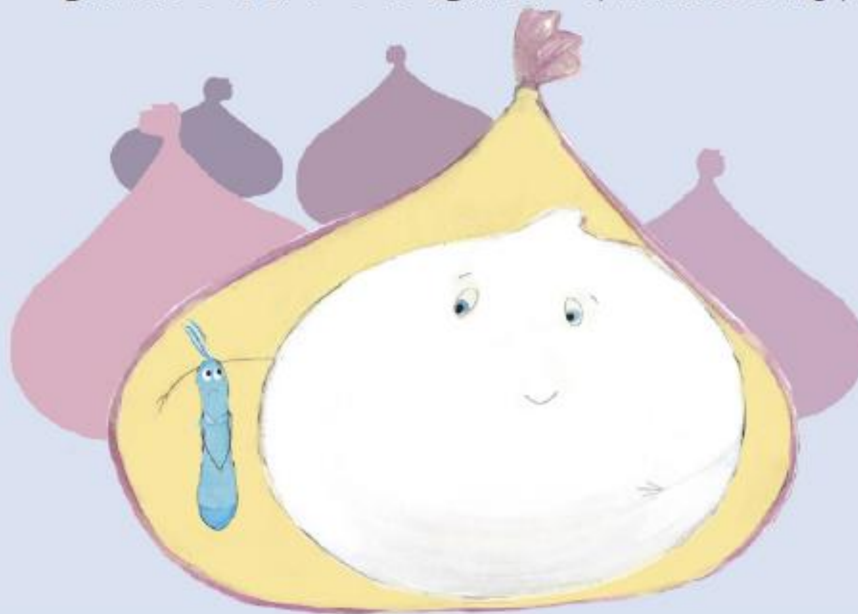
The same cannot be said of Flo.

"What's happening Bianca?" asked Flo with terrified eyes.

"Oh, Flo, there is nothing to be worried about! This is a normal journey for a mozzarella! We will leave soon from the dairy plant. A lorry will carry us in a supermarket. I am sure that a beautiful family will adopt us and I will feed them:
my only desire is to make them happy!
This is my destiny, the reason for my existence!" exclaimed Bianca.

...And with dreaming eyes, she imagines what delicious recipes she and her adopted family could prepare together: pizza, sandwiches, salads...

"How can you be so relaxed Bianca? The bag is so tight,
I cannot breathe! I need more oxygen" complained Flo.
"Keep calm Flo! There is enough air for the trip.
Humans reduce air content in the bag so that bad bacteria
can't grow inside" explained Bianca.
"Bad bacteria don't exist, Bianca" replied blustering Flo.



...And while he is just saying it, a maleficent laugh is heard in the plastic bag...

"You are so naïve, you bluish little thing!"

exclaimed a bacterium that was

hiding just behind Bianca.

It's so amusing to be a naughty boy!"

"Oh, no! That's not good, Flo." screamed Bianca.

"This kind of bacteria can cause infections and illness in humans.

If nobody notices him he might get eaten and do someone harm!

Flo, you must do something!"

" I cannot do anything Bianca. I am only a young bacterium

and I don't know anything about the world!" replied Flo.

...and Flo begins to cry, but his tears have a strange power...

"Stop crying, little bug! You are making me ill!" said the nasty bacterium.

"Don't stop, Flo! There is something in your tears making him sick!" screamed Bianca.

...and in few seconds the bad bacterium disappears, killed by the tears of a frightened Flo...

"Oh, Flo, luckily you were here with me!" said relieved Bianca.

"Someone told me that some bacteria are really powerful.

You killed that bad guy! How did you do it?"

"I don't know, my friend! I feel so strange now. My cheecks are blushing and I feel as I have fever" replied Flo.

"That's not just you Flo. It's getting hotter in here!

That shouldn't be happening, someone must have forgotten to put us in the fridge" said Bianca.

"It feels like a sauna! I feel swollen,

what's happening to me?" cried Flo.

...and within a few minutes Flo's body is duplicated…

"Keep calm, Flo. There is nothing to worry about.
You are just becoming a parent! That happens to bacteria when they get hot. When bacteria divide, they produce a new copy of themselves, like parents giving birth to children" she explained.

...and within minutes, Flo's body divides in two...

"If it doesn't cool down soon then it will happen again!" said Bianca.
"That's such a nice feeling!" sighted Flo.
"I am so happy not to be alone anymore!"

...and after 2 hours Bianca is surrounded by hundreds of Flos...

"Flo, stop please! With so many bacteria nobody will want to eat me!
Someone will notice that you're here! " said Bianca.
"I cannot stop, Bianca. I am so sorry" said Flo.

...and Flo is so sad about that that he starts crying, again...

"Oh, my friend, stop please! Shhh, lower your voice and listen!
Someone is buying us!" said Bianca.
"Mum, please, can we buy the mozzarella?
I would really like to have pizza tonight! Please, Mummy!!!"
implored a young girl's voice from outside the plastic bag.
"That's a good idea, Marty" said the mum
"you can even help me to make it!"

....and, looking around and seeing thousands of Flos,
Bianca doesn't know if to be happy or to be worried...

