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INVESTIGATION ON THE BLUE PHENOTYPE IN *PSEUDOMONAS* SPECIES INVOLVED IN BLUE DISCOLORATION DEFECT OF FRESH CHEESE

Scientific field AGR/16

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A Gabriele,
che ha pazientemente sopportato le mie ansie,
dolcemente stemperato le mie tensioni,
dato risalto a ogni risultato positivo,
mi ha sostenuta e incoraggiata.

"Further up and further in"
(The Last Battle, C.S. Lewis)

LIST OF CONTENTS

ABSTRACT

In 2010 the occurrence of blue spots on Mozzarella cheese was reported from several consumers in Italy and highlighted by local and international media and by RASFF alert system (RASFF Annual Report 2010). *P. fluorescens* spp. was identified as the causing agent of this blue pigmentation.

In this phD thesis work a collection of about 69 *Pseudomonas* spp. isolates (listed in Appendix 1) was used: from these 59 were isolated from spoiled samples of Mozzarella cheese presenting the blue coloration defect and identified by 16S rDNA sequencing. The production of the blue pigment was confirmed by incubation of the isolates in Mozzarella Preserving Fluid (PF) centrifuged and sterilized by filtration 0.22 µm; incubation was made at 4°C. The medium turned blue after 7 days in 30 samples (33.7% of the isolates). To investigate the genetic relationship between blue pigment-producing and blue pigment not-producing isolates, genome restriction was performed using *SpeI* enzyme, coupled with pulsed gel field electrophoresis (PFGE). From bands profile it was seen that the 30 blue producing isolates were grouped in 12 genotypes. From each genotype one representative strain was chosen for MultiLocus Sequence Typing analysis (MLST) to confirm a phylogenetic relationship among the blue pigment-producing strains. For 3 blue pigment-producing strains (200188/6, UMB247 and UMB248) the whole genome was sequenced and compared with 2 further blue pigment-producing strains genome (PS77 and PS20) and with the genomes of 5 blue pigment not-producing strains (PS40, PS20, Pf01, A506, SBW25). From this comparison a unique region of about 10kbp present in the blue pigment-producing strains and not shared by the blue pigment not-producing strains was found. This region is composed by 15 CDS, most of them (53.7%) coding for phage related elements. To investigate the relationship between the presence of prophages and the development of the blue phenotype the 30 blue pigment-producing isolates were induced by two different antibiotics (norfloxacin and ciprofloxacin). The presence of induced bacteriophages was assayed by measuring an inhibitory effect on the growing curves of blue pigment-producing *Pseudomonas* spp., by spot test assay and by plaques formation on double layer agar. For samples with a positive result from spot test, TEM photographs were made, showing two phage morphologies from a sample induced by ciprofloxacin. It was not possible to isolate these phages because they were not plaque producing.

The other main topic of this job was the identification of the blue pigment and of its role in the ecology of *P. fluorescens*. To determinate the environmental requirement for its production several assays were made incubating the blue strains in PF at different temperature (4°C, 14°C and 30°C) and in M9 minimal medium at different pH (5.7, 6.3, 7.2) with (a) different carbon source (glucose, galactose, succinic acid, lactic acid), (b) different metals (Mo, Cu, Zn, Ca, Mg, Bo, Co, Mn, Fe) and (c) 18 different amino acids. From these phenotypic tests it resulted that the blue production occurred only at refrigeration temperature (lower than 14°C) in medium with pH 5.7. The presence of Cobalt or lysine inhibited the blue synthesis, while it was increased when proline was present. The individuation of the blue molecule/s from blue samples of PF and M9+proline incubated with strains 200188/6, UMB248 and UMB254 was made by UPLC/MS without leading to a definitive result. Trying to identify the function of the blue molecule/s, its possible connection with *quorum sensing* signals and its role as bacteriocine were investigated. *Quorum sensing* signals were found not to be related with blue production; as for the bacterial growth inhibiting effect it was noticed that the presence of the blue molecule/s, contrary of what expected, was able to promote the growing of *Pseudomonas* spp.

RIASSUNTO

La presenza di macchie di colore blu su Mozzarella è una problematica attuale per le industrie produttrici di questa tipologia di formaggio fresco. In particolare dal 2010 sono stati segnalati diversi casi in Italia con grande risalto nei notiziari nazionali ed europei, causando anche una segnalazione da parte del sistema di allerta RASFF. La causa della formazione del colore blu è stata identificata nella contaminazione di ceppi appartenenti al genere *Pseudomonas*.

Per questo lavoro di tesi di dottorato è stata costituita una collezione di 69 isolati appartenenti al genere *Pseudomonas* (elencati nel capito Appendix 1): di questi, 59 sono stati isolati da campioni di Mozzarella con difetto blu e identificati mediante sequenziamento della regione 16S rRNA. La produzione del pigmento blu è stata confermata incubando gli isolati nel liquido di governo di Mozzarella, precedentemente centrifugato e sterilizzato per filtrazione 0.22 µm (PF), a 4°C. Trenta campioni diventarono blu dopo 7 giorni (33.7% degli isolati). Su tutti gli 89 ceppi è stata fatta la restrizione del genoma usando l'enzima *SpeI* seguita dalla corsa elettroforetica in campo pulsato (PFGE) per valutare un'eventuale correlazione genetica tra gli isolati produttori del pigmento blu. Dai profili ottenuti i 30 isolati presentati il fenotipo blu sono stati raggruppati in 12 genotipi. Per ogni genotipo è stato scelto un ceppo rappresentativo su cui è stata fatta una tipizzazione attraverso il sequenziamento di 7 *loci* conservati (MultiLocus Sequence Typing) per confermare l'esistenza di una relazione filogenetica comune ai ceppi produttori del blu. Per 3 ceppi produttori del blu (200188/6, UMB247, UMB248) è stato sequenziato l'intero genoma. Le sequenze dei genomi ottenuti sono state confrontate con quelle di altri 2 ceppi produttori del blu (PS77 e PS22) e di 5 ceppi non presentanti la formazione del pigmento blu (PS40, PS20, Pf01, A506, SBW25). Da questa analisi di comparazione dei genomi è stata individuata una regione di circa 10 kbp presente unicamente nei genomi dei ceppi produttori del blu, composta da 13 CDS, la maggior parte delle quali (53.7%) codificante per proteine costitutive di batteriofagi. Per indagare la relazione tra la presenza di profagi integrati nel genoma dei ceppi produttori del blu e lo sviluppo di questo particolare fenotipo, i 30 isolati produttori del blu sono stati sottoposti a induzione con due diversi antibiotici (norfloxacina e ciprofloxacina). La presenza di batteriofagi indotti dal trattamento con gli antibiotici è stata verificata misurando un'eventuale attività inibente sulla crescita di ceppi produttori del blu di *Pseudomonas* spp., seguita dalla conferma mediante spot test e isolamento con la formazione di

placche di lisi in doppio strato di agar. Alcuni campioni che hanno causato una zona di lisi negli spot test sono stati fotografati con microscopio elettronico a trasmissione. Attraverso le immagini sono state individuate due tipologie di particelle virali esclusivamente nei campioni indotti con ciporfloxacina. Non è stato però possibile procedere all'isolamento dei batteriofagi in quanto non sono state ottenute singole placche di lisi.

L'altra tematica affrontata durante questo progetto di dottorato è stata l'identificazione della/e molecola/e blu e del suo ruolo nell'ambiente. Per determinare i requisiti necessari per la produzione del pigmento blu sono state allestite delle prove fenotipiche in liquido di governo a tre diverse temperature (4°C, 14°C e 30°C) e in terreno minimo M9 a diversi pH (5.7, 6.3, 7.2) addizionato con (a) differenti fonti di carbonio (lattosio, glucosio, galattosio, acido lattico e acido succinico), (b) diversi metalli (Mo, Cu, Zn, Ca, Mg, Bo, Co, Mn, Fe) e (c) 18 diversi amminoacidi. La produzione del pigmento blu è stata osservata solo quando i campioni sono stati incubati a temperature di refrigerazione (al di sotto dei 14°C) in liquido di governo o nel terreno minimo a pH 5.7. La presenza di Cobalto e di alcuni amminoacidi, ad esempio la lisina, hanno avuto un effetto inibente sulla produzione del pigmento blu, mentre l'aggiunta di prolina ne ha aumentato l'intensità. L'analisi UPLC/MS dei campioni 200188/6, UMB247 e UMB248 in liquido di governo e dei campioni in M9 + prolina non ha portato all'identificazione univoca della/e molecola/e che danno la colorazione blu. Per comprendere la funzione del pigmento blu sono state verificate una possibile correlazione con i segnali di *quorum sensing* e un possibile ruolo come batteriocina. I segnali di *quorum sensing* non sono risultati legati alla produzione del blu, mentre per quanto riguarda l'attività batteriostatica è risultato che, al contrario delle aspettative, il pigmento blu possiede un effetto positivo sulla crescita dei ceppi blu di *Pseudomonas* spp..

STATE OF ART

Pseudomonas fluorescens

Pseudomonas spp. classification

Pseudomonas spp. are rod shaped, Gram-negative, mobile, aerobic bacteria. It consists in a large genus within the γ-Proteobacteria, known for its ubiquity in the environment and for its ability to use a wide variety of organic compounds as energy sources. It also includes phytopathogenic species (for example *P. syringae*) and human pathogenic species (*P. aeruginosa*) (*14*).

One of the first classification of pseudomonads was made in 1960s (even if the first studies date back to the end of 19th century) when Flügge distinguished two biotypes, later named *P. fluorescens* and *P. putida* (*30*). Since then several other species were ascribed to *Pseudomonas* genus, classified according to their physiology and metabolism. From 1970s the study on *Pseudomonas* spp. was increased deepened with genotypic comparisons (DNA homology, DNA-RNA hybridization), revealing a high genetic distance between the species (*8*). For this reason, the number of the species ascribed to *Pseudomonas* genus was narrowed. In 1996 Moore *et al*. through the sequencing of 16S rRNA gene identified two intrageneric clusters: *P. aeruginosa*, where four different lineages (*P. aeruginosa*, *P. resinovorans*, *P. mendocina* and *P. flavescens*) were grouped and *P. fluorescens* cluster, which gathered five species (*P. fluorescens*, *P. syringae*, *P. putida*, *P. cichorii*, *P. agarici*) (*18*). In 2000 Yamamoto *et al*. proposed an alternative phylogenetic tree based on the sequences of *gyrB* and *rpoD* genes because these targets showed a higher discriminatory power than 16S rRNA, confirming the presence of the two main clusters, but redefining the relationship between the different species; in particular the cluster two was divided in three subclusters (*P. putida*, *P. syringae* and *P. fluorescens* complex) (*31*). Because of the high level of biodiversity different "finger print" techniques have been used for the identification of *Pseudomonas* spp. in the environment (*13*, *23*).

In recent years the availability of whole genome sequencing techniques have led to a more complete understanding of *Pseudomonas* spp., confirming that to a high heterogeneity of ecological, metabolic and biochemical characters corresponds a high diversity at genomic level, sharing core genes that occupy between 25% to 35% of the genome for each strain. Many of the variable regions consist of horizontally-acquired DNA (transposons, plasmids, prophages) reflecting the ecological development of the strain in its evolutionary time (*14*).

Pseudomonas fluorescens group

Among *Pseudomonas* spp., *P. fluorescens* is characterized by the ability to grow at low temperatures (below 7°C) being psychrotrophic. This feature make it frequently

involved in spoilage of fresh foods such as vegetables, meat, fish and dairy products, where it can cause alterations given by the production of lipolytic and proteolytic enzymes (*22*), or by the developing of off-flavours and pigmentation (*16*). Some strains of *P. fluorescens* can produce pyoverdine, a yellow green siderophore, in iron limiting growth conditions (*19*). Despite these negative effects on fresh food *P. fluorescens* can have also a positive role in plant ecology, since it may protect them from pathogenic moulds, producing biofilm and plant hormones (*26*) or other metabolites as pyrrolnitrin, phenazine, hydrogen cyanide and volatile compounds, as well as cell wall degrading enzymes (*9*). Even the production of siderophores has a protective action towards plants, chelating iron and making it not accessible to plant pathogens (*24*).
At a genetic level the comparisons among the genomes of four strains within the *P. fluorescens* group (*P. protegens* Pf-5 and *P. fluorescens* strains SBW25, Pf0-1 and WH6) highlighted the wide diversity of these bacteria, with a core genome representing only the 52% to 54% of each strain. The variable regions have been associated with phenotypical characteristics developed by specific strains; for example they confer to *P. fluorescens* strain Pf-05 the ability to produce different secondary metabolites such as lipopeptide, bacteriocine and insect toxins giving it a competition advantage for the colonization of the rhizosphere environment (*12, 15*).

Pseudomonas spp. bacteriophages

Pseudomonas spp. bacteriophages have been isolated mainly from soil and waste water, reflecting the wide variety of ecological environment in which their hosts are presents. About the 97% of *Pseudomonas* spp. bacteriophages described so far belong to *Caudovirales* order according to ICTV (International Committee on the Taxonomy of Viruses) classification (*7*). More precisely, *Myoviridae* family phages (PB1 and ΦKZ-like type) have been isolated active against *P. plecoglossicida, P. putida*, *P. fluorescens* and *P. aeruginosa* species; *Shipoviridae* family has been found only in *P. aeruginosa* temperate bacteriophages but it represents the biggest rate (47%) of *Pseudomonas* spp. bacteriophages described; *Podoviridae* family members have been isolated infecting *P. putida*, *P. fluorescens* (T7-like virus typology) and *P. aeruginosa* (ΦKMV and LUZ24-like type) (*7*). This list miss all the complete and partial prophage sequences integrated into hosts chromosome, carrying in addition to phage-related genes also non-essential genes that can modify the phenotype of the host (*17*). One of the most studied example of this is the production of R-type and F-type pyocines by *P. aeruginosa*, coded by ancestral phage-related genes (*20*).

P. fluorescens bacteriophages

P. fluorescens strains genomes have been founded to contain multiple prophage-like regions (six in Pf_05, four in Pf_01, two in SBW25, three in A506) (*14*, *17*, *26*) but there are still no report in literature of prophage induction and isolation in this species. On the other hand lytic bacteriophages active on *P. fluorescens* have been widely isolated and studied. *P. fluorescens* SBW25 and its phage SBW25Φ2 have been used as model to study coevolution strategies in the bacterium-phage system since 2002 (*4*, *5*, *21*, *25*). Other lytic bacteriophages were isolated to be used in industrial and clinical environment, like the sequenced ΦUFV-P2, isolated from a Brazilian dairy industry (*11*); for example phage ΦIBB-PF7A and ΦS1 had been tested for the removal of biofilm formed by *P. fluorescens* (*27*, *28*)

P. fluorescens contamination in dairy products

Depending of the hygienic quality of milking procedure, *Pseudomonas* spp. may represent about 10%-50% of the microflora present in raw milk, but it becomes the dominant genus in spoiled raw milk and cheese obtained from raw milk having the shortest generation time at refrigeration temperature (1-7°C)(*29*). The species is not resistant to pasteurization and UHT treatment but it produce heat-resistant enzymes persisting after the processing of the milk (*10*). *P. fluorescens* can cause UHT milk clumping and sedimentation by the production of strain specific proteolytic enzymes that hydrolyse caseins (*3*), metallo-proteases (usually containing zinc and calcium), lipases and esterases. Calcium also stimulates enzymes production and it is necessary for their stability at high temperature (*29*). These enzymes can be founded not only in milk but also in cheese, where they can cause bitterness, unpleasant end products and, in some cases, the decreasing of cheese yield (*2*).

In recent years the development of a blue coloration, in particular on fresh cheese, has been reported as consequence of *P. fluorescens* contamination. As the proteases production, the defect appears to be strain specific (*16*). Several studies have been made on the nature of this blue coloration: Caputo *et al*. identified it as a derivate of leucoindigoidine (*6*), while Andreani et al. through the genome sequencing of two blue producing strains hypothesized that the blue synthesis comes from indole(*1*) but a certain identification of the blue pigment is still not available in literature.

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AIM OF THE STUDY

The aim of this Ph.D. thesis is the advancing in knowledge about the blue phenotype of some *Pseudomonas fluorescens* strains isolated mainly from dairy products, involved in the spoilage of Mozzarella cheese.

The investigation was made regarding different perspectives:

- the genotyping of 69 isolates, of which 30 blue pigment-producing isolates through the whole genome restriction analysis (REA-PFGE) and the phylogenetic correlation of each genotype by Multi Locus Sequence Typing (MLST) analysis through the sequencing of seven conserved genes;
- the definition of the nutritional requirements for the production of the blue pigment to improve the understanding of its function and its identification;
- the whole genome sequencing and analysis of blue pigment-producing strains and the comparison with sequenced blue not-producing *P. fluorescens* to identify the DNA region coding for the blue phenotype;
- the phage induction of blue pigment-producing *P. fluorescens* strains, considering a virus as vector of DNA horizontal transfer in an ancestor strain and as possible carrier of the blue phenotype.

1 BIODIVERSITY IN BLUE PRODUCING *PSEUDOMONAS FLUORESCENS* ISOLATES

1.1 Introduction

The phylogeny and the reliable identification of *P. fluorescens* isolates is not easily achievable: actually, *P. fluorescens* is regarded as a group (*8*, *11*) rather than a well-defined species within *Pseudomonas* genus and a consistent classification can be reached only using different target genes for sequencing, in addition to the usual 16S rRNA gene (*1*, *10*, *20*). From the comparative analysis of the complete genomes of different strains of *P. fluorescens*, it resulted that this complexity in *Pseudomonas* spp. identification is given by a small conserved core genome (representing only half of the genome of each strain) and a large pangenome (*8*, *15*). For this reason the conventional method for subtyping *P. fluorescens* is still based on phenotypical characteristics such as substrate utilization (API 20 NE profiles), even if it needs a high standardization because it is susceptible to the risk of low reproducibility (*17*, *18*). Molecular methods have been also proposed and used, such as Ribotyping (using *EcoRI* (*18*), *SmaI* and *HincII* enzymes), or the restriction enzyme analysis of the whole genome coupled with pulsed-field gel electrophoresis (PFGE) (*12*), while the typing through the amplification of conserved region such as 16S rDNA or the 16S–23S intergenic spacer have been proposed associated with further phenotypic analysis to confirm the molecular result obtained (*13*, *18*).

In this first part of the work, a collection of *P. fluorescens* isolated from dairy samples was examined in order to evaluate the strain diversity and the correlation between the blue pigment production and the genotype.

1.2 Bacterial strain collection.

1.2.1 Material and methods

Sixty-nine isolates belonging to *Pseudomonas fluorescens* group, listed in Appendix 1, were investigated. Three strains were purchased from international collections (*P. fluorescens* ATCC 13525, 50154, 50108), eight isolates were kindly provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (200188/1, 200188/2, 200188/6, 200188/8, 176673/1, 9AG, 9BG, 9AP). *P. fluorescens* strains SBW25, H and A506 were kindly supplied by academic collections, respectively from University of Exeter (United Kingdom), Universidade do Minho (Portugal) and Oregon State University (USA). The remaining 55 isolates were recovered from spoiled dairy products with blue coloration, collected between 2010

and 2014 as follows: approximately 10 g of fresh cheese sample were homogenized in 2% (w/v) sodium citrate and decimally diluted in ¼Ringer solution according to FIL-IDF standard 050:2008 (*7*). Appropriate aliquots were plated on Tryptic Soy Agar (TSA) (Sigma-Aldrich, St. Louis, USA) and on *Pseudomonas* Agar base added with CFC supplement medium (Merck, Darmstadt, Germany) and incubated at 30°C for 48 hours. Pure cultures were obtained from single colonies at the highest dilutions by twice striking on TSA and stored in Tryptic Soy Broth (TSB) added with 20% glycerol (Sigma-Aldrich) at -80°C.

1.2.1.1 Isolate identification

Fresh cells of each isolate were obtained by overnight culture in TSB at 30 °C; after centrifugation at 4000 *g* for 10 min, they were washed twice in deionized water and resuspended in 400 µl 1x TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA extraction was carried out by using GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich). The identification of the isolates was done by partial sequencing of the 16S rRNA gene. For the amplification reaction the universal bacterial primers BSF-8/20 and BSR-1541/20 were used according to Wilmotte *et al.* (1993) (*19*). The amplified products were partially sequenced by an outdoor provider (Eurofins Genomics, Ebersberg, Germany) and the obtained sequences were compared with GenBank database (http://www.ncbi.nlm.nih.gov).

1.2.1.2 Determination of the blue phenotype.

All the isolates of the collection were checked for the production of the blue pigment by striking on Mascarpone Agar (MA) according to Cantoni *et al.* (*3*) and incubating at 30°C for 72 hours. The preserving fluid (PF) of retail Mozzarella cheese obtained by biological maturation was also used as cultural medium to reproduce the discoloration phenomenon. It was centrifuged at 9000 *g* for 30 min and then filtered 0.22 µm, getting a competitor free broth comparable with the real environment in which the blue defect occurs. The isolates that showed a dark blue discoloration on MA were selected and fresh cells from overnight culture were 1% inoculated in 2mL of PF in 24 wells microplates. The incubation was carried out at three different temperatures (4°C, 14°C and 30°C) to confirm the pigment production. Color development was checked daily.

1.2.2 Results

1.2.2.1 Isolates identification

The new 55 isolates from dairy products were identified by partial sequencing of 16S rRNA. Results confirmed their belonging to *P. fluorescens* group; actually, most of them (52.7%) were ascribed to *P. fluorescens*, whereas 9.1% was attributed to *P. fragi*, 9.1% to *P. gessardii*, 9.1% to *P. libanensis*, 7.3% to *P. cedrina*, 7.3% to *P. costantinii* and *P. meridiana* and 1.8% to *P. azotoformans*, *P. grimontii and P. poae* (figure 1.1).

1.2.2.2 Determination of the blue phenotype

A blue or dark blue pigment was produced at 30°C on Mascarpone agar plates by 30 isolates (Table 1), whereas the remaining 38 showed a variable coloration from yellow to dark green or black, so they were dropped from subsequent analysis. In particular, none of the strains taken from international or academic collections was able to produce the blue phenotype. In the positive cases, the blue pigmentation occurred after 72h hours and it was diffusible in the MA medium, as reported by Cantoni *et al.* (*3*). The results observed after 7 days of incubation in preserving fluid of Mozzarella cheese at 30°C and 14°C, and after 10 days of incubation at 4°C are summarized in Table 1. Interestingly, the color production of the isolates grown in PF was different from that detected on MA medium. At the incubation temperature of 30°C no blue coloration was developed by any strain, while the PF was turned yellow (Table 3.1). At 14°C the blue pigment was produced after 72h, only by few isolates (UMB247, UMB248, UMB253, UMB254, UMB288 and 200188/6) but the coloration changed in yellow-green during the following days of incubation. At 4°C most of the strains produced a brilliant blue or dark blue coloration after 10 days. Some isolates (UMB249, UMB258, UMB260, UMB292, 176673/1, 9AP) which produced the blue darkening in MA did not reply the blue pigmentation in PF (Table 1.1)

					MA[1]		PF[2]	
strain code	**source**	**place**	**year**	**PFGE pulsotype**	**30°C**	**4°C**	**14°C**	**30°C**
UMB247	mozzarella cheese	Italy	2013	XXXIX	dark blue	blue	blue	yellow
UMB248	preserving fluid	Italy	2013	XXXIX	dark blue	blue	blue	yellow
UMB249	preserving fluid	Italy	2013	XLII	dark blue	colourless	yellow	yellow
UMB253	mozzarella cheese	Italy	2010	XXI	blue	blue	blue	yellow
UMB254	mozzarella cheese	Italy	2010	XIV	blue	blue	blue	yellow
UMB255	mozzarella cheese	Italy	2010	XIV	blue	blue	blue	yellow
UMB256	mozzarella cheese	Italy	2010	XLII	blue	blue	blue	yellow
UMB257	mozzarella cheese	Italy	2010	XIV	blue	blue	colourless	yellow
UMB258	mozzarella cheese	Italy	2010	XLII	blue	colourless	blue	yellow

UMB260	mozzarella cheese	Italy	2010	XLII	blue	colourless	blue	yellow
UMB261	mozzarella cheese	Italy	2011	XLII	dark blue	blue	blue	yellow
UMB268	mozzarella cheese	Italy	2010	XLII	blue	blue	colourless	yellow
UMB287	mozzarella cheese	Italy	2011	XXXV	dark blue	blue	blue	yellow
UMB289	mozzarella cheese	Italy	2011	XLII	dark blue	blue	blue	yellow
UMB290	mozzarella cheese	Italy	2011	XLI	dark blue	blue	blue	yellow
UMB291	mozzarella cheese	Italy	2011	XLI	dark blue	blue	blue	yellow
UMB292	mozzarella cheese	Italy	2011	XLII	dark blue	colourless	yellow	yellow
UMB293	mozzarella cheese	Italy	2011	IV	dark blue	blue	blue	yellow
UMB294	mozzarella cheese	Italy	2011	IV	dark blue	blue	blue	yellow
UMB295	mozzarella cheese	Italy	2011	III	dark blue	blue	blue	yellow
UMB296	mozzarella cheese	Italy	2011	III	blue	blue	blue	yellow
UMB309	ricotta cheese	Italy	2014	XI	dark blue	blue	blue	yellow

176673/1	mozzarella cheese	Germany	2010	XI	dark blue	colourless	yellow	yellow
200188/1	mozzarella cheese	Germany	2010	XXXVII	dark blue	blue	blue	yellow
200188/2	mozzarella cheese	Germany	2010	XXXVII	dark blue	blue	blue	yellow
200188/6	mozzarella cheese	Germany	2010	XXXII	dark blue	blue	blue	yellow
200188/8	mozzarella cheese	Germany	2010	XXXVII	dark blue	blue	blue	yellow
9BG	mozzarella cheese	Germany	2010	XI	dark blue	blue	blue	yellow
9AP	mozzarella cheese	Germany	2010	XI	dark blue	colourless	yellow	yellow
9BP	mozzarella cheese	Germany	2010	XI	dark blue	blue	blue	yellow

Table 1.1: List of the 30 blue pigment--producing isolates investigated in this work with their isolation details, PFGE profile and their color development in Mascarpone Agar medium (MA) and in preserving fluid (PF) of Mozzarella cheese.

1.3 Strain typing by Restriction Enzyme Analysis by using PFGE technique.

1.3.1 Material and methods

The genomes of all 69 isolates were analyzed by Restriction Endonuclease Analysis using Pulsed-Field Gel Electrophoresis (REA-PFGE). Pure cultures were grown overnight on Nutrient Agar (Merck, DE) at 30°C, then single colonies were dissolved in Cell Suspension Buffer (0.1M TRIS HCl , 0.1M EDTA, pH 8) to reach an absorbance value at OD_{600nm} between 0.6 and 0.8. Then, 200 µL of the cell suspension were mixed with an equal amount of 2% agarose gel melt in TE buffer (0.01M TRIS HCl, 0.01M EDTA, pH 8) and kept in water bath at 55°C. Each plug was immersed in 5 mL of Cell Lysis Buffer (0.05M TRIS HCl, 0.05M EDTA, pH 8, 1% (w/v) Sarcosyl, 0.2 mg/mL Proteinase K (Sigma-Aldrich)) and incubated overnight at 37°C with shaking at 80 rpm. Lysis solution was removed and rinsing steps were made adding 8.5 mL of TE buffer pre-warmed at 50°C and incubating at 50°C for 10 minutes. This step was repeated 4 times. Genome digestion was made with *SpeI* enzyme (ThermoScientific, Waltham, USA), in the following solution: *SpeI* 20 U, Tango buffer 1x, TE 1x to reach 200 µL volume. The enzymatic digestion was made at 37°C for 6 hours. Digested plugs fragments were placed in a 1% pulsed field certified agarose gel in TBE buffer (0.09M TRIS HCl, 0.09M Boric Acid, 2mM EDTA, pH 8). Run conditions on CHEF Mapper (BioRad Laboratories, Hercules, USA) were 14°C, 6 volt, initial switch 1s, final switch 25 s, runtime 22 h. This protocol is a slight modification of the one proposed by Martin *et al.*, (*9*) and Nogarol *et al.*, (*12*). The gel was then stained by diving in ethidium bromide solution (1µg/mL) for 10 minutes and rinsed with distilled water for 20 minutes. Images were captured with Gel DOC XR (BioRad Laboratories) and were analyzed with GelJ software (*6*) to align band profiles. A similarity tree was created by using Dice similarity method with a 1% tolerance and UPMGA linkage.

1.3.2 Results

Genomic patterns generated from all isolates by *SpeI* digestion are reported in Figure 3.1. The similarity percentage joining the restriction patterns of *P. fluorescens* ATCC 13525^T strain, which was used as marker and replicated in all runs, was chosen to assess the ability of the protocol to discriminate among strains. In our experimental conditions, this similarity value stood at 80% and it was considered the threshold above which it was not possible to distinguish among isolates of a same strain (data not shown). This cut-off of discrimination agrees with that reported by Nogarol *et al.*,

(2013)(*12*). From 69 isolates, 43 genome patterns were recognized, corresponding to different genotypes defined as "pulso-types". Blue pigment-producing strains belonged to 12 different pulso-types (number III, IV, XI, XIV, XXI, XXXII, XXXV, XXXVII, XXXIX, XLI, XLII, XLIII), as reported in Table 1.1, and they were placed in different clusters of the UPMGA tree (Figure 3.1). Isolates grouped in pulso-types XIV, XXI and XLIII were recovered from the same Mozzarella cheese sample (except for isolate UMB248), as well as for isolates gathered in pulso-types III, IV, XXXV, XLII that were collected from another sample of Mozzarella cheese. In these cases, the contamination was polymicrobial, namely characterized by the coexistence of different strains of the same species. Conversely, five blue pigment-producing isolates (UMB249, UMB256, UMB258, UMB260, UMB261), recovered in different years, are joined together in a same pulso-type (XLIII); similarly, five other isolates (9BG, 9BP, 9AP, 176673/1, UMB309) collected in different times and places, were positioned in the same pulso-type (XI). These cases confirm the hypothesis of the presence of resilient strains in dairy factory environments (*12*).

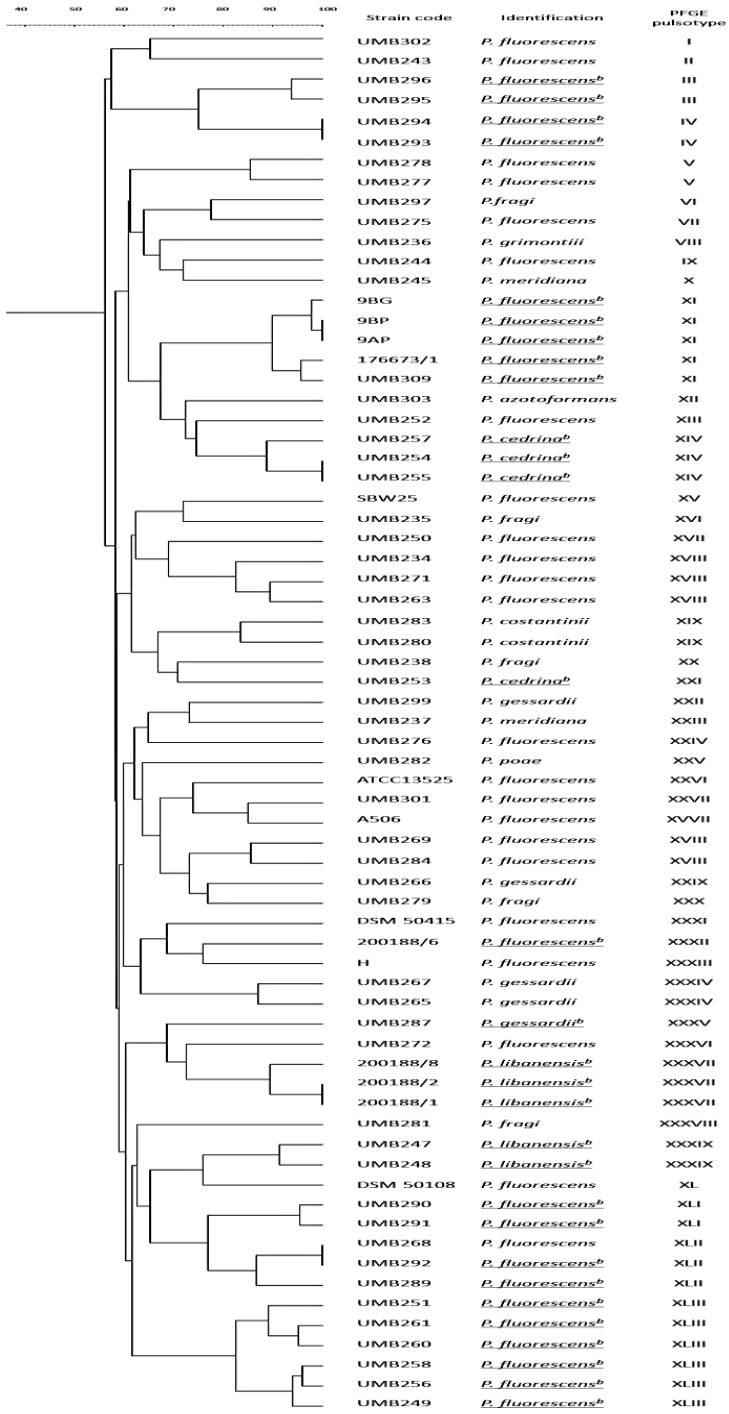

Figure 1.1:Restriction profiles of the 69 isolates obtained by PFGE and their specie attribution. Blue pigment-producing isolates are underlined and marked with b

1.4 Multi-Locus Sequence Typing

1.4.1 Material and methods

A Multilocus Sequence Typing scheme was performed on representative isolates according to the clustering analysis obtained with PFGE profiles. The protocol of Andreani *et al.* (*2*) was followed. Seven *loci* of different housekeeping genes (*gyrB, glnS, ileS, nuoD, recA, rpoB, rpoD*) were amplified in 25 µL reaction mixture composed of Buffer 1x, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM each primers, 1 U Taq (5prime,USA), 10 ng of sample DNA. The amplification protocol was performed in a Mastercycler ep® thermal cycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: initial step at 94°C for 2 min, 35 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 30 s and extension at 72°C for 1 min, final extension step at 72°C for 7 min. The amplification products were visualized by electrophoresis on 1.8% (w/v) agarose gels, stained with ethidium bromide and sequenced for both DNA strands. The obtained sequences were trimmed and aligned using CLC software (Qiagen, Venlo, Netherlands). Sequences were then concatenated following the alphabetical order of the *loci*, aligned and compared with the all sequenced strains in the MLST database obtaining a phylogenetic tree based on Maximum Likelihood algorithm with MEGA software (*16*).