"Be quiet and don't move! I hope they won't notice you Flo!"
whispered Bianca. But then something strange happened...
Marty opened their plastic bag...
Air got in the bag and Flo felt comforted as he can breathe again...
Then, Marty screamed...

...and Flo is so scared by the shout that he does not notice
the water turning blue...

"Marty, what happened?" screamed Mum.

"Look mum, this mozzarella is blue!" said Marty pointing the bag.

Don't be silly, Marty! A mozzarella can't be blue!"

exclaimed Marty's mum.

...and looking at the blue mozzarella cheese, mum gasps...

"Don't touch it, Marty!" said the Mum worriedly.

"I am calling Aunt Penny. She knows all about strange things like this".

"We are in trouble, Flo! Your tears reacted with the air and turned blue.

Look at me. I look like a Smurf" whined Bianca.

..but Flo is not listening to her.

He is thinking at his mum's words:

"You are a special baby, Flo!

don't leave the colony.

Let me protect you!"

Now he understands what she meant...

Out of breath, Professor Penny arrived at her sister's house, prepared as always with her big bag of useful scientific equipment.

"Don't be scared, Marty! Pass me the gloves in my bag" instructed Penny "Come here, my dear, I will explain everything"

...and sitting next to aunt Penny, Marty starts listening...

and Flo and Bianca are curious to hear to the words of the nice girl that they met previously in the dairy plant...

"Marty, sniff the mozzarella, do you smell anything strange?" said Penny.

"No, Aunt Penny" answered Marty.

"Now, take a fork and pierce it" said Penny

"Is there something wrong in this mozzarella?"

"No" replied Marty." But Aunt Penny, look at the color!!" she exclaimed.

...Blanca and Flo are now really curious to understand what happened in their plastic bag...

"What you see is caused by a bacterium, something so incredibly small
that you can't see him. He is smaller than a puppy dog,
than a kitten, than a mouse, a fly or a flea.
In fact, he is even tinier than the naked eye can see.

He is a good bacterium because he doesn't make people sick.
We scientis call these kinds of bacteria **Spoilers**.

Some of them are very useful, as they can fight against bad bacteria and kill them. You can find several of these good bacteria in food, but usually you don't notice them because they are uncolored.

I suppose that something happened in this plastic bag: maybe the temperature rose and the bacterium increased in the water.

For some unknown reasons, this baterium can produce a substance that becomes blue with air contact" explained Penny
"Isn't that funny?"

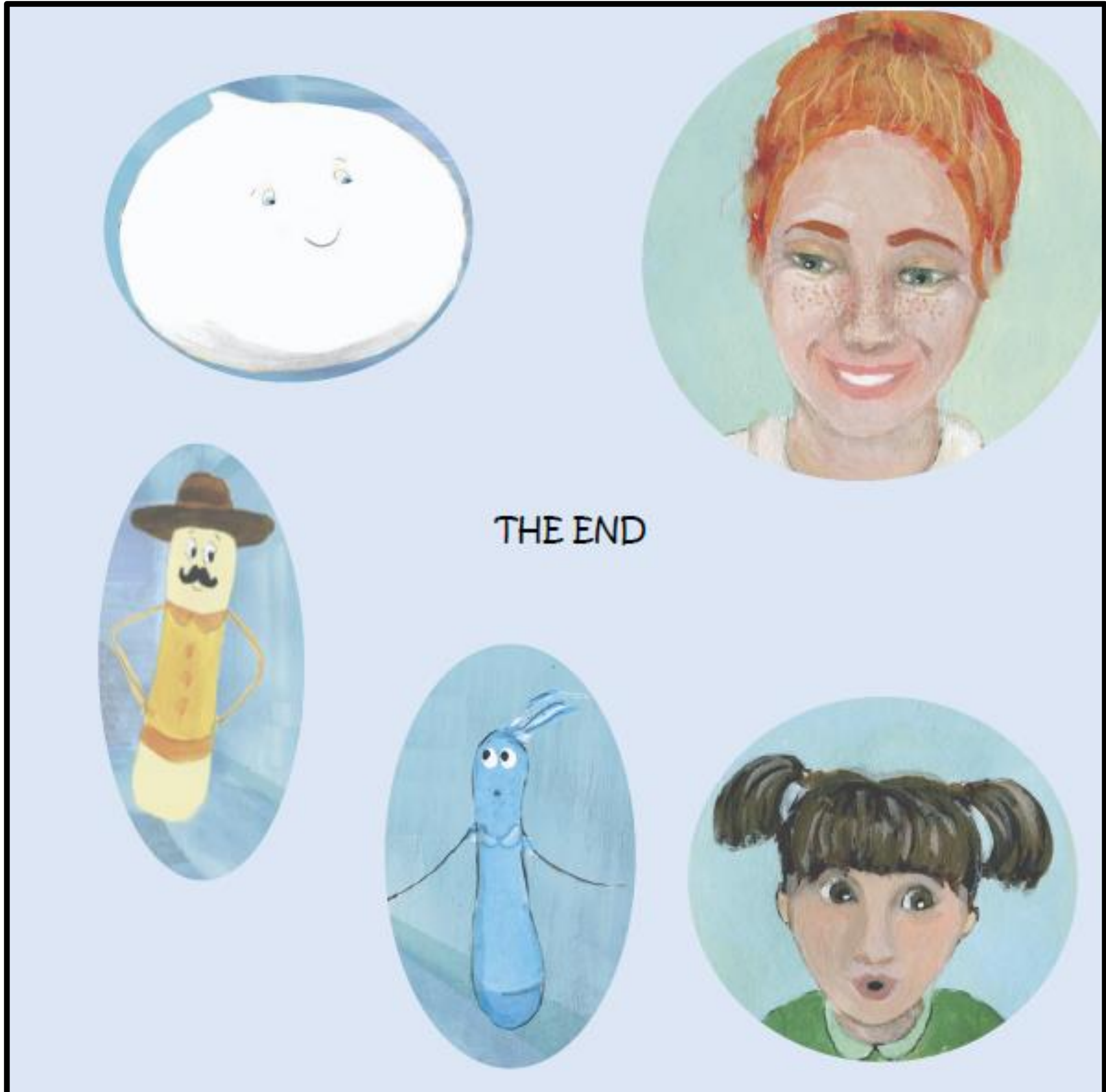
...And Marty immediately has a liking for this funny bacterium...