1.4.2 Results

From each of the twelve pulso-types that included blue pigment-producing isolates, one representative strain with the blue phenotype was selected for MLST analysis. The sequences obtained from each of the seven *loci* were compared with the related sequences available in *P. fluorescens* MLST database (http://pubmlst.org/pfluorescens), obtaining the corresponding alleles and ST profiles (Table 1.2). Six strains out of twelve (200188/6, 200188/8, 9BG, UMB253, UMB254, UMB260), had an already known allelic profile. Of the remaining six, three strains (UMB287, UMB289, UMB293) exhibited the same new allelic profile (ST 99), whereas the others (UMB 248, UMB291 and UMB295) revealed new single allelic profiles (STs 102, 100 and 101, respectively). The sequences of the seven *loci* for each of the twelve selected blue-producing strains were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the accession number from KU512209 to KU512285.

strain code	*glnS*	*gyrB*	*ileS*	*nuoD*	*recA*	*rpoB*	*rpoD*	ST profile
200188/6	29	29	29	29	29	29	29	29
200188/8	29	29	29	29	29	29	29	29
9BG	26	26	26	26	26	26	26	26
UMB248	26	26	88	26	26	83	30	102
UMB253	25	25	25	25	25	25	25	25
UMB254	25	25	25	25	25	25	25	25
UMB260	25	25	25	25	25	25	25	25
UMB287	90	25	25	91	87	69	25	99
UMB289	90	25	25	91	87	69	25	99
UMB291	90	84	25	92	88	45	25	100
UMB293	90	25	25	91	87	69	25	99
UMB295	91	25	25	25	87	69	25	101

Table 1.2: Allelic profile corresponding to the DNA sequences of each locus and ST profile obtained from concatenated sequences of the 12 strains used for MLST analysis

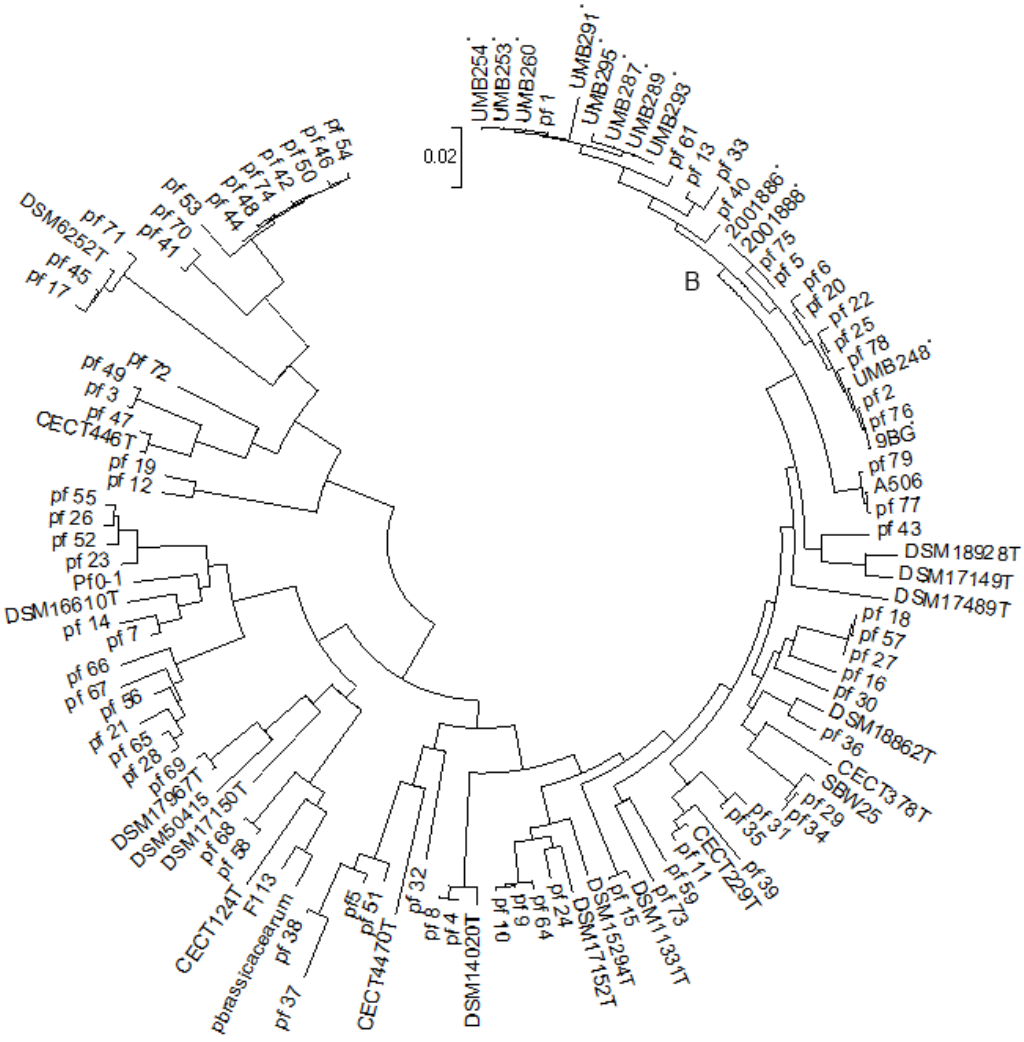

Figure 1.2: Maximum Likelihood tree obtained from the comparison of concatenated sequences from all - *loci*. Isolates sequenced for the 7 MLST genes in this work are marked with a dot following the name. The "blue branch" is indicated by letter B

The comparison with the sequences of strains investigated by Andreani *et al.* (*2*) showed that the strains with the same allelic profile were frequently isolated from the same geographic region. In particular, all the strains belonging to the allelic profile 25 were isolated in North East Italy, while those attributed to the allelic profile 29 were collected from Germany. The concatenated sequences obtained from the twelve selected strains of this work were aligned with those available in the *P. fluorescens* MLST database to build a phylogenetic tree (Figure 1.2): all our new strains were assembled within the cluster formed by the former blue pigment-producing strains. This outcome corroborates the hypothesis of the existence of a "blue branch" in *P. fluorescens* species, meaning that all the isolates producing the discoloration share a common evolutionary development, as already suggested by Andreani *et al.* (*2*).

1.5 Discussion and conclusion

After the cases of blue discoloration in Mozzarella cheese that occurred in Italy since 2010, the interest in *P. fluorescens* as a contaminant of dairy plants and fresh dairy products is increased (*4*, *9*, *12*, *14*). The attribution of the defect to the above-mentioned species is well-founded and it's known that it depends on the growth conditions of bacterial cells (*5*). Our results confirm these observations and highlight how, in spoiled samples, populations of blue-producing strains may coexist with those that do not generate the discoloration. Moreover, a same pulso-type can be isolated years later in the same place, settling the possibility that a same strain may persist in a dairy working environment for a long time. As regards a specific isolate, the appearance of the blue pigmentation in the preserving fluid (PF) is affected by the incubation temperature and, particularly, it occurs at 4°C and at 14°C for 80% of the isolates, while is not taking place at 30°C.

Because of the high heterogeneity of *P. fluorescens* genome, the REA PFGE protocol proves to be unsuitable as discriminatory technique to identify blue pigment-producing strains, since isolates grouped in the same pulso-type may exhibit a different phenotype. This agrees with what reported by Nogarol *et al.* (*12*). On the other hand, the results of the MLST analysis endorse the supposition that blue-producing strains have a common ancestor, as already suggested (*2*). The meaning of the phylogenetic split-up of this cluster respect to other strains *P. fluorescens* is unknown. However, it must be noted that these strains are all deriving from the dairy environment and that the phenomenon occurs in products subjected to chill storing.

1.6 References

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Microbiology 146: 85–89

2 BLUE PIGMENT INVESTIGATION: NUTRITIONAL REQUIREMENTS, FUNCTION AND IDENTIFICATION

2.1 Introduction

The production of the blue pigment from *P. fluorescens* strains has still an unknown function. The identification of the blue molecule/s became even more important when this particular phenotype of this species was recently found in fresh cheeses (*7*, *9*, *16*). From literature research it is known that *Pseudomonas* spp. produce colored molecules as secondary metabolites, for example siderophores (*2*, *20*) phenazines or bacteriocins like the famous pyocianin produced by *P. areuginosa* (*11*). In *Pseudomonas* spp. the production of these molecules is frequently related to *quorum sensing* signals (*20*), extracellular low molecular mass molecules present in a concentration depending on the cellular density and the growth phase of the producing organism, sensed by surroundings cells and able to modulate their physiological processes. The most common of these molecules are N-acyl derivates of homoserine lactone (*12*, *21*).

The first hypothesis on the nature of the blue coloration developed in fresh cheese was made by Cantoni *et al*. (2003) (*6*) that supposed it could be indigoidine, by basing on the studies made in 1960's on two blue producing *P. indigofera* and *P. lemonnieri* species. This assumption was confirmed later by Caputo *et al*. (2015) by ESI- Orbitrap-based mass spectrometry (*8*). Indigoidine is an intracellular pigment, not soluble in water, but this characteristic is not shared by the blue pigment produced on fresh cheese considering that it is highly diffusible in cheese structure and also (when it occurs in Mozzarella) in preserving fluid, as shown in figure 2.1.

Another recent identification of the blue pigment was made by Andreani *et al.* (2015). Using a transcriptomic approach coupled with MALDI-TOF mass spectrometry an indigo analog was identified, probably derivative by indole, related to tryptophan metabolism. Anyway the molecule structure was not yet defined (*1*).

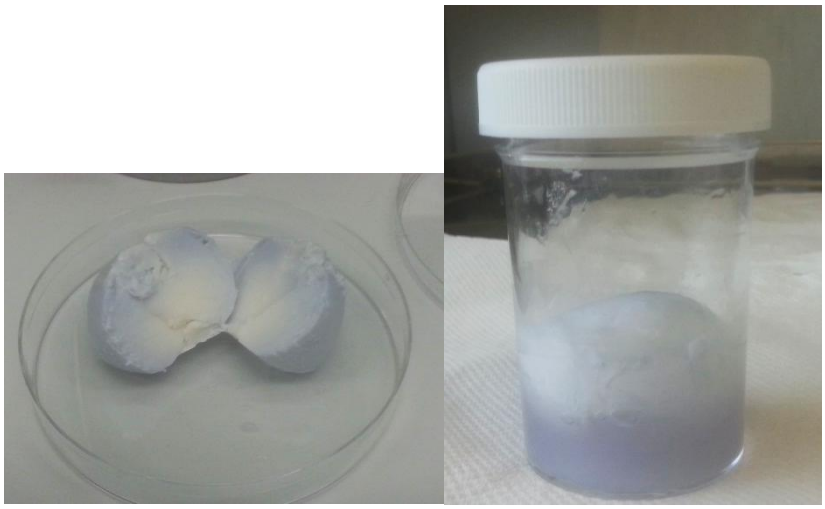

Figure 2.1: images of blue discoloration spoilage on Mozzarella cheese and relevant preserving fluid by *P.fluorescens* reproduced during laboratory experiments

In this work the investigation approach to identify the blue color was reversed. Instead of searching directly the molecule structure, factors influencing the blue production were investigated. First it was observed that the blue pigment was produced in liquid medium only when the strains were grown at low temperature 4-14°C (chapter 1) and that when grown at 30°C the coloration was not formed. So it was examined if there were other growing factors (carbon source, metals, amino acids source) influencing the blue color development. It was also investigated if the blue pigment could be related to *quorum sensing* signals, considering that its development occurs when the bacterial load reaches high concentration (> 10^6 UFC/mL). It could bear a negative effect for other *P. fluorescens* populations, giving a competitive advance to the blue pigment-producing strains. Then the chemical properties of the unpurified blue color (pH stability) were assayed and UPLC coupled with MS analysis were performed.

2.2 Nutritional requirements for the blue phenotype expression

2.2.1 Materials and methods

To evaluate the nutritional requirements for the production of the blue pigment, 12 blue pigment-producing *P. fluorescens* group strains (chosen according to REA-PFGE genotyping results, see paragraph 1.3) and two blue pigment not-producing *P. fluorescens* strains were inoculated in M9 minimal medium (12.8g/L Na_2HPO_4 $7H_2O$, 3g/L KH_2PO_4, 0.5g/L NaCl, 1g/L NH_4Cl) with the addition of different carbon sources, metals and amino acids. Final pH was set at 5.7, reflecting the environmental conditions in which the blue defect was observed. All the trials were made in 24 wells micro-plates with 2mL of liquid medium in each well. The list of the strains used is reported in Table 2.1. A506 and DSM50415 strains were chosen as negative control.

Strain	16S rDNA identification	Genotype according to REA-PFGE
200188/6	*P.fluorescens*	XXXII
200188/8	*P. libanensis*	XXXVII
9BG	*P. fluorescens*	XI
UMB247	*P. libanensis*	XXXIX
UMB248	*P. libanensis*	XXXIX
UMB253	*P. cedrina*	XXI
UMB254	*P. cedrina*	XIV
UMB258	*P. fluorescens*	XLIII
UMB260	*P. fluorescens*	XLIII
UMB287	*P. gessardii*	XXXV
UMB291	*P. fluorescens*	XLI
UMB293	*P. fluorescens*	IV
DSM50415	*P. fluorescens*	XVVII
A506	*P. fluorescens*	XXXI

Table 2.1: *Pseudomonas spp*. strains selected for nutrient assays

2.2.1.1 Test with different carbon sources

For the determination of the role of carbon source for the blue color development, M9 medium was added separately with a final concentration of 30mM glucose, galactose, sodium citrate and sodium lactate solutions adjusted at pH 5.7 and filtered 0.22 µm.

Twenty µL of each strain grown at 30°C overnight in Nutrient Broth (Sigma-Aldrich) were inoculated in 2mL of M9 medium supplemented with micronutrients (1mM $MgSO_4$, 100µm $CaCl_2$, 3nm $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.4µM H_3BO_3, 30nm $CoCl_2\cdot 6H_2O$, 10nm $CuSO_4$, 80nm $MnCl_2\cdot 6H_2O$, 10nm $ZnSO_4\cdot 7H_2O$, 1µm $FeSO_4\cdot 7H_2O$) and different carbon source. Samples were incubated at 4°C, 14°C and 30°C until the development of the blue coloration.

2.2.1.2 Test with different amino acids

For the determination of the influence of single amino acid on the blue production each strain was incubated in M9 minimal medium 0.2% (w/v) glucose with the addition of one of the 19 amino acid listed in Table 2.2 at 1mM final concentration.

Amino acids			
Tyrosine	Isoleucine	Glycine	Valine
Histidine	Lysine	Histidine	Glutamine
Proline	Alanine	Phenylalanine	Glutamic acid
Asparagine	Arginine	Serine	Tryptophan
Leucine	Cysteine	Threonine	

Table 2.2: amino acids used

Assays were made with the same procedure described in carbon source paragraph. In this case, the micro-plates were incubated at 4°C for 10 days.

2.2.1.3 Test with different mineral salts

For the determination of the influence of single micronutrient on the blue production each strain was incubated in M9 minimal medium 0.2% glucose pH 5.7 including just one mineral salt from the M9 micronutrient mix. To verify if also the lack of one micronutrient could affect the blue color synthesis assays using different mixes of micronutrient were done as reported in Table 2.3.

MIX NUMBER	Micronutrient composition
1	Mo, Bo, Mn, Ca, Fe, Mg
2	Bo, Mn, Ca, Fe, Mg
3	Mo, Mn, Ca, Fe, Mg
4	Mo, Bo, Ca, Fe, Mg
5	Mo, Bo, Mn, Fe, Mg
6	Mo, Bo, Mn, Ca, Mg
7	Mo, Bo, Mn, Ca, Fe

Table 2.3: Composition of the micronutrient mixes used

Plates were prepared as described in the previous paragraphs and incubated at 4°C for 10 days.

2.2.2 Results

2.2.2.1 Influence of the carbon source

Beyond the temperature, the carbon source affects the blue color development. Plates incubated at 30°C started to change the aspect of the minimal medium from colorless to yellow after 48h for each carbon source used. Plates observed after 96h showed a yellow-brown coloration; after 168h and 240h of incubation no further development of the well colors were detected (figure 2.2). When the incubation was made at 14°C the blue coloration was detected after 96h only in wells containing the medium supplemented with glucose, while in the other media the coloration was turned grey-brown. During the observation made after 168h and 240h it was noticed that the blue pigment deteriorated first in dark blue and then turned brown-green (figure 2.3). A brilliant light blue coloration was observed only in glucose containing medium incubated at 4°C. In this case the coloration development was seen after 240h for 5 strains (figure 2.4).

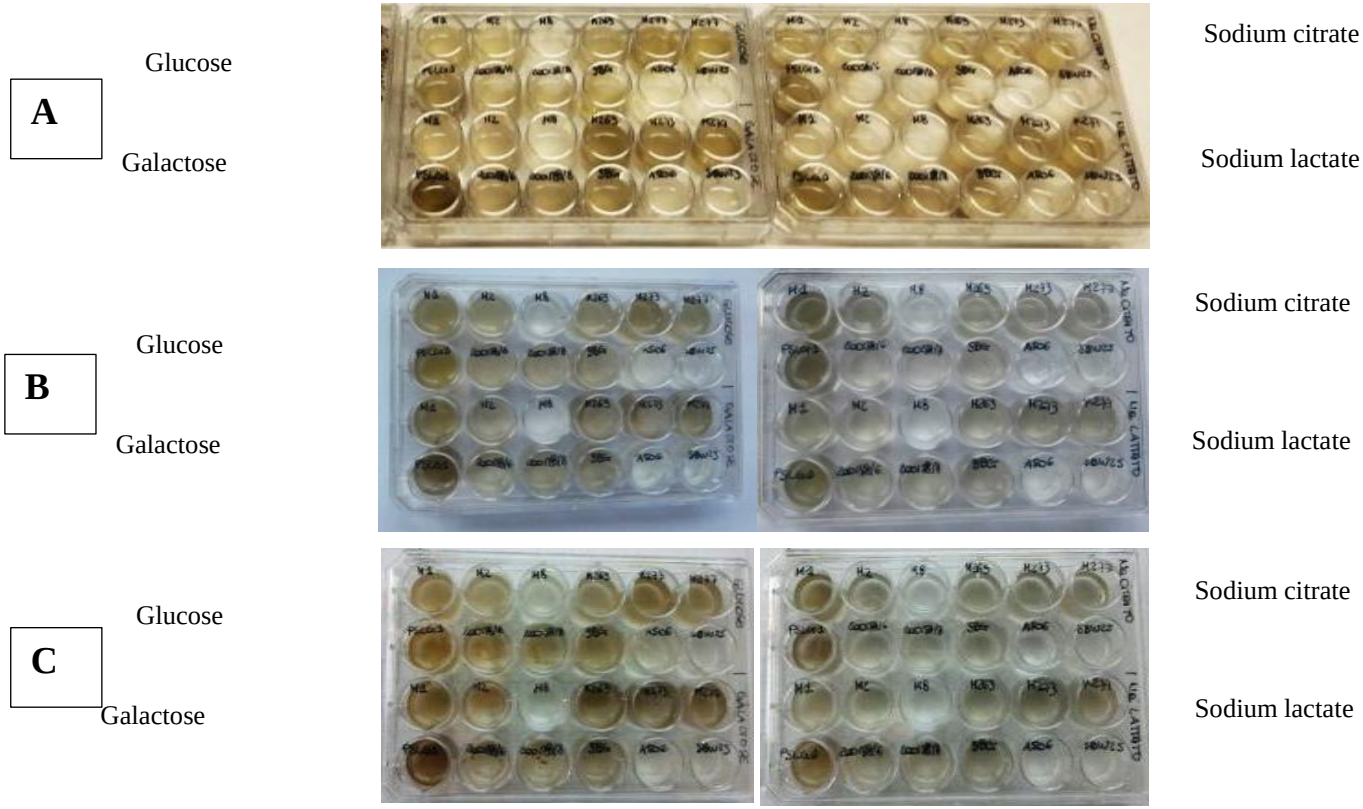

Figure 2.2: Colour development in plates of M9 supplemented with different carbon sources (pH 5.7) incubated at 30°C after 96h (A), 168h (B) and 240h (C). Strains for each double lines: UMB253, UMB254, UMB260, UMB287, UMB291, UMB293, UMB248, 200188/6, 200188/8, 9BG, A506, DSM50415

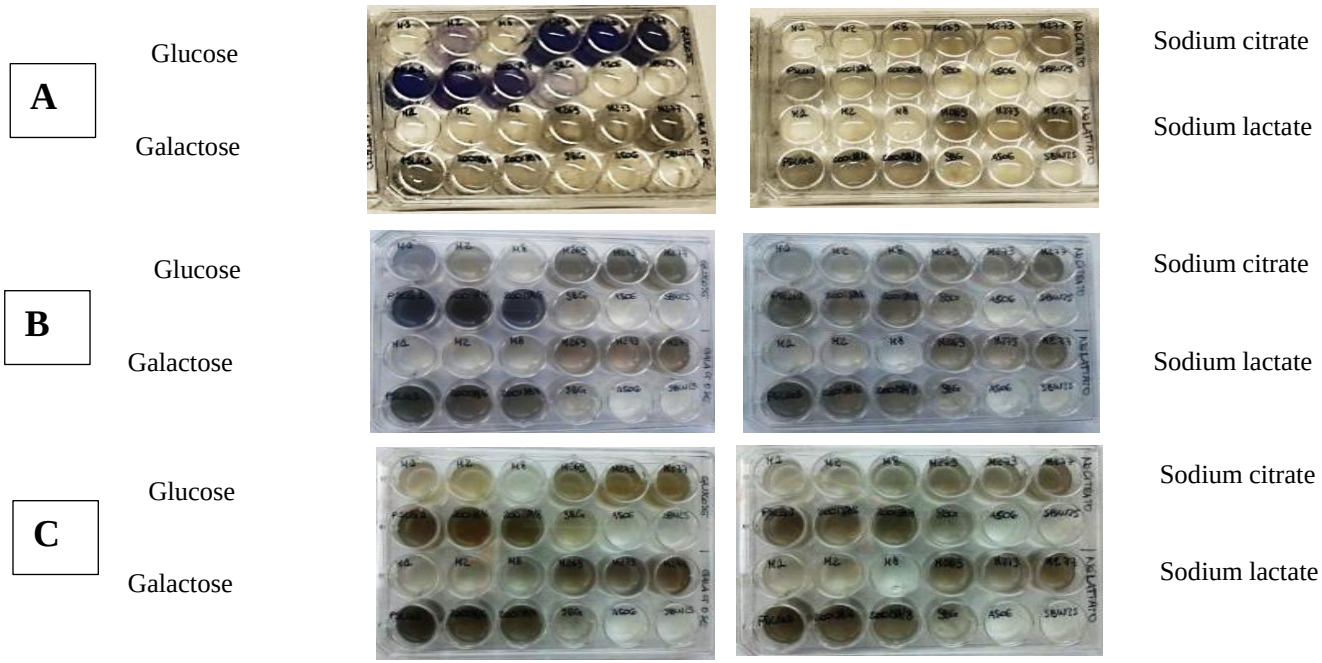

Figure 2.3: Colour development in plates of M9 supplemented with different carbon sources (pH 5.7) incubated at 14°C after 96h (A), 168h (B) and 240h (C). Strains for each double lines: UMB253, UMB254, UMB260, UMB287, UMB291, UMB293, UMB248, 200188/6, 200188/8, 9BG, A506, DSM50415

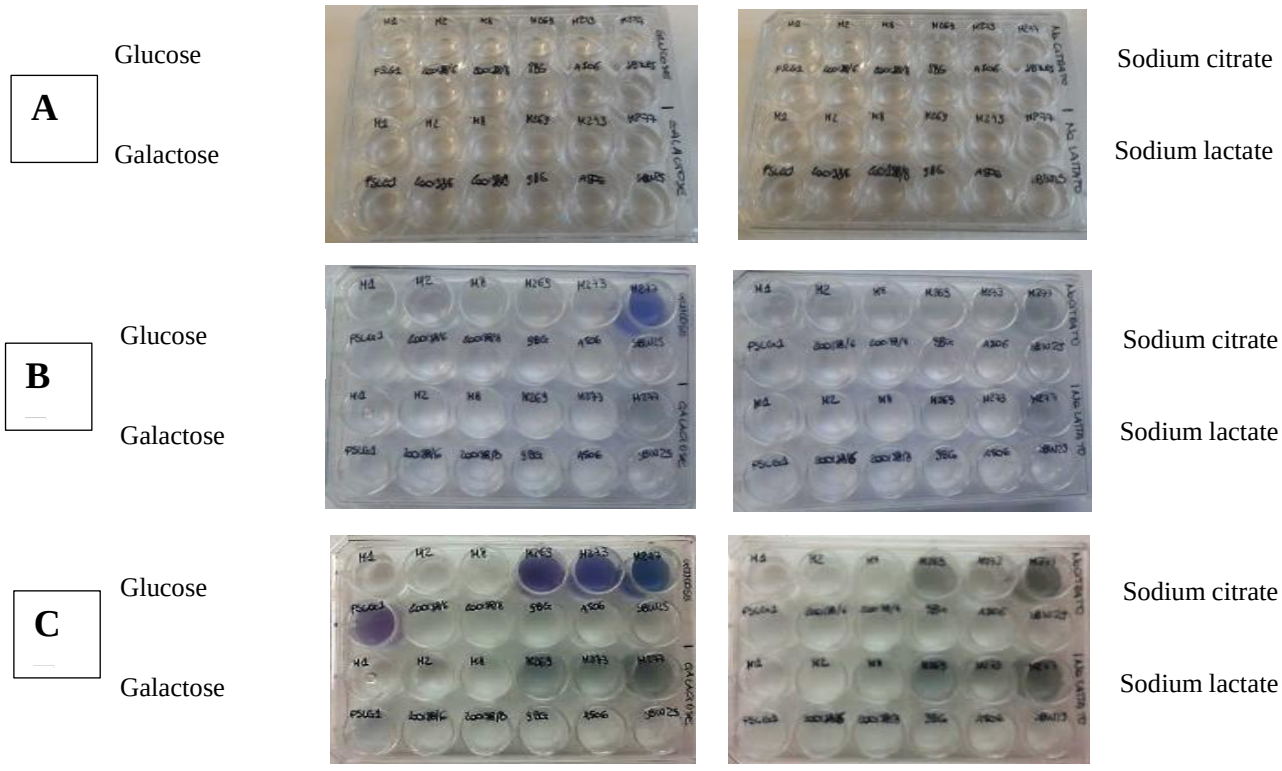

Figure 2.4: Colour development in plates of M9 supplemented with different carbon sources (pH 5.7) incubated at 4°C after 96h (A), 168h (B) and 240h (C). Strains for each double lines: UMB253, UMB254, UMB260, UMB287, UMB291, UMB293, UMB248, 200188/6, 200188/8, 9BG, A506, DSM50

2.2.2.2 Influence of amino acids

The incubation of blue strains with different amino acids showed that there is not a single amino acid responsible for the blue production and that the coloration occurs in presence of different amino acids. It was possible instead to identify some amino acids with an inhibitory effect on the synthesis of the blue pigment such as leucine and isoleucine, while the unique presence of glutamic acid, tyrosine and cysteine didn't allow the growth of the *Pseudomonas* spp. strains, as shown in figure 2.5. The most intense blue coloration was obtained when proline was added. Again, it was noticed that after 240h the pigment deteriorate resulting dark grey.

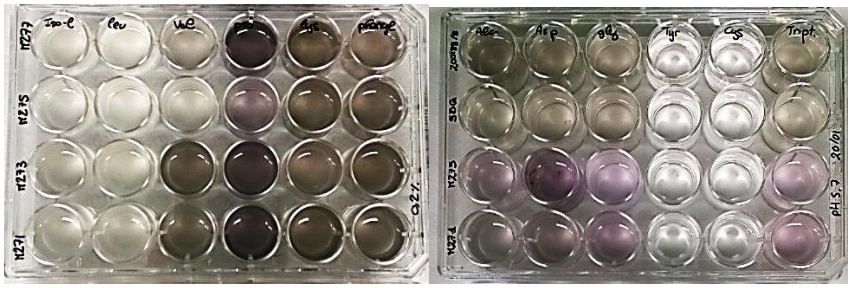

Figure 2.5**: Effect of some of the different amino acids on the production of the blue pigment. Amino acids order: isoleucine, leucine, valine, proline, lysine, phenylalanine, alanine, arginine, glycine, tyrosine, cysteine, tryptophan. Strains for each lines: UMB295, UMB293, UMB291, UMB289 (right), 200188/6, 9BG, UMB293, UMB289 (left)**

2.2.2.3 Influence of mineral salts

As occurred for the amino acids assays also with the micronutrients it was not possible to identify a specific element able to regulate positively the blue production, but it was observed that the presence of cobalt and copper as the only minerals could have an inhibitory effect on the *Pseudomonas* spp. strains growth (figure 2.6 and figure 2.7).

Figure 2.6: Effect of the single micronutrients on the blue pigment production. Test on strains UMB248, UMB258 and 200188/6 are shown. Metals order: Mo, Bo, Mn, Cu, Zn, Co, Ca, Mg, Fe.

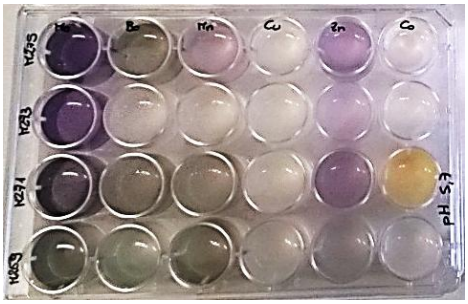

Figure 2.7: Inhibitory effect of Cu and Co on blue production. Metals order: Mo, Bo, Mn, Cu, Zn, Co. Test on strains UMB293, UMB291, UMB289, UMB287 are shown.

As cobalt and copper showed not to promote the blue synthesis, they were not included into the micronutrient mixes used to determinate if one specific element could be highly influencing in the blue pigmentation. In the plates containing M9 medium with the different mixes there was no difference in the rate of blue production.

2.2.3 Discussion and conclusion

From these phenotypical assays it was confirmed the evidence of the requirement of the refrigeration temperature (below 14°C) for the developing of the blue coloration, as happened when strains were inoculated into Mozzarella preserving fluid. Moreover, the need of glucose as carbon source was stated, while it was not possible to locate a specific element (both regarding amino acids and metals) that could be necessary for the blue pigment production. From these test made on 12 blue producing strains it was also noticed that in synthetic media the pigment production resulted to be highly different for each strain with different intensities and shades (data not shown).

2.3 Exploring the function of the blue pigment

2.3.1 Materials and methods

2.3.1.1 Quorum sensing test

The potential *quorum sensing* effect on the blue production was tested on 12 blue pigment-producing strains, listed in table 2.4, as follows: 100 µL of an overnight culture were inoculated in 10 mL of PF and incubated at 4°C for 7 days to allow the production of the blue pigment. Thereafter samples were centrifuged at 9000 *g* for 20min and filtered 0.22 µm. In a 24 wells microplate, 2mL of Mozzarella PF were inoculated with 20µL from each overnight culture in NB. Five hundred µL from serial dilutions of the prepared supernatants from the same strain were added, to verify if *quorum sensing* signals have a promoting effect on the blue production. Negative controls were made without adding supernatants. Microplates were incubated at 4°C and observed every 24h for 10 days.

Strain	16S rRNA identification
UMB248	*P. libanensis*
UMB253	*P. cedrina*
UMB254	*P. cedrina*
UMB260	*P. fluorescens*
UMB287	*P. gessardii*
UMB289	*P. fluorescens*
UMB291	*P. fluorescens*
UMB293	*P. fluorescens*
UMB295	*P. libanensis*
9BG	*P. fluorescens*
200188/6	*P. fluorescens*
200188/8	*P. libanensis*

Table 2.4: Blue pigment-producing Pseudomonas spp. strains used for quorum sensing and bacteriocin tests

2.3.1.2 Bacteriocin test

To evaluate the role of the blue molecule as a bacteriocin, blue supernatant from strain 200188/6 was prepared in Mozzarella PF as previously described. Blue producing *Pseudomonas* spp. listed in table 2.4 were grown overnight in NB at 30°C; from these cultures different bacterial suspensions were prepared washing cells with phosphate buffer. Twenty-five mL of NB were inoculated with bacterial suspensions reaching a final OD_{600} of 0.1, and 500µL of blue supernatant from 200188/6 strain were added. Negative controls were prepared in the same way without adding the blue supernatant. Samples were then incubated at 30°C. Bacterial growing was monitored by reading OD_{600} every 2h for 26h.

2.3.2 Results

2.3.2.1 Quorum sensing relation

The blue pigment production occurred after 7 days of incubation at 4°C without differences between the wells in which were added the supernatants and the control wells, as shown in figure 2.8.

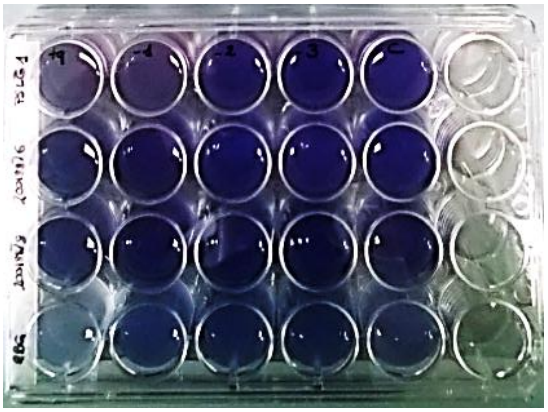

Figure 2.8: *Quorum sensing* assay for strains UMB248, 200188/6, 200188/6 and 9BG. Ten-fold supernatants dilution (undiluted, -1, -2, -3) were added in the first 4 rows. In the fifth row no supernatants were added.

2.3.2.2 *Bacteriocin effect*

Growth kinetics obtained from measuring OD_{600} values at different times are reported in figure 2.9.

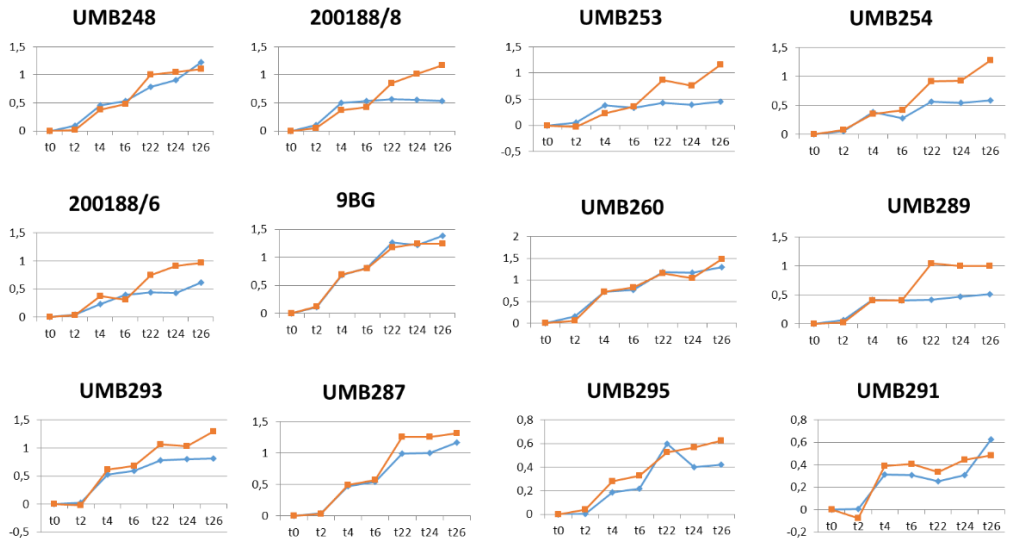

Figure 2.9: Blue pigment-producing*Pseudomonas* spp. strains growth in presence of the blue pigment. Values on y-axis represent the OD_{600} measure, x-axis reports the measure time (hours). Orange lines represent the samples where blue supernatant was added, while blue lines represent the control, without the addition.

The presence of the blue pigment did not have a negative effect on the growth of other *Pseudomonas* spp. strains, contrary it resulted in a promoting activity on eight strains, and it didn't affect the growth of the remaining four strains.

2.3.3 Discussion and conclusion

Pigment production in *Pseudomonas* spp. can be related to siderophores or bacteriocins molecules (*11*, *20*), whose production is also linked to *quorum sensing* signals, like phenazines produced by *P. aureofaciens* or the pyoverdine produced by *P. aeruginosa* (*21*). The production of antibacterial pigment related to *quorum sensing* molecules had been found also for other Gram-negative bacteria, as for example, violaceine production from *Chromobacterium violaceum* (*13*). On this knowledge the relation between the blue pigment and *quorum sensing* mechanism and its possible anti-bacterial function were investigated. From the obtained results it was observed that the presence of eventual *quorum sensing* signals, that should be contained into well-grown culture supernatants, did not influenced the blue pigment production. The blue

molecule didn't show any inhibitory effect on *Pseudomonas* spp., but it seems to own growth stimulating properties. This could give to blue *Pseudomonas* spp. an ecological advance, but this should be further confirmed.

2.4 Identification of pigments produced by *P. fluorescens*

2.4.1 Materials and methods

2.4.1.1 Sample preparation and pigment production

In order to identify the blue molecule produced by *P. fluorescens*, four blue pigment-producing strains (200188/6, UMB248, UMB251 and UMB258) were selected. As controls, two blue not-producing strains (A506 and DSM50415) were chosen. Strains were pre-enriched in NB at 30°C overnight. Subsequently, Mozzarella PF, M9 minimal medium supplemented with 0.2% (w/v) glucose and M9 minimal medium supplemented with 0.2% (w/v) glucose and 1mM proline were inoculated with 1% of the pre-enriched culture. Cultures were incubated in dark at 4°C for 7 d (PF) or 10 d (M9). Thereafter they were centrifuged (9000 *g* for 20 min), filtered through a 0.22µm-pore size cellulose acetate syringe filter and kept at -20°C before the UPLC-PDA-ESI-HR-MS analysis.

2.4.1.2 Instrumentation

The Ultra Performance Liquid Chromatography - Photo Diode Array - High Resolution - Mass Spectrometry (UPLC-PDA/ESI-HR-MS) analyses were carried by coupling an Acquity UPLC separation module (Waters, Milford, MA, USA) to an Acquity PDA eλ Detector (Waters) and a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer through a HESI-II probe for electrospray ionisation (Thermo Scientific, San Jose, CA, USA).