"I will take this bacteria to my university so we can study it!"
said Penny
"and then we will go out for dinner! Pizza for everyone!"

...but Flo feels so confused...
"I am so sorry, Bianca. Everything that happened is all my fault"
said Flo.

"Oh Flo, don't say that: now I understand how special Flo are you
and you made me special, too;
nobody will eat me, but we will be friends forever"

...and hugging each other, they think at where their friendship
might take them...


THE END

Glossary

Bacterium: An organism made of a single cell.
With few exceptions, bacteria can be seen only with the aid of a microscope, and millions of them would fit on the head of a pin.

Colony: A group of individual organisms living closely together, usually for mutual benefit.

Dairy Plant: An estabilishment where milk is received, processed and used to make dairy products (such as cheese or yoghurt).

Spoiler: A bacterium usually found in food.
It doesn't induce illness in people, but it can change the color, the flavour, the smell and other characteristics of food.

LIST OF ORIGINAL PUBLICATIONS

Book chapters

- **Andreani N.A**, Fasolato L., 2016. *Pseudomonas* and related genera, in "The Microbiological Quality of Food". Submitted.

Journal papers

- **Andreani, N.A.,** Martino, M.E., Fasolato, L., Carraro, L., Montemurro, F., Mioni, R., Bordin, P., Cardazzo, B., 2014. Tracking the blue: a MLST approach to characterise the *Pseudomonas fluorescens* group. Food Microbiology, 39, 116-126.
- **Andreani, N.A.,** Carraro, L., Martino, M.E., Fondi, M., Fasolato, L., Miotto, G., Magro, M., Vianello, F., Cardazzo, B., 2015. A genomic and transcriptomic approach to investigate the blue pigment phenotype in *Pseudomonas fluorescens*. International Journal of Food Microbiology, 213, 88-98.
- **Andreani, N.A.,** 2016. Transposon-induced pigment deficient mutants of a blue *Pseudomonas fluorescens* strain. In preparation.
- Magro, M., Fasolato, L., Bonaiuto, E., **Andreani, N.A.**, Baratella, D., Corraducci, V., Miotto, G., Cardazzo, B., Vianello, F., 2016. Oprf Porin as mineral iron sensor in *Pseudomonas fluorescens*: An adhesion mechanism by magnetic nanoparticles. Submitted to Environmental Microbiology in November 2015 (under review).
- Sattin, E., **Andreani, N.A.,** Carraro, L., Fasolato, L., Balzan, S., Novelli, E., Squartini, A., Telatin, A., Simionati, B., Cardazzo, B., 2016. Microbial dynamics during shelf-life of industrial Ricotta cheese and identification of a *Bacillus* strain as a cause of a pink discolouration. Food Microbiology, 57, 8-15.

- Fasolato, L., Carraro, L., Facco, P., Cardazzo, B., Balzan, S., Taticchi, A., **Andreani, N.A.,** Montemurro, F., Martino, M.E., Di Lecce, G., Gallina Toschi, T., Novelli, E., 2016. Agricultural by-products with bioactive effects: microbial changes in a fresh pork sausage enriched with phenolic compounds from olive vegetation water. Submitted at International Journal of Food Microbiology in December 2015 (under review).