2.4.1.3 UPLC-PDA analysis of the blue pigment

Five µL of 0.22 µm-filtered Mozzarella PF or bacterial growth medium were separated on an Aeris PEPTIDE XB-C18 column (150×2.1 mm, 1.7 µm, 100 Å) equipped with a SecurityGuard ULTRA cartridge (Phenomenex, Torrance, CA, USA) kept at 35 °C, and using 0.1 mL/100 mL of formic acid (FA) in MilliQ-treated water (solvent A) and 0.1 mL/100 mL of formic acid (FA) in acetonitrile or methanol (solvent B). For the UPLC separation, a linear elution gradient was applied (1% to 20% of solvent B in 10 min) at a flow rate of 0.2 mL/min. The LC eluate was analysed by a PDA detector: a wavelength range of 190–800 nm was applied for diode array spectra generation; a λ = 550–650 nm was extracted for the identification of "blue" peaks.

2.4.1.4 UPLC-PDA/ESI-HR-MS analysis of the yellow pigments

Five µL of Mozzarella preserving fluid were separated on an Aeris PEPTIDE XB-C18 column (150×2.1 mm, 1.7 µm, 100 Å) equipped with a SecurityGuard ULTRA cartridge (Phenomenex, Torrance, CA, USA) kept at 35 °C, and using 20 mM ammonium acetate (NH_4-Ac) in MilliQ-treated water (solvent A) and acetonitrile (solvent B). For the UPLC separation, a linear elution gradient was applied (1% to 20% of solvent B in 16 min) at a flow rate of 0.2 mL/min. The LC eluate was analysed by a PDA detector: a λ range of 190–800 nm was applied for diode array spectra generation; a λ = 380–470 nm was extracted for the identification of "yellow" peaks.

The LC eluate from a PDA detector was further directed to a mass spectrometer through a heated ESI (HESI) interface. The eluate was analysed by HR-MS operated in a positive ionisation mode. The source conditions were as follows: sheath gas flow rate 35, aux gas flow rate 15, spray voltage 3.0 kV, capillary temperature 320°C and aux gas heater temperature 250°C. Full MS and data dependent tandem MS analysis of ten the most intense ions [$ddMS^2$(Top 10)] was performed. The resolution was set at 70000 and 17500, the AGC targets were 1×10^6 and 5×10^5, and maximum ion injection times were 200 ms and 100 ms for Full MS and $ddMS^2$ scan types, respectively. The MS data were processed using the Xcalibur software (version 3.0, Thermo Scientific).

2.4.2 Results

2.4.2.1 Blue pigment production in Mozzarella Preserving Fluid

Blue pigment production by *P. fluorescens* strains was investigated in Mozzarella PF. As expected, blue pigment-producing strains incubated under refrigeration in Mozzarella PF generated a clear blue coloration after 7 d. All of them had an absorbance maximum (λ_{max}) at 595–600 nm (data not shown). Meanwhile, Mozzarella PF inoculated with blue not-producing strains preserved its natural colour (figure 2.10).

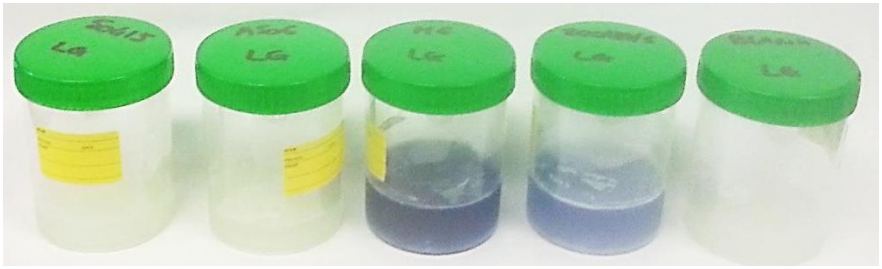

Figure 2.10: Mozzarella PF inoculated with *P. fluorescens* strains DSM 50415, A506, UMB258, 200188/6 and not inoculated

To further investigate these colorations produced by the same *P. fluorescens* strains in Mozzarella PF, the UPLC-PDA analyses were carried out, obtaining chromoatograms reported in figure 2.11.

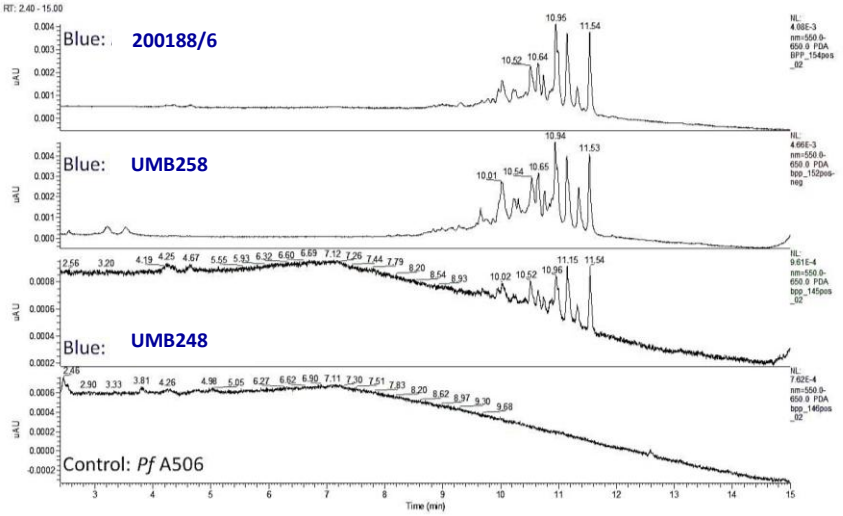

Figure.2.11: UPLC-PDA chromatograms (550-650 nm) of blue samples produced by incubation of *P. fluorescens* strains 200188/6, UMB248 and UMB258 in Mozzarella PF at 4°C. Sample obtained by incubation of the blue not-producing strain *P. fluorescens* A506 in the same medium was used as a control.

Blue-range (550–650 nm) chromatograms of the blue samples were characterized by a series of peaks with three the most intense ones: at retention times of 10.9, 11.1 and 11.5 min. The presence of different peaks in blue samples, absent in the control (sample obtained upon cultivation of *P. fluorescens* strain A506), could be potentially related to the produced blue molecules. Therefore, UPLC eluate was further directed into mass spectrometer. However, no significant masses, corresponding to the "blue" peaks were identified. This could be hypothesized as the "blue" pigment(s), produced by the investigated *P. fluorescens* strains is(are): 1) heat-labile and become disrupted during the introduction into the mass spectrometer through a HESI source; 2) poorly ionisable; 3) present in traces (*17*); 4) suppressed by food matrix (Mozzarella PF).

2.4.2.2 Blue pigment production in synthetic medium

To decrease the food matrix background (for the analytical purposes) we decided to produce the blue coloration in a synthetic medium. To this aim, we incubated the same blue pigment-producing strains in M9 minimal medium (*18*) supplemented with both glucose and proline. In this case, the obtained blue samples showed diverse blue shades: strains 200188/6 and UMB248 produced a dark blue-grey coloration, UMB258 turned clear blue, and UMB251 produced a violet-blue colour. All of them had an

absorbance maximum (λ_{max}) at 595–600 nm (data not shown). It is worth to note that these colorations were not stably produced, as they were not obtained from the same strains in the different replications of the experiment. For example, *P. fluorescens* UMB251 when incubated in M9 minimal medium supplemented with glucose and proline produced a blue-violet coloration in the first assay, while it produced a clear blue coloration in the second repetition. To further investigate these colorations produced by the same *P. fluorescens* strains in M9 minimal medium with glucose and proline, UPLC-PDA analyses were carried out. We performed the UPLC separation with acid eluents (containing 0.1% formic acid, pH 2.7) as the blue colour was found to be stable in acid pH (data not shown). The obtained results (UPLC-PDA chromatograms) are shown in Figure 2.12.

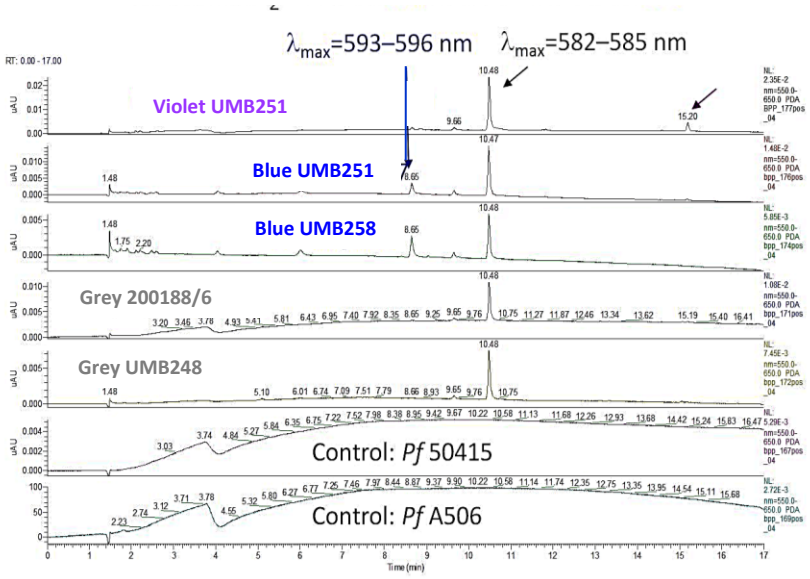

Figure.2.12: UPLC–PDA chromatograms (550–560 nm) of samples produced by incubation of *P. fluorescens* strains UMB248, UMB251, UMB258, 200188/6 in M9 minimal medium with 0.2% (w/v) glucose and 1mM proline at 4°C. Samples from the incubation in the same medium of blue not-producing *P. fluorescens* strains A506 and DSM50415 were used as controls.

Coloured samples (blue-violet, clear blue and blue-grey ones), produced by incubation of *P. fluorescens* strains in synthetic medium, were characterized by a single common peak with the highest intensity at a retention time of 10.5 min in UPLC-PDA chromatogram. For two different colour shades analysed (clear blue and blue-violet), different smaller peaks were found in addition: at a retention time of 15.2 min for blue-violet samples and at a retention time of 8.7 min for clear blue samples. No peaks were common between the samples incubated in Mozzarella PF and samples incubated in the synthetic medium.

UPLC eluate was further directed into a high-resolution mass spectrometer. No significant accurate masses of the negative ions, discriminating blue and control samples, were obtained. Several different accurate masses of the positive ions, discriminating blue and control samples, were identified (data not shown). However, none of these masses were yet attributed to any known blue pigment, produced by *P. fluorescens*. The present work is in progress in collaboration with a research group of prof. Helge Bode (Goethe Universität, Frankfurt am Main, Germany).

2.4.2.3 Identification of yellow pigments produced by P. fluorescens

Pseudomonas fluorescens species is also characterized as a producer of yellow pigments, including the ones known as pyoverdins (*3*). Moreover, there could be a relation between the blue pigments and the yellow pigments produced by the strains of this species. Indeed, already in 1958 the research group of prof. R. P. Elliott demonstrated a correlation between pyoverdine concentration and its fluorescent colour, which was blue when a low pyoverdine concentration was present (*10*). To this purpose, we investigated also the production of yellow pigments, produced by the blue pigment-producing *P. fluorescens* strains. The strain A506, used in this study, is an example of a pyoverdin producer (*15*), and we applied it as a control. Using this strain, we developed the UPLC chromatographic separation and ESI-HR-MS detection method for the identification of the pyoverdins (see paragraph 2.4.1.4). To verify whether the strains, presenting the blue phenotype, could also produce a yellow pigment, we measured the λ_{max} of the culture broths at the yellow emission wavelength range. The blue pigment-producing strain broths had a second λ_{max} of 381–386 nm (data not shown), which is characteristic for ferri-pyoverdins (*19*).

To identify the yellow pigments, we adopted the UPLC separation of culture broths (M9 minimal medium supplemented with glucose) at pH 5 instead of pH 2.7 (used for blue pigment separation) as a higher pH favours the pyoverdin identification in mass spectrometry (*4*, *5*, *14*). We selected the wavelength region 380–470 nm for PDA analysis. Blue pigment-producing strains were found to have a single common major peak at a retention time of 9.8 min in UPLC-PDA chromatogram (Figure 2.13).

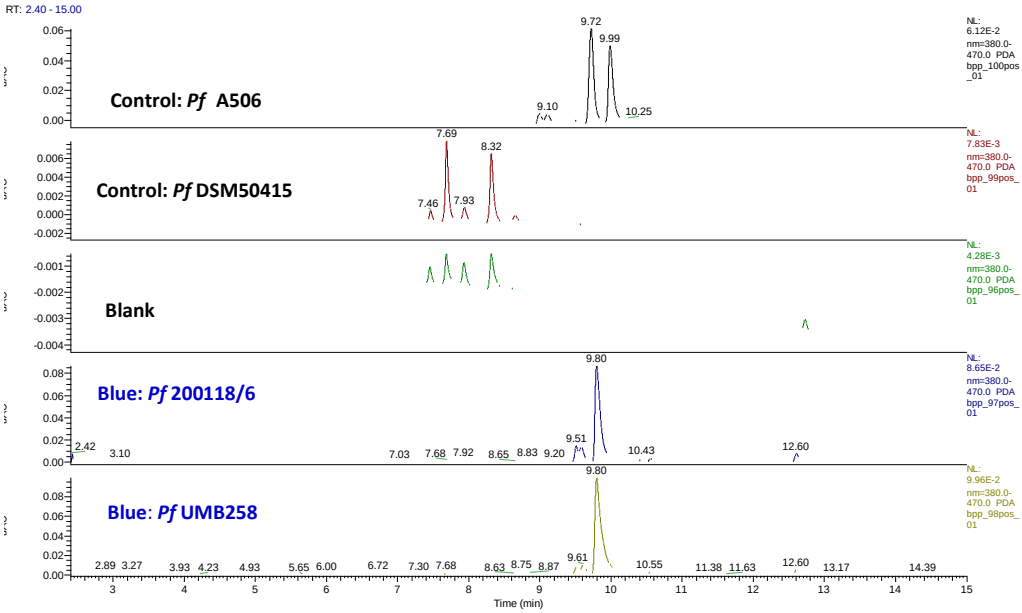

Figure2.13: UPLC–PDA chromatograms (380–470 nm) of blue samples produced by incubation of *P. fluorescens* strains UMB258 and 200188/6 and control samples produced by incubation of *P. fluorescens* strains A506 and DSM50415 in Mozzarella PF at 4°C.

For both blue pigment-producing strains, 200118/6 and UMB258, HR-MS analysis attributed it to an accurate mass of the positive ion of 1242.423 *m/z*, which is characteristic for ferri-pyoverdin with malic amide side-chain (Figure 2.14, 200118/6 is shown as an example) (*14*)

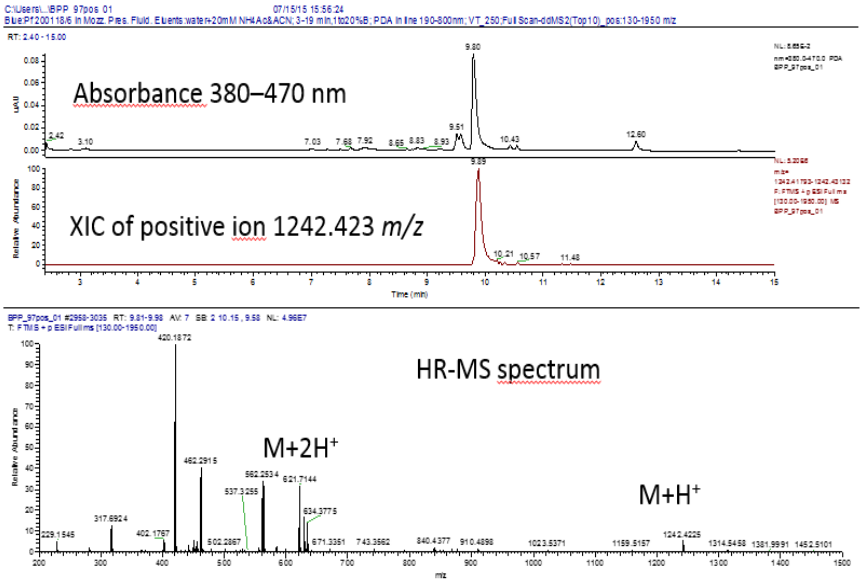

Figure 2.14: Identification of an accurate mass, corresponding to ferri-pyoverdin with malic amide side-chain, in *P. fluorescens* 200118/6 culture broth: UPLC-PDA chromatogram (A), extracted ion chromatogram (XIC) (B) of the positive ion 1242.423 and HR-MS spectrum (C) of the "yellow" peak (eluting at the 9.9 min). The difference of 0.1 min between the PDA and XIC chromatograms is due to the physical distance of the two detectors.

2.4.3 Discussion and conclusions

Identification of the blue coloration causing the spoilage of fresh cheese is an active subject. In recent years, two different methods to uncover the identity of this blue molecule have been proposed, giving two distinct answers. Caputo et al. (2014) identified the blue pigment as a leucoindigoidine/indigoidine, while Andreani et al. (2015) hypothesized an indole derivate without reaching a precise identification. The UPLC-PDA/ESI-HR-MS analyses performed in this study did not reveal neither indigoidine nor indoles. In Mozzarella PF, a group of three major peaks characteristic for blue samples was observed. However, it was not possible to unveil any blue pigment structures corresponding to them. The low reproducibility of the blue coloration in M9 minimal medium did not allow the identification of any blue pigment. However, it showed that the reason for this difficulty could be attributed to the presence of more than one coloured molecule produced by the strains.

As the production of a "blue" pigment in *P. fluorescens* is known to be potentially related to the production of the "yellow" pigment pyoverdine (*10*), the blue pigment-producing strains were tested for pyoverdine production. For both blue pigment-producing strains, 200118/6 and UMB258, HR-MS analysis attributed the "yellow" peak in UPLC-PDA chromatogram to an accurate mass of the positive ion, which is characteristic for ferri-pyoverdin (*14*). This could potentially explain the yellow coloration developed in Mozzarella PF by these blue pigment-producing strains when incubated at 30°C. However, this fact cannot be related to the identification of the blue pigment even if a blue pyoverdin nature was described (*10*).

2.5 References

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3 GENOME SEQUENCING AND COMPARISON

3.1 Introduction

In recent years the whole genome sequencing of *P. fluorescens* strains related to rhizosphere environment has been widely used to understand their specific ecological traits, such as phenazine or HCN production (12, 13, 15), colonization abilities and microbial biocontrol activities (11). In January 2015, five complete and 38 draft genome sequences of *P. fluorescens* were available in NCBI GenBank database (10). In the last year, at least five more *P. fluorescens* whole genome data were added from strains isolated in dairy products (3, 10), showing the interest for this species also in a completely different environment, where it plays a negative role as spoilage agent of fresh cheeses.