Abstracts

- Martino, M.E., Fasolato, L., Montemurro, F., Boldrin, P., Mioni, R., **Andreani, N.A**., Cardazzo B., 2012. A novel MLST scheme for the characterisation of *Pseudomonas fluorescens* strains isolated from food matrices (Foodmicro2012, Istanbul, Turkey).
- **Andreani, N.A**., Martino, M.E., Fasolato, L., Cardazzo, B., 2013. Tracking the blue: MLST and phenotypic characterisation of *Pseudomonas fluorescens* strains isolated from food (Spoilers13, Quimper, France).
- **Andreani, N.A.,** Carraro, L., Fasolato, L., Martino, M.E., Magro, M., Vianello, F., Cardazzo, B. Dissection of spoilage phenotypes of *Pseudomonas fluorescens* in food matrices using a transcriptomic approach (Foodmicro2014, Nantes, France).
- Carraro, L., Fasolato, L., Cardazzo, B., **Andreani, N.A.,** Lucchini, R., Balzan, S., Dalsanto, M.L., Servile, M., Novelli, E. Recycling of waste: effect of polyphenols extract from olive oil water waste on *Listeria monocytogenes* and possible use as natural preservative in food (Foodmicro2014, Nantes, France).
- Fasolato, L., Carraro, L., Cardazzo, B., Balzan, S., Taticchi, A., **Andreani, N.A.,** Martino, M.E., Novelli, E., 2014. Phenols Extract from olive vegetation water (PEOW) as ingredient in raw sausages and salami (Foodmicro2014, Nantes, France).
- Sattin, E., Donà, S., **Andreani, N.A**., Balzan, S., Telatin, A., Zerbato, E., Simionati B., Cardazzo B., 2014. Following food microbiome development during shelf-life in the Nextgen era (Foodmicro2014, Nantes, France).

- **Andreani, N.A.,** 2015**.** Into the blue: Dissection of spoilage phenotypes of *Pseudomonas fluorescens* in food matrices (Exploring research in Cornwall 2015, Penryn, the UK).
- **Andreani, N.A**., Carraro, L., Cardazzo, B., Maifreni, M., Innocente, N., Marino, M., 2015. Extreme environment ecology and food safety of cheese brine microbial communities (ProkaGENOMICS15, Göttingen, Germany).

Other scientific contributions:

- **Andreani, N.A**., 2015. The strange case of blue mozzarella. PLaNCK!, 5, 8-9.
- **Andreani, N.A**., 2016. The Adventures of Flo, the Special Bacterium. Illustrations by Kate Nelms.

CURRICULUM VITAE

Nadia Andrea Andreani was born in Montichiari (BS, Italy) in the 13th of November 1988.

Academic studies

2013-2015: PhD student at the University of Padua (Università degli Studi di Padova) at School of Veterinary Science.

Dissertation title: INTO THE BLUE: Spoilage phenotypes of *Pseudomonas fluorescens* in food matrices.

Supervisor: Cardazzo Barbara.

2010-2012: master degree at the University of Padua (Università degli Studi di Padova).

Biotechnologies for Food Science (LM-9 - 2nd level degree in Pharmaceutical, veterinary and medical biotechnologies).

Final degree mark: 110/110 cum laude.

Dissertation title: Molecular typing and phenotypic characterisation of *Pseudomonas* fluorescens group strains isolated from food.

Thesis supervisor: Cardazzo Barbara.

2007-2010: 1st level degree at the University of Padua (Università degli Studi di Padova).

Health Biotechnologies (L-2 - 1st level degree in Biotechnologies).

Final degree mark: 108/110.

Dissertation title: Development of a Real-time PCR assay for the detection of allergens in food matrices.

Thesis supervisor: Patarnello Tomaso.

Congress attendance

1-3 July 2013: International Scientific Symposium: SPOILERS 2013-Microbial Spoilers in Food (Quimper, France).

Oral Presentation: “Tracking the blue: characterisation of strains of *Pseudomonas fluorescens* group from food.”

Winner of the Best Presentation Award.

1-4 September 2014: International Scientific Symposium: Food Micro 2014 (Nantes, France).

Oral Presentation: "Dissection of spoilage phenotypes of *Pseudomonas fluorescens* in food matrices using a transcriptomic approach".

27th March 2015: Exploring research in Cornwall 2015 (Penryn, the UK).

Oral Presentation: "Into the blue: Dissection of spoilage phenotypes of *Pseudomonas fluorescens* in food matrices”.

Winner of the 3rd prize of the Best Presentation Award.

29 September-2 October 2015: International Scientific Symposium: PROKAgenomics15.

Poster presentation: "Extreme environment ecology and food safety of cheese brine microbial communities".

RINGRAZIAMENTI

Di recente la mamma di un amico, leggendo i suoi ringraziamenti, ha detto: “Hai dimenticato di ringraziare il cane!”, scherzando sulla lunga lista di ringraziamenti. Io non lo farò… non mi dimenticherò di ringraziare nemmeno il cane!

Questi 3 anni di Dottorato sono stati una delle esperienze piu incredibili della mia vita.
Sono grata alla **Scuola di Dottorato in Scienze Veterinarie** dell’Università di Padova che ha supportato la mia educazione: grazie per questa grande opportunità.