DNA sequences comparison among strains of the same species can be useful to locate and identify genes that give a specific phenotypic character. In classical approach of genetic studies (1), one way to find out what a specific gene does is to see what happens when the microorganism acquires or lose it. In some cases, phages can act as vector to transfer genes that are functional to the bacterial host for its survival or dominance in an environment rather than for viral life.

3.2 Materials and methods

3.2.1 Strains used for genome sequencing

According to results obtained by the phylogenetic analysis of the *P. fluorescens* isolates previously described (chapter 3), three blue producing strains (UMB247, UMB248 and 200188/6) where selected for whole genome sequencing. Species identification was confirmed by the sequencing and comparison of *gyrB* and *rpoD* genes (2, 17).

3.2.2 Whole genome sequencing and assembly

Purified DNA was used for sequencing using Illumina MiSeq (300 paired-end bp) platform. Library preparation was performed with Nextera® XT DNA Library preparation kit (Illumina Inc (US)) according to manufacturer's instructions. The raw data of three new genomes of *Pseudomonas fluorescens* UMB247, UMB248 and 200188/6 were quality filtered using Trimmomatic (5) and error correction and assembly were performed using Spades 3.1 (4). Contigs with length inferior to 500 bp and coverage less than 2 were removed. The new sequenced genomes were submitted to NCBI with accession number JXMI00000000, JXLI00000000 and LYXI00000000

3.2.3 Assembly and comparative genomics

Coding DNA sequences for the strains UMB247, UMB248 and 200188/6 were predicted using Prokka pipeline (14). The whole genome sequence of seven strains (Table 5.1) of *Pseudomonas fluorescens*, two presenting the blue phenotype and five not producing the blue pigment, were downloaded from NCBI and included in the comparative analysis. "All against all" approach was performed using blastp (6) for all the CDS in all the genomes. All the distances between each gene in each genome against all the genes in all the genomes were used to construct a panmatrix using the R packages (available at http://cran.r-project.org/). CDS were grouped in clusters using a threshold of 0.75 and complete linkage. Core and pangenome size were calculate using binomial-mixture model (16).

3.2.4 Research of prophage sequences

A preliminary investigation was performed to look for genetic elements indicating the presence of prophages by using PHAST (PHAge Search Tool) web server (18). The annotation was then confirmed and completed with the sequence analysis in Phagonaute database (9).

3.2.5 Primer design and PCR amplification

The presence of common genetic regions in the blue pigment-producing isolates was checked by the amplification of a 900 bp segment (coding for two hypothetical protein in CDS2 and CDS3 according to PROKKA pipeline annotation listed in table 5.2) on 30 blue pigment- producing and 30 blue not-producing *P. fluorescens* isolates listed in Appendix 1. Primers HYP1_F (GATTCACACCGCAATCGTCG) and HYP1_R (GGTCGCGTTCTTCAATCAGC) were designed using NCBI Blast web tool. PCR amplifications were performed using a final volume of 25 µL containing 1x Taq Buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.4µM of each primer, 1 U Taq enzyme (5prime, De) and 50 ng of genomic DNA. A classic three step thermal cycle was used with an initial step at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing 60°C for 1 min and extension at 72°C for 1 min, and a final step of extension at 72°C for 5 min. The amplified products were analysed by electrophoresis on 1.5% agarose gels stained with Etidium bromide and visualised on a UV transilluminator (Gel Doc XR®, Biorad).

Strain	Genome length (Mbp)	% GC	Contigs	Plasmid	CDS	Source/Accession number
200188/6[b]	6.2	60.1	171	-	5059	This study
UMB247 [b]	6.2	60.2	108	-	5079	This study
UMB248 [b]	6.2	60.2	75	-	4891	This study
PS77[b]	6.1	59.7	63	-	5523	LCYB00000000
PS22[b]	5.1	58.3	357	-	6370	LCYA00000000
PS40	6.3	59.2	496	-	6459	LCYD00000000
PS20	5.9	60.1	154	-	5281	LCYC00000000
A506	6.0	59.9	2	1	5267	NC_017911.1
Pf01	6.4	60.5	1	-	5722	NC_007492.2
SBW25	6.7	60.5	1	-	5921	NC_012660.1

Table 3.1: List and information of the *Pseudomonas fluorescens* genomes used for comparative analysis; blue pigment-producing strains are marked with letter "b"

3.3 Results

3.3.1 Whole genome sequencing and assembly

Results of the genome sequencing are reported in table 5.1: the three blue producing *P. fluorescens* strains have a genome length of 6.202.177 bp (200188/6), 6.225.186 bp (UMB147) and 6.228.552 (UMB148), respectively. From the annotation 12180 gene clusters were found.

3.3.2 Comparative genomics of blue pigment producing and blue not-producing *P. fluorescens*

A comparative genomics approach was used in this study to identify gene clusters which differed between the blue pigment-producing strains and the blue not- producing strains. A total of 10504 gene clusters were detected among the 10 genomes and of these 2851 were present in all the isolate (core-genome). Clustering analysis obtained from the panmatrix showed the relationship between the gene clusters identified among the isolates included in this investigation (figure 5.1).

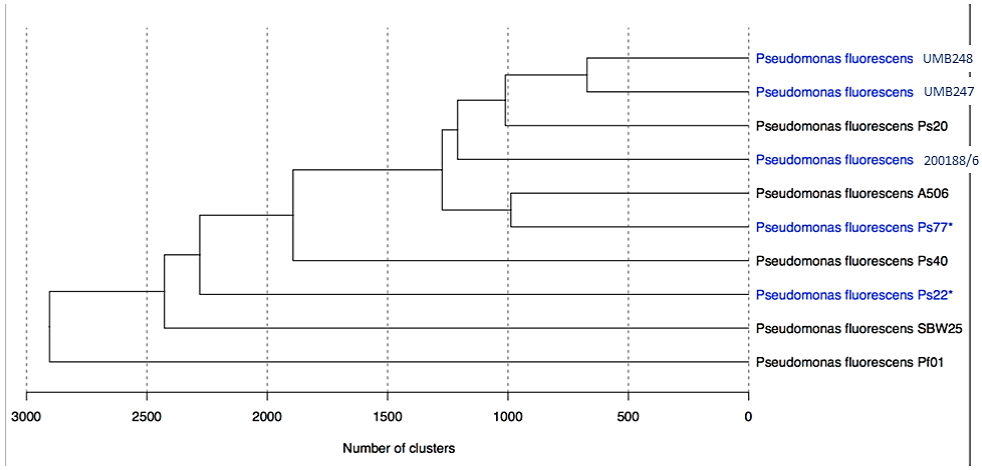

Figure 3.1: Clustering analysis tree constructed from the panmatrix using Manhattan distances between the ten isolates of *P. fluorescens* strains, of which five were able to produce the blue pigment.

A total of 24 genes were recognized as specific for the five isolates which were able to produce the blue pigment. Of these, 15 were identified as a cluster and were positioned in a specific region of the genomes with an average length of 10kbp. From the annotation made with PROKKA pipeline resulted that some of the CDS present in these regions, as shown in figure 5.2, code for phage genes, in particular for a part of

the capsid (CDS12) and a part of a tail (CDS15). So, in order to identify the product of the surrounding genes annotated as hypothetical, the aligned nucleotide sequence was searched with PHAST webtool and compared into Phagonaute web interface. In this way new gene functions were founded completing the annotation, as reported in table 5.2

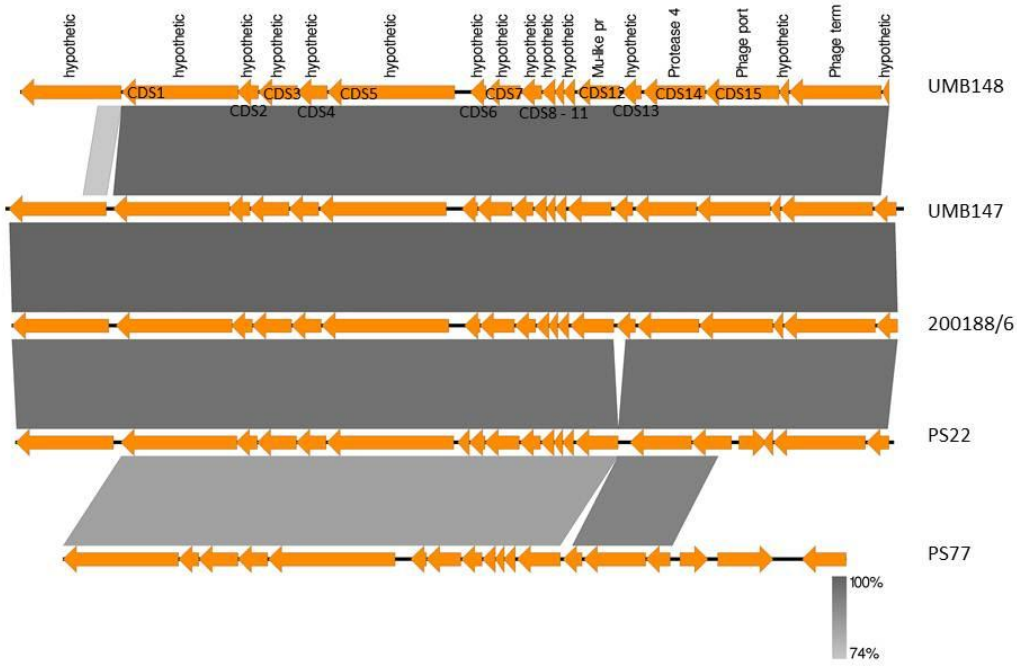

Figure 3.2: Alignment of the unique region present in 5 sequenced blue pigment-producing strains; strain name is reported on the right, grey scale represent the homology percentage.

CDS	PROKKA pipeline annotation	PHAST annotation	PHAGONAUTE annotation
1	hypothetical protein	putative cell wall peptidase	amidase 5
2	hypothetical protein	hypothetical protein	phage minor tail
3	hypothetical protein	hypothetical protein	phage minor tail (hydrolase)
4	hypothetical protein	putative tail protein	tail tape measure (transglycosilase)
5	hypothetical protein	putative phage associated protein (hypothetical)	hypothetical protein
6	hypothetical protein	hypothetical protein	hypothetical protein
7	hypothetical protein	hypothetical protein	hypothetical protein
8	hypothetical protein	putative phage associated protein (hypothetical)	Phage tail protein
9	hypothetical protein	hypothetical protein	hypothetical protein
10	hypothetical protein	hypothetical protein	hypothetical protein
11	hypothetical protein	hypothetical protein	hypothetical protein
12	Mu-like prophage major head subunit gpT	putative major capsid protein	capsid coat protein (limocin)
13	hypothetical protein	capsid protein	capside protein
14	Peptidase_S49 family		(head maturation protease)
15	Phage portal protein, lambda family	portal protein	phage portal protein

Table 3.2: Different functions predicted by the nucleotide sequence comparison in different databases

By comparing the nucleotide sequence and its protein translations in phage specific databases it was possible to identify the product of 9 CDS over 15. Six CDS are still unidentified. The presence of structural phage genes endorse the hypothesis of a prophage integrated into the genome of blue pigment-producing *P. fluorescens* strains.

3.3.3 Primer design and PCR amplification

After a first test on sequenced strain UMB147, UMB148 and 200188/6 DNA, where a thick band at 900bp was obtained, the amplification was made on all the blue isolates included into the *Pseudomonas* spp. collection made up for this work and on 30 *Pseudomonas* spp. not presenting the blue phenotype.

The expected fragment was amplified in 29 blue isolates out of 30, even if the band obtained had a minor intensity than the one resulted from the sequenced strains on which the primer couple were designed. From the 30 blue not-producing isolates no signal was detected. The designed primers HYP1_F and HYP1_R confirmed, with 98,3% reliability, the presence of that fragment in all the isolates presenting the blue phenotype, and its absence in the blue not-producing isolates tested.

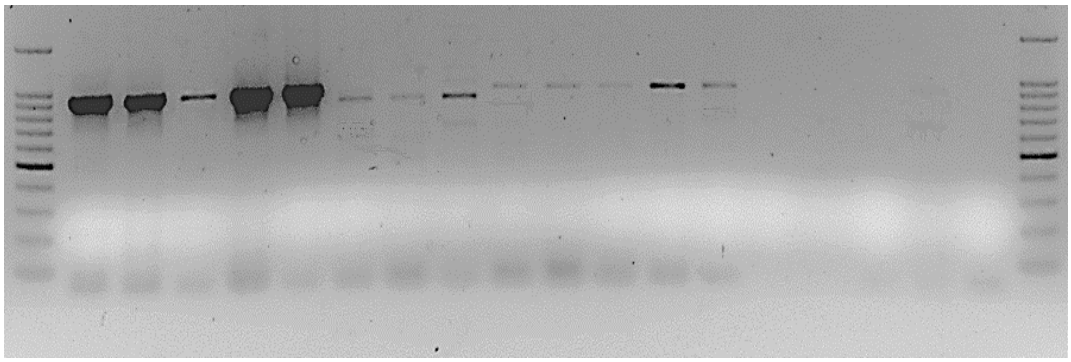

Figure 3.3: Results of amplification with HYP_F andd HYP_R primers. Samples order: UMB248, UMB247, 9BG, 200188/6, 200188/8, UMB253, UMB254, UMB287, UMB289, UMB291, UMB293, UMB295, UMB258, A506, SBW25, Pf_01, ATCC13525. From line 1 to 11 DNA was extracted from blue pigment-producing strains, while from line 12 to 15 strains used do not produce the blue pigment

3.4 Discussion and conclusion

Blue producing *P. fluorescens* strains were already found to be related phylogenetically from MultiLocus Sequence Typing analysis, clustering together in the so-called "blue branch"(2, 8). From the whole genome sequencing of three blue pigment-producing strains and their comparison with two other blue pigment-producing *P. fluorescens* sequenced by Andreani *et al*.(2015) (3) and five blue not-producing *P. fluorescens* strains, an unique region shared into the genome of the blue pigment-producing strain was found. Unfortunately, the most of the coding sequences in this region coded for unknown (hypothetical) protein, but significantly related to the presence of genes coding for phage elements. This outcome was further investigated, obtaining the identification of other phage-related elements into this sequence. This endorsed the hypothesis that this sequence shared only in the genome of the blue producing *P. fluorescens* could originate from a bacteriophage integrated into the bacterial genome of an ancestor strain, that lost some of its functional genes becoming a defective prophage (7). The presence of this phage elements into blue pigment-producing *Pseudomonas* spp. genomes could mean a relation between the prophage acquisition by the bacteria and the developing of the blue phenotype, but further studies, above all the individuation of the blue molecule and its coding genes, are required to confirm this hypothesis.

3.5 References

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4 BACTERIOPHAGE INDUCTION

4.1 Introduction

With the increasing of sequenced bacterial genomes has become more clear that they exhibit a high rate of modification, and that a substantial part of bacterial DNA is acquired by horizontal (or lateral) transfer through transformation, conjugation or transduction. Another means of this kind of gene transfer is the integration in the bacterial genome of viral DNA by lysogenization. From the study of *Pseudomonas* genomes has been revealed that the presence of one or more integrated prophages is a common character. In addition to their functional genes, phages can carry also extra genes able to modify the phenotype of bacterial host ('lysogenic conversion genes', LCG), sometimes carrying characters to respond to environmental conditions. These genes are transcription units with their own promoters and terminators, regulated independently from the rest of the prophage (3). As consequences from the phage attack bacterial defence strategies are activated causing point mutation or DNA deletion of the integrated prophage, leading to obtain defective prophages or isolated phage genes in bacterial genomes. Some bacteria had been succeeded in modifying these residual genes to gain an advantage; for example in *Pseudomonas aeruginosa*, two phage-tail gene-clusters were developed into bacteriocins (6).

Considering the correlation between the bacterial phenotype modification and the integration of prophages we tried to induce and isolate phages eventually integrated into blue pigment-producing *P. fluorescens* strains in order to verify if they could be the carrier of this new phenotype.

4.2 Material and methods

4.2.1 Phage induction

Phage induction was made for 30 blue producing strains (listed in Appendix 1). Fifty µL of each strain, grown overnight at 30°C, were inoculated in 50 mL of Nutrient Broth (Sigma-Aldrich) added with 0.2% glucose, 10mM $CaCl_2$ and 10mM $MgSO_4x7H_2O$ (NB+), shaking at 120 rpm at 30°C, until the culture reached OD_{600} nm of 0.5. Then the proper concentration of antibiotic was added. All the strains were induced with norfloxacin (50µg/mL) or with ciprofloxacin (4µg/mL) (4). The culture with the antibiotic was further incubated at 30°C overnight by shaking at 120 rpm. The day after 50µL of $CHCl_3$ were added to the samples followed by a centrifuge step at 9000 *g* for 20 min. Supernatants were filtered 0.45µm and maintained at 4°C.

4.2.2 Growth inhibiting activity test

The presence of induced bacteriophages was verified on 31 blue pigment-producing *P. fluorescens* group isolates (listed in Appendix 1) as follows: In a 96 wells microplate, 10µL of an overnight culture and 10µL of the previous filtered supernatant were inoculated in 180µL of NB+ for each well. A positive control was made for each strain inoculating 10µL in 190µL of NB+. Plates were incubated in Tecan Infinite PRO200 (Tecan) reader at 30°C for 24h monitoring the bacterial growth measuring OD_{600} every hour. Samples showing a growth inhibiting activity were tested by spot-test assay on a soft TSA (agar 0.4% w/v) layer inoculated with 100µL overnight culture of host strain (1). Plates were incubated inverted at 30°C overnight.

For the potential phages isolation, a double agar plaque assay was made as follows:10µL of the sample were added to 100µL of a fresh bacterial suspension in 100µL of NB+ broth added with 10mM $MgSO_4$ and $CaCl_2$ and incubated at 25°C for 20 min. Then 3mL of TSA 0.4% agar kept warm at 50°C were added, gently mixed and poured on a TSA plate. After agar solidification plates were incubated inverted at 30°C overnight (2).