Devo ringraziare la mia relatrice, **Barbara Cardazzo:** grazie per avermi dato la possibilità di intraprendere il meraviglioso viaggio del Dottorato. Non mi sono mai sentita sola, mantenendo comunque la mia indipendenza intellettuale.
Non solo Barbara, ma tutto il gruppo di ricerca di “Ispezione degli alimenti” è stato fondamentale per lo sviluppo del mio sguardo critico e il mio *background* intellettuale. Un grande grazie va alla mia insegnante e amica **Maria Elena**: non hai mai smesso di motivarmi, anche da lontano. Grazie a **Luca Fasolato,** detto “Il Maestro”: i tuoi insegnamenti, la tua franchezza, la tua ironia e il tuo modo di essere hanno reso la nostra collaborazione un’amicizia dolce-amara. Grazie alla mia compagna **Lisa Carraro**: lavorare con te è sempre bellissimo! Grazie per i consigli, la tua gentilezza e la tua timidezza. È stato proprio come lavorare con una sorella.
Voglio poi ringraziare tutto il gruppo Pat-Barg: **Serena, Massimo, Rafaella, Roberta, Francesco, Massimiliano, Giulia**, ma anche altri visi amici del Dipartimento come **Matteo, Giulia e Eleonora.** Un ringraziamento speciale va a **Marianna** per l’ospitalità e per avermi nutrito durante la stesura della tesi.
Devo dire grazie anche al **Prof. Gianfranco Gabai** per i suoi consigli durante questo percorso di Dottorato: il Suo aiuto nelle diverse situazioni mi ha permesso di sentirmi libera di chiedere consiglio quando necessitavo di fiducia e aiuto.

Infine, devo riconoscere che questi 3 anni non sarebbero stati gli stessi senza i miei studenti. Tutti voi avete dato un pezzo del complesso puzzle della mia tesi di Dottorato. Sono stata molto fortunata ad avervi incontrato sulla mia strada **Alberto, Andrea** (i ragazzi *Pseudomonas*)**,** ma anche **Alessia, Valentina, Luca, Silvia, Angelica, Raissa…**

Un passo importante della mia crescita è stato il periodo all'estero in Cornovaglia da gennaio a luglio 2015.

Innanzitutto, devo ringraziare la **Fondazione Ing. Aldo Gini** che lo ha finanziato economicamente.

Durante questa esperienza ho incontrato delle persone straordinarie, a partire da **Michiel Vos**, il mio supervisor Inglese-Olandese: grazie per avermi accettato nel Suo gruppo di ricerca e per avermi permesso di lavorare sulle mie amate *Pseudomonas fluorescens*. Alla fine di questi sei mesi ne sono uscita arricchita sia professionalmente che come persona.

Vorrei anche ringraziare tutto il gruppo di Michiel: **Prof**. **William Gaze**, **Lihong** e **Andy,** nonché la pazza componente femminile (**Abigail**, **Aimee**, **Anne** e **Amy**). È stato un grande piacere e, soprattutto, divertimento lavorare con voi.

La Cornovaglia mi ha dato anche una seconda famiglia, composta da **Jo, Simon, Ruby** e **Isaac**: mi sono sentita a casa per tutta la permanenza da voi! Grazie per avermi fatto sentire una Cain!

L'amicizia è stata una grande scoperta, dal momento in cui non potevo immaginare che importanti relazioni avrei potuto creare in un periodo così breve all'estero. Devo dire grazie a **Leo** per essere stato il mio porto sicuro: grazie per il tuo abbraccio quando la nostalgia di casa era in agguato e per avermi insegnato a sorridere. Dall'altro lato, **Tomasa** è stata il mio porto incerto: la sorellanza che ci lega va oltre l'incompatibilità della nostra natura. È stato un enorme piacere che le nostre strade si siano incrociate, Tom: grazie per la persona che mi hai reso. Un grazie speciale al gruppo dei "Launchers" composto da **Sidan**, **Sam**, **Dave** e **Atta,** per il tempo passato insieme. Vorrei inoltre ringraziare **Francesca**, **Giulia**, **Alice** e **Iris** (il mio braccio sinistro e il mio braccio destro), **Claudio**, **Daniela** e **Pawel**, **Eduardo, Prabhu, Shivangi, Adeline, Faryal…**

Comunque, il mio cuore è in Italia perché qui ho i migliori amici che io possa desiderare.

Un primo ringraziamento va a **Elena** per essere tutto per me, un'amica, una sorella, una mamma e una fan. Non mi sento mai sola. Grazie a sua figlia **Gloria**, la mia sorellina adottiva, per il suo sorriso speciale e per avermi insegnato che tutto dipende dagli occhi con cui guardiamo il mondo.

Grazie a **Jessica** per essere sempre lei stessa, ovvero la mia pazza sorellina minore, e per lasciare che io mi prenda cura di lei. Grazie per non aver rinunciato e per la splendida amicizia con **Elena** che lega tre menti così differenti in una relazione indissolubile.

Devo ringraziare **Vale** e **Nico** per ricordarmi sempre quanto sono amata, per le nostre vacanze insieme e perché, guardando a loro, mi ricordo sempre quanto fortunate sono ad avere loro e ad avere Simo.

Grazie **Paolo**, **Daniela**, **Francesco**, **Enrica** e **Padre Giovanni** per dire sempre sì ai nostri incontri.

Non posso dimenticare gli amici di sempre **Letizia**, **Erika**, **Fabio e Monica, Mario,** per essere sempre al mio fianco.

Un grazie speciale a tutti i miei parenti, ma uno particolare alla **zia Elena** per il tempo passato a leggere il giornale sulle sue ginocchia.

Ho una famiglia fantastica che mi ha dato un supporto totale in questo lungo percorso: spero davvero che **Paolo** e **Giulia** siano fieri di me e che il loro sorriso, la loro intelligenza e il loro entusiasmo non vengano mai affievoliti. Siete due ragazzi speciali e vi amo piu di me stessa.