4.2.3 Transmission Electron Microscopy

Some of the samples forming a clear area from the spot test were then analysed by TEM to confirm that the inhibition activity was due effectively to a bacteriophage and not to other antimicrobial molecules produced by the induced strains or by a residue presence of the antibiotic used for the induction. Phage morphology was observed by transmission electron microscopy EFTEM Leo 912ab (Zeiss) with a 100kV voltage. Ten µL of viral suspensions were placed on 300 mesh copper specimen grids coated with carbon film; after 30 min samples were dried, washed with three drops of distilled water and then a drop of in uranyl acetate (2% w/v, pH 4.5) was added to negatively stain the viral particles. After 10 min the stain solution was removed. After drying, the preparations were observed at different magnitudes. Images were acquired with a CCD camera at 1024x1024 pixel resolution. Data are averages of 10 measurements carried out on at least two different microscopic preparations.

4.3 Results

4.3.1 Growth inhibiting activity

Results of the effect of the supernatants from the induced strains on growth curves of blue pigment-producing isolates are summed up in Figure 6.2 and 6.3.

From the induction with norfloxacin supernatants of isolates UMB248, UMB253, UMB254, UMB256, UMB261, UMB287, UMB289, UMB295 isolates were selected, while from the induction with ciprofloxacin supernatants of UMB248, UMB253, UMB254, UMB256, UMB261 isolates were selected. Supernatants were renamed and spotted on the isolates that showed to be sensitive as reported in table 4.1 and 4.2.

Despite the clear halo formed by samples 2N, 3N, 4N, 1C, 2C, 3C, 4C, and 5C in the spot test, no plaques were further detected, not allowing the bacteriophage isolation.

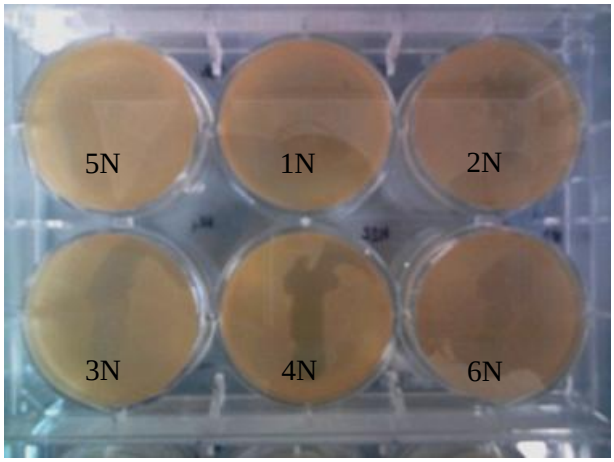


Figure 4.1: Spot test of samples 1N, 2N, 3N, 4N, 5N, 6N on UMB295 strain

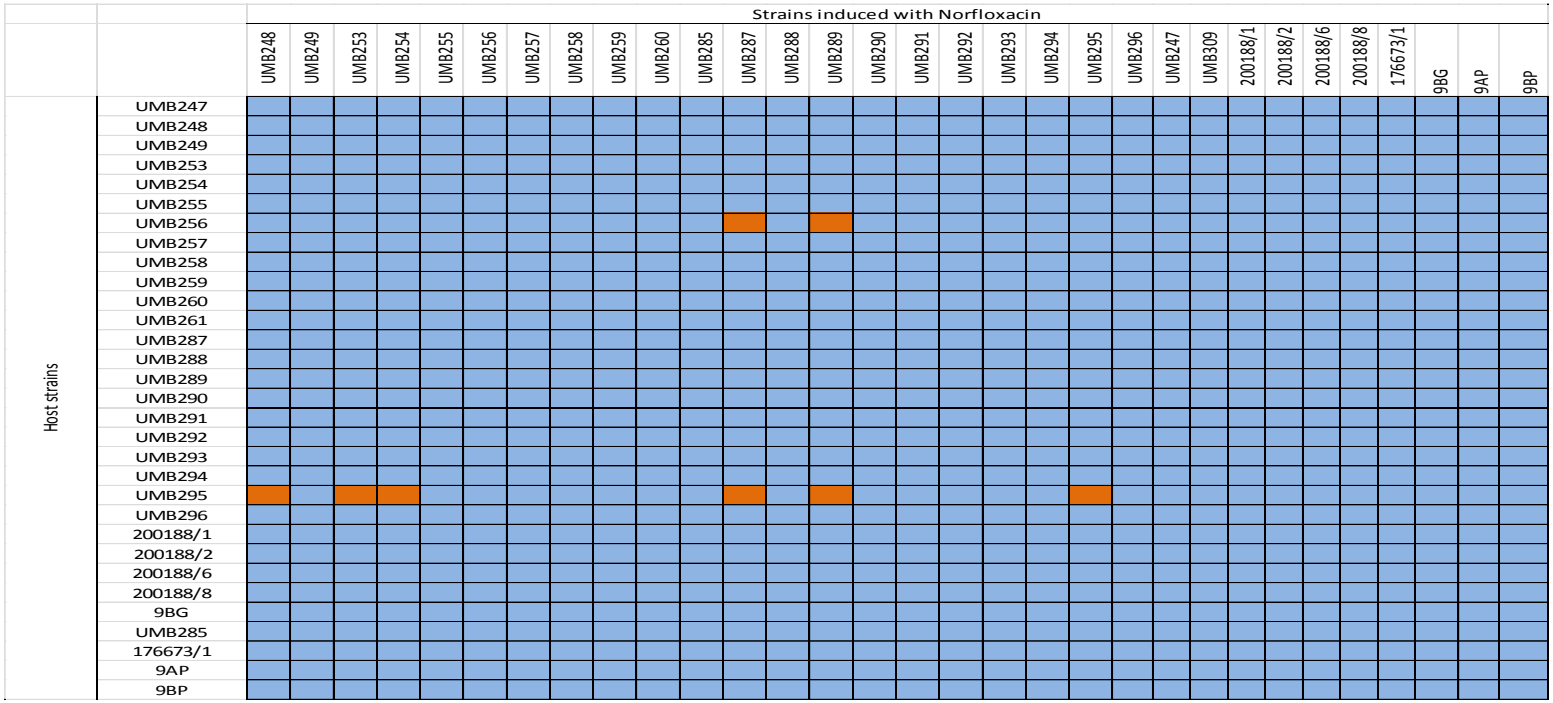


Figure 4.2: Heatmap representation of the inhibiting activity of the supernatants obtained from norfloxacin induction; orange = detection of growth inhibiting activity, blue = no difference of growth between the sample with the supernatant and its control.

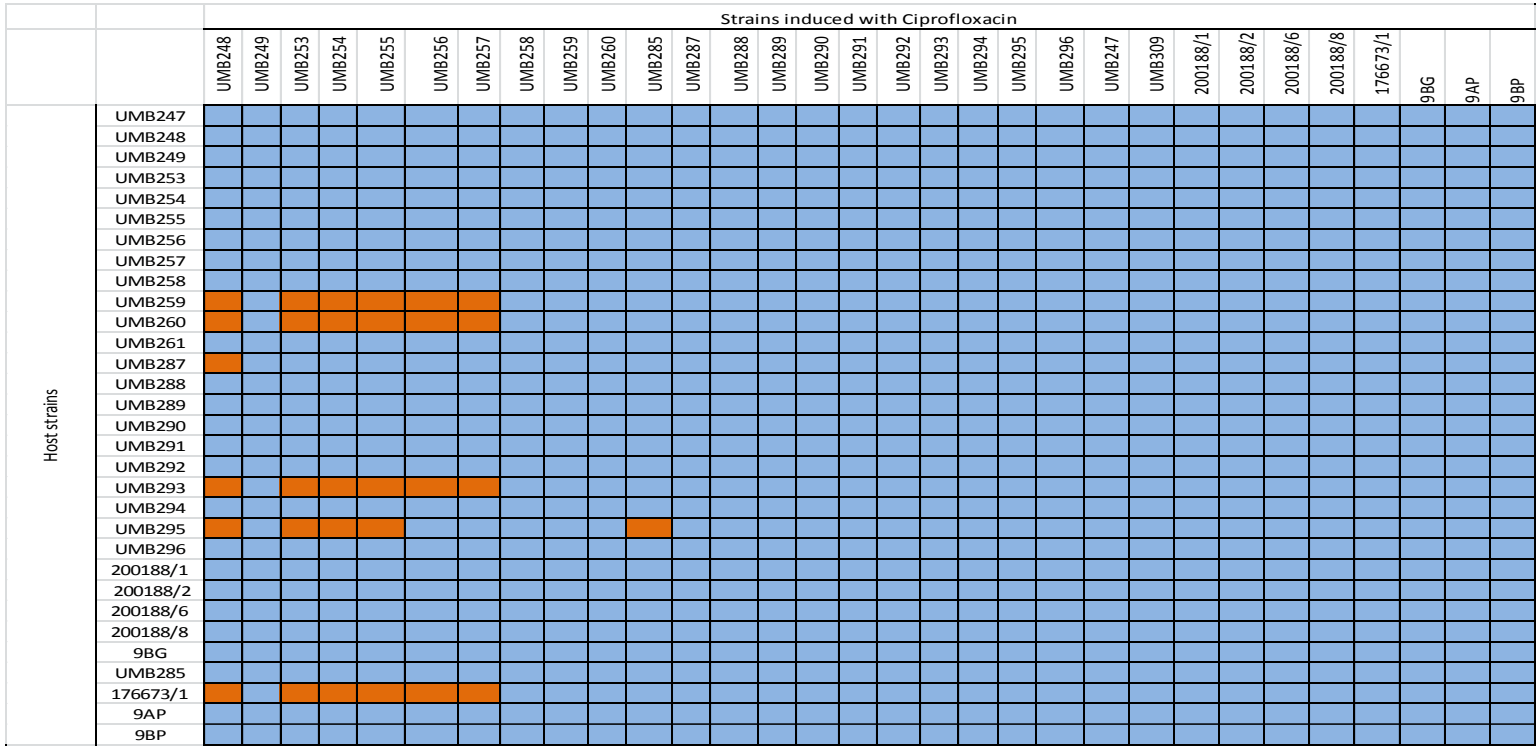


Figure 4.3: Heatmap representation of the inhibiting activity of the supernatants obtained from norfloxacin induction; orange = detection of growth inhibiting activity, blue = no difference of growth between the sample with the supernatant and its control.

NORFLOXACIN INDUCED			
Strain induced	Supernatant code	Associated host strain	Spot result
UMB248	1N	UMB295	NEGATIVE
UMB253	2N	UMB295	POSITIVE
UMB254	3N	UMB295	POSITIVE
UMB256	4N	UMB295	POSITIVE
UMB261	5N	UMB295	NEGATIVE
UMB287	6N	UMB256	POSITIVE
UMB289	7N	UMB256	NEGATIVE
UMB295	8N	UMB256	NEGATIVE

Table 4.1: Spot test results of the supernatant from norfloxacin induction

CIPROFLOXACIN INDUCED			
Strain induced	Supernatant code	Associated host strain	Spot result
UMB248	1C	UMB259, UMB260, UMB293	POSITIVE
UMB253	2C	UMB259, UMB260, UMB293	POSITIVE
UMB254	3C	UMB259, UMB260, UMB293	POSITIVE
UMB256	4C	UMB259, UMB260, UMB293	POSITIVE
UMB261	5C	UMB295	POSITIVE

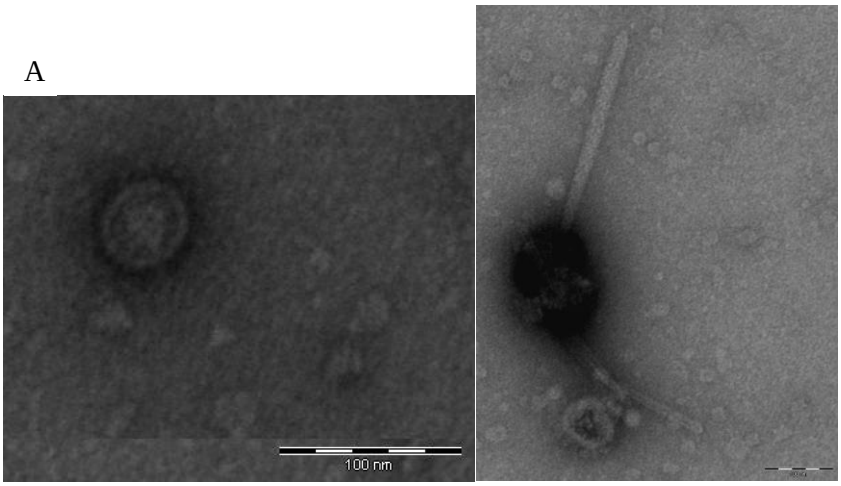
Table 4.2: Spot test results of the supernatants from ciprofloxacin induction

Despite the unsuccessful isolation by plaque assay, some of the samples confirming the inhibiting activity (e.g. with a positive spot test, as shown in figure 4.1) were selected for TEM visualization.

4.3.2 Microscopic observation

TEM visualization was made on samples 2N, 7N, 1C, 2C, 3C. Bacteriophages were detected only in one of ciprofloxacin induced samples (2C). In particular, there were found two different phage morphologies (Figure 4.4) one consisting in a head of 75 ± 12 nm (A), while the other is composed by a head of 100 ± 17nm and by a tail of 227 ± 4nm length and 22 ± 5nm diameter (B). This second morphology makes the phage ascribable to *Siphoviridae* family, while no certain characterization could be made for the first phage. Considering its head diameter and its temperate life-style it could be identified as belonging to *Tectiviridae* family, but a further investigation should be made (8).

A



B

.

Figure 4.4: Bacteriophages visualized by TEM analysis of sample 2C

4.4 Discussion and Conclusion

The isolation of prophages from blue pigment-producing strains belonging to *P. fluorescens* group was not successful; this could had been for several reasons. Temperate bacteriophage induction can be obtained with different methods (different antibiotics, UV light, hydrogen peroxide) at different efficiencies (7). In our study for example the phage induction was obtained using ciprofloxacin and not when norfloxacin was used.

This is consistent with what reported by Fothergill et al. (2011); the phage production from the same strain can be significantly different according to the antibiotic used (4). In this work norfloxacin and ciprofloxacin were used because they showed the highest rate of induction in *P. aeruginosa* (4).

The good result of the induction is also related to the growth state of the bacterial culture and the growing temperature. Moreover, is not easy to find suitable indicator strain or the conditions needed for phage propagation. For all of these motifs it can occur that the only evidence of the induction of phage like particles is their visualization by transmission electron microscopy (TEM) (7).

Although the presence of prophage elements is well-known in *P. fluorescens* genome (5) there is still no evidence of temperate phages isolation and characterization from this species.

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SCIENTIFIC PRODUCTS

Chierici M. (2014) Investigation on *Pseudomonas* spp. producing blue discoloration in mozzarella cheese and their bacteriophages. Poster in 19th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, Bari, September 24th-26th, 2014
2nd Annual PhD report

Chierici M. Biodiversity in *Pseudomonas fluorescens* strains causing blue discoloration in Mozzarella cheese. Oral communication in 20th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Perugia, Perugia, September 23rd-25th, 2015
3rd Annual PhD report

Chierici, M., Picozzi, C., La Spina, M. G., Orsi, C., Vigentini, I., Zambrini, V., Foschino, R. 2016. Strain diversity of *Pseudomonas fluorescens* group with potential blue pigment phenotype isolated from dairy products. *Journal of Food Protection* doi:10.4315/0362-028X.JFP-15-589 (in press)

Chierici, M., Porcellato, D., Mondin, C., Orsi, C.,, Zambrini V., Petit M.-A., Foschino R. 2016. The blue phenotype in *Pseudomonas fluorescens* strains is related to genes conserved in a cryptic bacteriophage. *Microbiology* (in submission).

Investigation on Pseudomonas spp. producing blue discoloration in mozzarella cheese and their bacteriophages

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The first part of the PhD thesis project concerned the recovery and the isolation of *Pseudomonas* spp. strains producing the blue pigment and their identification. The blue colour production was investigated on different media and at different temperature. Latent bacteriophages were induced from the selected strains by the addition of Norfloxacin, and then the searching of sensitive strains was accomplished among the blue producing strains. The recovered *Pseudomonas* spp. strains sensitivity to one bacteriophage active on *P. fluorescens* was also tested.

Studio di Pseudomonas spp. causa della colorazione blu in mozzarella e dei loro batteriofagi

La prima parte del progetto di tesi di dottorato ha riguardato il recupero e l'isolamento di ceppi appartenenti al genere *Pseudomonas* produttori di pigmento blu e la loro identificazione. La produzione del colore blu è stata verificata su differenti terreni colturali e a diverse temperature. Dai ceppi selezionati sono stati indotti i batteriofagi latenti mediante l'aggiunta di Norfloxacina, ed è stato fatto uno screening tra i ceppi produttori di pigmento blu per individuarne i ceppi sensibili. L'attività di un batteriofago attivi su *P. fluorescens* è stata verificata sui ceppi di *Pseudomonas* spp. che costituiscono la collezione.

Key words: Mozzarella, Pseudomonas spp., blue, bacteriophages.

1. Introduction

This poster reports the main results of the first part of this PhD project concerning the study of the blue discoloration on mozzarella cheese caused by *Pseudomonas* spp.. The activities scheduled for the first year were:

(A1) the recovery of *Pseudomonas* spp. producing blue colour: their identification and the production of blue colour on different media and at different temperature;

(A2) the blue pigment separation and characterization

(A3) the bacteriophages recover and the assays for sensitive strains.

2. Materials and Methods

The strains in use in this work were supplied by academic collections, international collections or they were recovered from samples of dairy and vegetables products.

The production of the blue pigment was assayed by plating each strain on TSA (Triptic Soy Agar, Oxoid) and on Mascarpone Agar (Cantoni *et al*., 2011). Plates were incubated at 30°C and 9°C until the observation of the pigment production. Blue producing strains were identified by the amplification of 16S rDNA region. To characterize the blue pigment the strains were grown in clear TSB at 10°C. After 20 days cultures were centrifuged and the supernatant was recovered. Sulfosalicylic acid was added at 3% final concentration and the samples were centrifuged. The supernatant was then filtered 0,22 and the UV absorbance was read from 200 to 700 nm.

To induce temperate bacteriophages, blue producing strains were inoculated in TSB (Triptic Soy Broth, Oxoid) added with $CaCl_2$, and incubated at 30°C until they reached their exponential growth, then Norfloxacin was added. The culture was incubated overnight, filtered 0,45 µm and stocked at 4°C.

To assay strain sensibility to induced bacteriophages, overnight cultures were mixed with each phage suspension in a 96-wells titre and the absorbance at 600nm was recurring measured for four days. For a better identification of bacteriophages, the phage suspensions were spotted on soft TSA inoculated with the strain at its exponential phase. The same method was used to test the activity of bacteriophage phi-IBB PF7A on all the strains in use.