Luisa mi ha reso consapevole che le distanze non importano, perché niente può separare due sorelle. Ti voglio bene!

Mamma e Papà sono immensi: sono il centro della mia esistenza. Mi hanno supportato su tutti i fronti durante questa carriera accademica senza fine, senza mai chiedere di piu di ciò che sono, ma convincendomi sempre che io potessi fare tutto con la mia intelligenza, il mio sorriso e il mio modo di essere. Questa tesi è soprattutto per voi. Grazie per avermi insegnato che il lavoro paga sempre. Ora, il mio desiderio più grande è che voi siate orgogliosi di me.

Questa tesi è dedicata a voi, mamma, papà e fratelli, perché vi voglio infinitamente bene!

Ultimo ma non meno importante, questa tesi trova le sue origini nella mia famiglia che sarà… in colui che mi ha spinto a fare il Dottorato, pur consapevole che questa scelta mi avrebbe tenuta lontana da casa per altri 3 anni… la pazienza che hai mostrato in questi 8 anni è piu di ciò che mi potessi aspettare (e di ciò che mi merito, probabilmente). Grazie per l'opportunità di partire e intraprendere il viaggio in Cornovaglia. Grazie per avermi resa la tua "Bimba Prodigio": in queste due parole leggo sempre l'orgoglio che talvolta tu non sai come dimostrare.

Grazie per avermi sempre aspettato, per il supporto in ogni passo di questo meraviglioso viaggio, per l'amore che vedo nei tuoi occhi silenziosi, per il sogno di una vita insieme. Grazie **Simone** per la persona che mi hai reso da quando stiamo insieme. Mi hai reso completa e non vedo l'ora di diventare la tua famiglia, per sempre.

Un grazie speciale a **Claudio** e **Luciana** per avermi dato l'uomo migliore che io potessi desiderare e per trattarmi sempre come una figlia.

Grazie a tutti!

Nadia

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ		HELLENIC REPUBLIC
ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΤΡΟΦΙΜΩΝ & ΔΙΑΤΡΟΦΗΣ ΑΝΘΡΩΠΟΥ ΕΡΓΑΣΤΗΡΙΟ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ & ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ ΤΡΟΦΙΜΩΝ ΙΕΡΑ ΟΔΟΣ 75, ΑΘΗΝΑ 11855, Τηλ/fax: 529-4693 E-mail *gjn@aua.gr*		AGRICULTURAL UNIVERSITY OF ATHENS DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION LABORATORY OF MICROBIOLOGY & BIOTECHNOLOGY OF FOODS IERA ODOS 75, ATHENS 11855 tel\fax: 30-1-529-4693 E-mail *gjn@aua.gr*

16/01/2016

TO WHOM IT MAY CONCERN

<u>Statement – Evaluation report</u> regarding the candidate Andreani Nadia Andrea for a PhD degree at UNIVERSITÀ DEGLI STUDI DI PADOVA SCUOLA DI DOTTORATO IN SCIENZE VETERINARIEand her THESIS with Title: "***INTO THE BLUE: Spoilage phenotypes of Pseudomonas fluorescens in food matrices"***

<u>Overall scientific level – originality</u>

The candidate has acquired significant knowledge within the following scientific fields: food microbiology both conventional and molecular tools: It needs to be stressed that within this thesis 2 papers already have been published, 3 submitted (under evaluation) and 1 is in progress to be submitted. She has presented different parts of this study (8 abstracts presentations in International level, e.g. FoodMicro 2012,2014 & Spoilers, Quimper, France). This is a more than a very good indication of the academic acceptance of this study, and as such it counts to Thesis merit.

In particular, her PhD thesis is structured in 4 Chapters i.e. covering literature review (Section 1, Introduction) while the experimental design, plans and results were covered within the following Chapters [e.g. Chapter 2. Tracking the blue: A MLST approach to characterize the *Pseudomonas fluorescens* group where details concerning the phenotype of this spoilage is provided Chapter 3. A genomic and transcriptomic approach to investigate the blue pigment phenotype in Pseudomonas fluorescens; specifically the study goes to 'omics' a field extremely important and fast growing in the food science area Chapter 4. Transposon-induced pigment deficient mutants of a blue *Pseudomonas fluorescens* strain, while a general discussion & conclusion follows]. In this way the PhD thesis is very well structured and well documented in most cases with high quality and recent (within 2015) references

The work that has been performed is huge and there is no doubt that the candidate has been under stress to complete this target. It is more than evident that there was a great effort to put in order all these data and this was proven to be difficult in some cases, but very successful at the end. As it was mentioned earlier the high quality of this study is evident, since 2 papers already have been published and 4 are in progress to be submitted or under evaluation. Once again I would like to emphasize the fact that the academic community has accepted the findings; these are shown in the statistics section of my recommendation.

All these publications are within the area of Food Microbiology - molecular biology and more specifically are related to food spoilage. The subject is of great importance since almost 1/3 of the foods are wasted and for this reason the scientific community should understand and propose solutions. The Thesis does contribute to this direction (understanding spoilage) and

can be considered as excellent work. It should be mentioned that food spoilage should be considered as very complex phenomenon, and worldwide few laboratories have been involved in this type of studies. This approach should be considered to add value and merit to the Department of Comparative Biomedicine and Food Science.

I am sure that the knowledge of the subject that the candidate developed through this exercise is extensive. The fact that results have been discussed in depth was evident in the text, while the Introduction (preparation of the state of the art) was also very well written and interpreted.
Finally it is evident that a very wide set of methods/tools and methodologies (mainly form the area of molecular biology and cell physiology) to achieve and to conclude the goals, have been used by the candidate. This is an additional merit for her future career and/or research interested.

Statistical data: The candidate in respectable and high impact scientific journals has published four articles. In many (3 out of 6) articles Ms Andreani is the first author. This is evident of her vital contribution on the study.

Andreani, N.A., Martino, M.E., Fasolato, L., Carraro, L., Montemurro, F., Mioni, R., Bordin, P., Cardazzo, B., 2014. Tracking the blue: a MLST approach to characterise the Pseudomonas fluorescens group. Food Microbiology, 39, 116-126.

Andreani, N.A., Carraro, L., Martino, M.E., Fondi, M., Fasolato, L., Miotto, G., Magro, M., Vianello, F., Cardazzo, B., 2015. A genomic and transcriptomic approach to investigate the blue pigment phenotype in Pseudomonas fluorescens. International Journal of Food Microbiology, 213, 88-98.

Andreani, N.A., Carraro, L., Cardazzo, B., Zhang, L., 2016. Transposon-induced pigment deficient mutants of a blue *Pseudomonas fluorescens* strain.

Magro, M., Fasolato, L., Bonaiuto, E., Andreani, N.A., Baratella, D., Corraducci, V., Miotto, G., Cardazzo, B., Vianello, F., 2016. Oprf Porin as mineral iron sensor in Pseudomonas fluorescens: An adhesion mechanism by magnetic nanoparticles Submitted to Environmental Microbiology in November 2015 (under review).

Sattin E., Andreani N.A., Carraro L., Fasolato L., Balzan S., Novelli E., Squartini A., Telatin A., Simionati B., Cardazzo B., 2016. Microbial dynamics during shelf-life of industrial Ricotta cheese and identification of a Bacillus strain as a cause of a pink discolouration. Submitted to Food Microbiology in November 2015 (under review).

Fasolato, L., Carraro, L., Facco, P., Cardazzo, B., Balzana, S., Taticchi, A., Andreani, N.A., Montemurro, F., Martino, M.E., Di Lecce, G., Gallina Toschi, T., Novelli, E., 2016. Agricultural by-products with bioactive effects: microbial changes in a fresh pork sausage enriched with phenolic compounds from olive vegetation water. Submitted at International Journal of Food Microbiology in December 2015 (under review).

Pre-examiner: George – John NYCHAS, Agricultural University of Athens, Athens, Greece

École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes Atlantique
SECALIM UMR - INRA 1014
INRA

!
Monique ZAGOREC, PhD
Tél : +33 2 40 68 78 46
Mail : monique.zagorec@oniris-nnantes.fr
http://www6.angers-nantes.inra.fr/secalim

ONIRIS, INRA,
UMR 1014 SECALIM, Groupe 5
Site de la Chantrerie, Route de gachet
CS40706
44307 Nantes cedex 3, France

Reference : 1061930

Report on the PhD Thesis manuscript entitled
"Into the blue: spoilage phenotypes of *Peudomonas fluorescens* in food matrices"
presented by Nadia Andrea ANDREANI, at the University of Padova, Doctoral School of Veterinary Sciences, Italy

Structure of the manuscript

The scientific part of the thesis manuscript encompasses a short abstract followed by a background part and one page explaining the aims of the study. It is then divided into four chapters written in English, each one corresponding to a published, submitted, or in preparation scientific article. Chapter 1 is a review on *Pseudomonas* and related genera, submitted as a book chapter. Chapter 2 reports results of a multilocus sequence typing (MLST) analysis of *Pseudomonas fluorescens* and was published in *Food Microbiology* in 2014. It is followed by a short appendix (half a page and one table) with additional data obtained after the publication and confirming the robustness of the method and the results presented in the published paper. Chapter 3 corresponds to genomic and transcriptomic results published in 2015 in *International Journal of Food Microbiology*. Here also, a short appendix (~4 pages with 4 figures) is added at the end of the chapter, which reports additional data obtained after publication. Chapter 4 is the manuscript of an article in preparation for its further submission. It reports the isolation and analysis of mutants deficient in pigment production. A short 1 page general conclusion ends the thesis. Several appendixes are listed at the end of the document, giving information on the production activity of Mrs. Andreani (CV, list of articles, posters, conferences, and additional production).

Nature of the scientific study and results

The subject of this PhD was a deep analysis of the mechanisms involved in the spoilage caused by *P. fluorescens* in dairy products, and characterized by the appearance of an undesired blue color caused by pigment production. A MLST-based typing method was developed indicating that some blue pigmented strains clustered together. Then genomics transcriptomics and functional genomics studies were performed to identify genes and functions involved in the synthesis of this blue pigment.

In the 1st chapter, a bibliographic study is proposed on *Pseudomonas*, and particularly *P. fluorescens*. The present knowledge about the difficult taxonomy of this genus, and the numerous species and group species it encompasses was reviewed. As well quite exhaustive listing of various spoilage defects (resulting from protease and lipase secretion or by pigment production) caused by *Pseudomonas* spp. was performed and a large documentation on the mechanisms (when known) responsible for it is also provided. This bibliographic introduction points out the large diversity of the *P. fluorescens* group species at the phenotypic and genotypic levels and the difficulty to classify strains depending on their spoilage capability.

This logically leads to chapter 2 which is dedicated to the analysis of a collection of 136 strains. Type strains and natural isolates from different ecological origins were analyzed for their phenotypic traits potentially associated to spoilage activity (extracellular production of lipase, protease, and lecithinase activities, pigment production on agar media). A MLST scheme with 7 genes was constructed, based on previous literature. Then after removing strain duplicates, typing was performed in order to construct phylogenetic clusters. This resulted in a tree based on the ST (sequence types) of strains on which were added their phenotypes: their ability to produce degradation enzymes, their ability to produce pigments, and their color characteristics or fluorescence detection. The tree was constructed with 100 strains, including 79 non redundant isolates. Interestingly the 12 strains producing a blue pigment clustered together in a "blue branch". Their ability to cause mozzarella cheese spoilage by the production of "blue mozzarella" was also shown. The other phenotypic traits (protease, lipase, and lecithinase production, and production of other pigments than the blue one) did not match with the MLST clustering. This study procures then an interesting method for identifying and tracking undesired strains belonging to the *P. fluorescens* group in food. This work was published in the review *Food Microbiology* in 2014. In a further study following the publication, 7 additional strains were analyzed. Six were blue pigmented and their MLST analysis revealed they clustered also in the "blue branch", confirming the robustness of the method.

Then in the following chapter (chapter 3) 4 phylogenetically related strains (2 blue and 2 non pigmenting strains) were studied. Their genomes and transcriptomes were compared to search for the functions putatively involved in the synthesis of the blue pigment. Further biochemical analyses were also performed to identify its structure. Only 194 genes were present in the 2 blue pigmenting strains and absent from the 2 non pigmenting ones, and could then encompass genes involved in the production of the blue pigment. Transcriptomic experiments showed that among the genes shared by all strains, 34 were up-regulated in the "blue" strains, and 51 were down-regulated. The analysis of the putative functions of these genes, as well as the determination of the molecular mass of the pigment and of its solubility characteristics, pointed out that this blue pigment was different from other pigments previously characterized and that it may be derived from tryptophan. In particular the presence of an additional copy of a *trpABCDF* operon in the "blue" strains was proposed as involved in the synthesis of the pigment. This part of the chapter led to an article published in *International Journal of Food Microbiology* in 2015. Further experiments performed on additional strains (18 producing the pigment and 18 non producers) confirmed the correlation between the pigment synthesis and the presence

of the additional *trp* operon as its presence was PCR detected only in the producing strains. The acquisition of these genes by horizontal gene transfer was hypothesized following sequence alignment of those 5 additional genes and phylogenetic tree reconstruction.

The 4th chapter relates another approach aiming at the identification of the genes involved in the blue pigment production. For that purpose a transposon mutagenesis was performed on a "blue" strain and 24 mutants that had lost the ability to produce the pigment and one hyper pigmented one were selected. Sequences flanking the transposon insertion were sequenced to identify the mutated genes. In addition, several phenotypic traits of the mutants were analyzed for investigating the putative biological role of the pigment. Results confirmed the involvement of the above mentioned *trp* gene cluster in the biosynthesis and pointed out other functions important for the regulation of its production (iron transport, oxidative stress, cell signaling, and biofilm formation) paving the way to elucidate the role and biosynthetic pathway of the blue pigment. Chapter 4 is presented as a manuscript in preparation for subsequent submission.

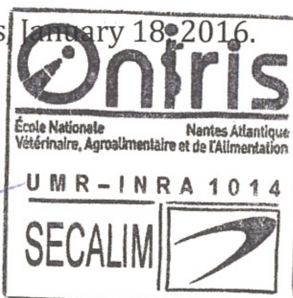
This 3 years study, performed by Mrs Andreani presents a great interest for fighting food spoilage and also to enhance our knowledge about bacterial metabolism *i.e.* for both applied and academic research fields. The approaches that were used were adapted to the research question and the results are convincing.

Valorization of the work

Mrs Andreani is the first author for the four publications presented in the thesis manuscript. In addition, she is also co-author of 3 manuscripts which have been submitted to *Food Microbiology*, *Environmental Microbiology*, and *International Journal of Food Microbiology.* All these scientific journals belong to the first quartile and are considered as excellent. She also cosigned 8 abstracts presented at international scientific congresses, among which she attempted 4 and got 2 prizes. This clearly shows the very high quality of the work that has been realized and certainly results from a very promising young scientist.

To conclude, this PhD manuscript with the excellent work it presents totally fulfills the requirements for obtaining the PhD title and therefore merits its defense.

Done in Nantes, January 18, 2016.



M. Zagorec