3. Results and Discussion

3.1 Recovery of *Pseudomonas* spp. producing blue colour

A collection of 86 *Pseudomonas* spp. strains was made by several sources: 58 strains were taken from academic collections, ten strains were recovered from dairy products, 11 strains were recovered form vegetables, seven strains were bought from

international collection DSM. All the strains were able to grow both on TSA and Mascarpone Agar at both incubation temperature, but only 29 strains spread a dark colour on TSA and a blue pigment on Mascarpone Agar. These strains were all formerly isolated from mozzarella cheese, except for one strain that was isolated from vegetables. The blue pigment production didn't occurred at the same time for all the strains, but at particular time for each strain among 48h and 20 days, according to previous findings (Martin *et al*. 2011). The composition of the medium didn't affect the time needed to detect the pigment formation. The blue pigment-producing strains were identified by 16S rDNA sequencing as belonging to the "*P. fluorescens* lineage" (Yamamoto *et al.* 2000), which correspond to the species revealed responsible for the blue discoloration of mozzarella cheese (Nogarol *et al*. 2013; Sechi P. *et al*. 2011). To identify the effective species more genotyping analysis are planned for the next year, including $(GTG)_5$ REP-PCR and PFGE.

3.2 Blue pigment characterization

The free-cell broth of all the 29 blue producing strains was scanned from 200 to 700 nm using the clear broth and the free-cell broth of a wild type strain as blank, and the obtained spectra showed a common double peak at about 380 and 415 nm, as expected considering that the wavelength for the detection of the blue colour is between 400 and 450 nm. Further analysis are going to be made with the purpose to identify this pigment such as colour turning depending on pH and HPLC/MS analysis.

3.3 Bacteriophages recover and the assays for sensitive strains

Regarding the bacteriophages induction and the searching for sensitive strains the method used didn't provide the expected results, and no bacteriophages have been isolated yet. It had not been possible to detect any decreasing or slowing of cell growth monitoring the OD at 600nm. From the spot test some plaques were obtained, but the possible presence of bacteriophages has to be confirmed yet. The filtered supernatant from the strains treated with Norfloxacin will continue to be assayed for the isolation of bacteriophages. Ten strains were found sensitive to phage phi-IBB PF7A, among them five are *P. fluorescens* blue-producing, and one is the DSM strain 50108, classified as *P. fluorescens* biovar II.

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Biodiversity in *Pseudomonas fluorescens* strains causing blue discoloration in Mozzarella cheese

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The aim of this PhD thesis was to study different strains causing blue discolouration defect in Mozzarella cheese. A first phenotypical investigation was made on *Pseudomonas* spp. strains isolated from different environment, followed by strains typing was using PFGE analysis. On selected blue producing strains a phylogenetic correlation was made by MLST analysis on seven constitutive genes.

Biodiversità in ceppi di *Pseudomonas fluorescens* responsabili della colorazione blu su Mozzarella

Lo scopo di questa tesi di dottorato è stato l'analisi di ceppi appartenenti al genere *Pseudomonas* spp responsabili di una colorazione blu nei prodotti lattiero-caseari. Dopo una preliminare indagine fenotipica su ceppi apparenenti al genere *Pseudomonas* isolati da matrici diverse, i ceppi sono stati tipizzati mediante PFGE. La correlazione filogenetica di sette ceppi produttori del pigmento blu è stata determinata mediante il sequenziamento di sette geni costitutivi (MLST).

1. Introduction

The blue discoloration defect given by *Pseudomonas* spp. in fresh cheese is a well-known problem for dairy industries. Since 2010 the occurrence of blue spot on Mozzarella cheese was reported from several consumers in Italy and highlighted by local and international media and by RASFF alert system. The microbiological analysis on spoiled products identified the *Pseudomonas fluorescens* species as the causing agent of this blue color, relating the blue coloration with a bacteria concentration of 10^6 UFC/mL, reached in less than 5 days of storage at 8°C (Cenci-Goga *et al.* 2014). The strains identification at specie and biovar level with 16S rRNA, *gyrB* and *rpoD* sequencing (Yamamoto *et al.* 2000) was not discriminating enough to exactly relate the blue production to a particular genotype, for this reason other molecular typing

methods have been proposed; PFGE profiles resulted a reliable methods to correlate the analysed strains with the source of contamination (Martin *et al*., 2011) giving also the possibility to characterize eventual cross-contamination (Nogarol *et al.* 2013).

A few hypothesis have been made on the nature of the blue dye, having as starting point the production of coloured molecules by *Pseudomonas* spp. (for example pyocianine, pyoverdine or pyomelanin) (Brown and Luke 2010), or the blue pigmentation produced by other bacterial genera (Newsome *et al.* 2014). The current thesis is the identification of the blue pigment as indigoidine (Cantoni *et al*., 2011; Caputo *et al*., 2015) but this is not really consistent with the blue chemical proprieties observed in spoiled cheese, for example regarding water solubility.

2. Material and methods

2.1 Strain isolation and identification

Pseudomonas spp. strains investigated in this work were isolated from food, mainly from dairy products, by cultural techniques (plate count on CFC agar). The identification of the species was obtained by 16S rDNA gene partial sequencing. The production of the blue coloration was verified by striking the isolates on Mascarpone agar (MA) (Cantoni *et al*., 2011) and on TSA medium by incubation at 30°C.

2.2 Blue production assays

The selected "blue" strains were inoculated in Mozzarella preserving fluid previously centrifuged (9000g x 10min) and filtered 0,45 µm (PF) The influence of the growing temperature was checked incubating the inoculated PF at 4°C and 30°C. Strikes on MA were repeated as positive control. To investigate the environmental requirements for the blue synthesis further trials were made in minimal medium (M9) added with different carbon sources (glucose, lactose, galactose, sodium citrate, sodium lactate in concentration 10mM). Trials on different carbon sources were made incubating tubes at 4°C.

2.3 REA by PFGE

Seventy-three isolates belonging to *Pseudomonas fluorescens* group, including 30 isolates that show the blue pigmentation, were compared by PFGE analysis after

genome digestion with 20U/sample of SpeI enzyme. Run conditions were 6 volt, initial switch 1, final switch 25s, 22h runtime (Martin *et al*., 2011; Nogarol *et al*., 2013).

2.4 MLST analysis

According to the previous work (Andreani *et al.* 2014) 7 *loci* of different housekeeping genes *gyrB, glnS, ileS, nuoD, recA, rpoB, rpoD* were amplified and sequenced for both DNA strands. The obtained sequences were trimmed and aligned using CLC software (Quiagen). Single loci were compared in *P. fluorescens* MLST database (http://pubmlst.org/pfluorescens). The concatenated sequences were aligned and compared with the all sequenced strains in the MLST database obtaining a phylogenetic tree based on Maxium Likehood algorithm (MEGA software).

3. Results

3.1 Strain isolation and identification

The strain collection used for this PhD work was made up by 90 isolates collected from different sources: dairy products (71), vegetables (11), soil and water (8). All the strains resulted belong to *P. fluorescens* group, except for eight strains belonging to *P. putida* group and five strains belonging to *P. chlororaphis* group. An unique specie ascription was not always possible given by the low resolution obtained with 16S rRNA sequencing, being not sufficiently discriminatory because of its slow evolution rate (Yamamoto *et al.* 2000). Among 90 isolates striked on MA, 32 strains, all isolated from dairy products (Mozzarella and Ricotta cheese) produced a blue-green pigmentation after 3 days at 30°C. They were ascribed to *P. fluorescens* group, identified as *P. libanensis*, *P. cedrina*, *P. gessardii*, *P. poae*, *P. fluorescens* and *P. azotoformans*.

3.2 Blue production assays

Strains inoculated in PF showed a different color production: when incubated at 30°C after 3 days ten strains showed no pigmentation while 16 strains produced a light green-yellow coloration; when the growing temperature was 4°C after 7 days 8 strains coloured the PF in dark green, 3 in dark blue, 7 in light blue, 2 in yellow while 7 had no colour production. Strikes on MA used as positive controls gave similar results: 18 strains showed dark blue pigmentation, 2 had yellow colonies and 3 had no colour (Table 1).

Table 1: *Results of coloration assays in PF after selection from the first strike on MA*

strain	Isolation	year	MA	PF 30°	PF 4°
200188/1	Mozzarella cheese	2010	white	yellow	dark green
200188/2	Mozzarella cheese	2010	white	white	dark green
200188/8	Mozzarella cheese	2010	white	white	dark green
176673/1	Mozzarella cheese	2010	dark blue	yellow	dark green
9AP	Mozzarella cheese	2010	dark blue	yellow	dark green
9BG	Mozzarella cheese	2010	dark blue	yellow	dark green
9BP	Mozzarella cheese	2010	dark blue	yellow	dark green
UMB253	Mozzarella cheese	2010	dark blue	yellow	light blue
UMB254	Mozzarella cheese	2010	dark blue	yellow	light blue
UMB255	Mozzarella cheese	2010	dark blue	white	light blue
UMB256	Mozzarella cheese	2010	dark blue	yellow	light blue
UMB257	Mozzarella cheese	2010	dark blue	white	white
UMB258	Mozzarella cheese	2010	dark blue	yellow	light blue
UMB260	Mozzarella cheese	2010	dark blue	yellow	light blue
UMB261	Mozzarella cheese	2010	dark blue	yellow	light blue
UMB278	Mozzarella cheese	2010	yellow	white	yellow
UMB288	Mozzarella cheese	2010	dark blue	white	white
UMB289	Mozzarella cheese	2010	dark blue	white	white
UMB290	Mozzarella cheese	2010	dark blue	white	white
UMB291	Mozzarella cheese	2010	yellow	white	yellow
UMB293	Mozzarella cheese	2010	dark blue	white	white
UMB294	Mozzarella cheese	2010	white	yellow	white
UMB296	Mozzarella cheese	2010	dark blue	yellow	white
UMB248	Mozzarella cheese	2013	dark blue	white	dark green
UMB249	Mozzarella cheese	2013	dark blue	yellow	dark blue
UMB247	Mozzarella cheese	2013	dark blue	yellow	dark blue
UMB309	Ricotta cheese	2014	dark blue	yellow	dark blue

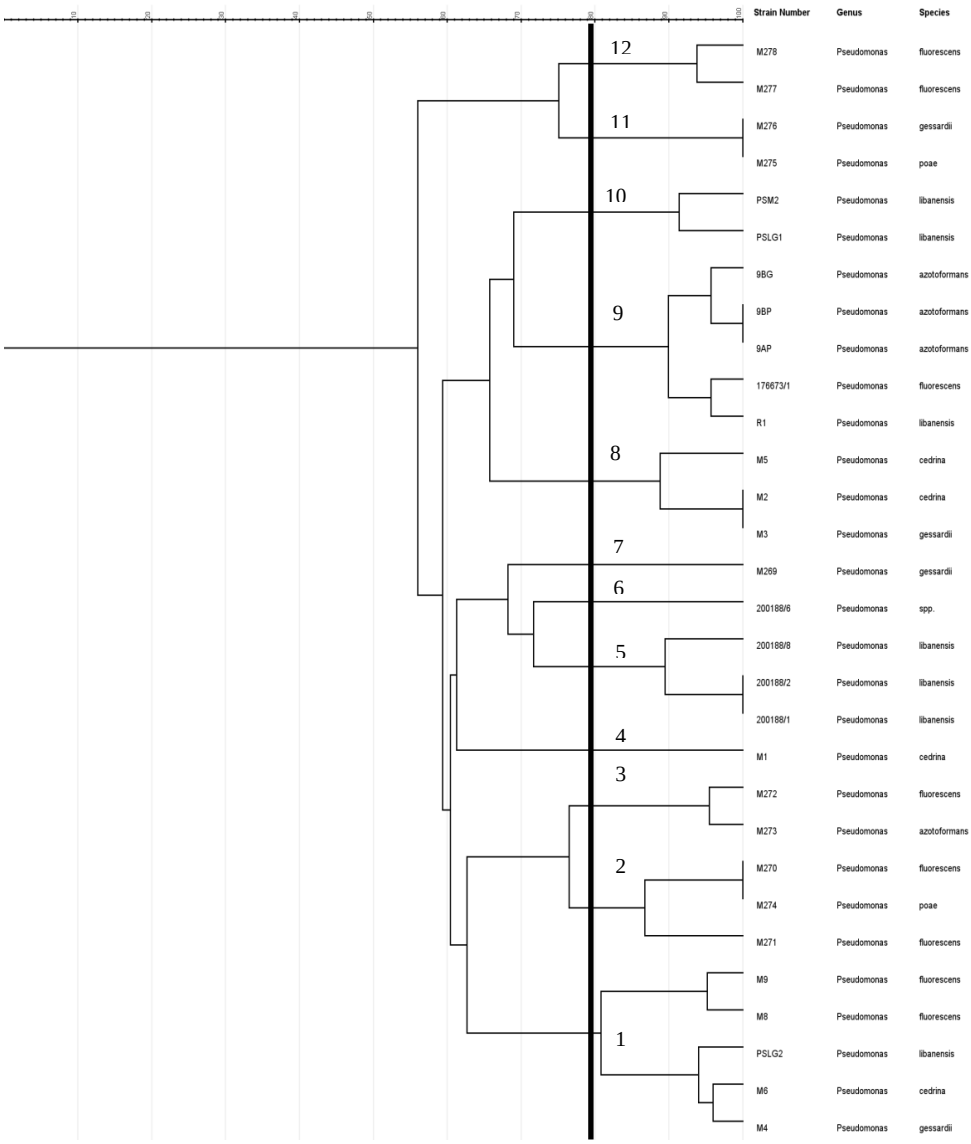
Strains inoculated in M9 showed no growth with lactose, good growth without pigmentation with sodium citrate and sodium lactate, and growth with blue production with glucose. The blue production was noticed after 20 days at 4°C, consisting with the slow rate of growth given by the low temperature and by the medium composition.

3.3 REA by PFGE

From band profiles obtained with PFGE techniques 45 clusters were identified from 73 strains analysed considering a similarity cut-off value of 80% (Nogarol C. *et al*., 2014) (data not shown). Blue producing strains gathered in 12 different clusters, mainly

according to isolation year and place, except for two clusters (identified as number 1 and number 9) where two strains isolated in 2014 (PSLG2 and R1) clustered with strains isolated in 2010 in different places (Figure 1). In cluster 1 is included also a strain (PS77) isolated from dairy that never showed any dark or blue pigmentation (data not shown). From the 12 “blue clusters” 7 strains were selected for MLST analysis (Table 2).

Figure 1: *PFGE profiles of "blue"pigment-producing strains with cluster indication; cut-off value fixed at 80% is marked with black line.*



3.4 MLST analysis

The obtained sequences were compared in *P. fluorescens* MLST database, assigning the number of the respective loci and ST profile. Some sequences of four strains were not ascribable to any locus and so the ST profile couldn't be estimated (Table 2). In the phylogenetic tree obtained from the alignment of the concatenated sequences (Figure 2) the strain analysed resulted phylogenetically near, confirming the hypothesis of the existence of a "blue branch" from Andreani *et al*. (2014).

Figure 2: *Phylogenetic tree obtained from MLST analysis: the "blue branch" is pointed out by letter "B"*

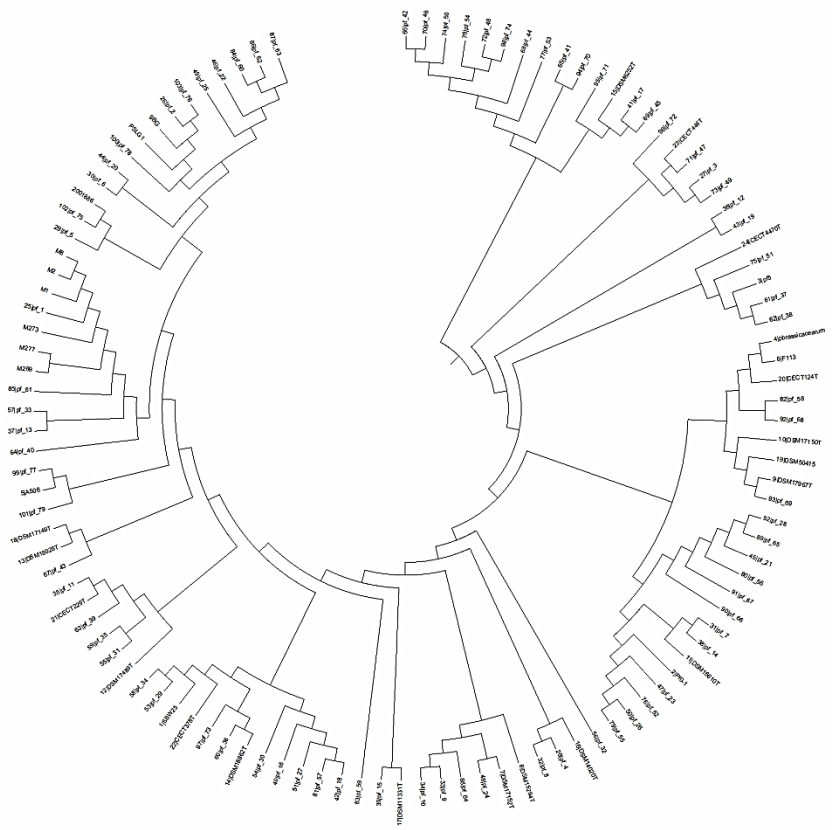


Table 2: *Strains used for MLST analysis with respective PFGE cluster and their loci and ST profile results. ND=not determinated*

name	PFGE cluster	glnS	gyrB	ileS	nuoD	recA	rpoB	rpoD	ST profile
200188/8	10	25	25	25	25	25	25	25	25
9BG	30	29	29	29	29	29	29	29	29
UMB253	19	ND	25	25	ND	ND	69	25	ND
UMB254	27	ND	25	25	ND	ND	69	25	ND
UMB260	1	ND	ND	25	ND	ND	45	25	ND
UMB287	13	25	25	25	25	25	25	25	25
UMB291	6	25	25	25	25	25	25	25	25
UMB248	6	26	26	ND	26	26	ND	30	ND

4. Discussion and conclusions

The results obtained in this PhD work show that the blue discoloration defect caused by *Pseudomonas fluorescens* strains is still a complex issue given that the reproducibility of the phenomenon appears strictly connected to the growth temperature and medium composition. Some strains lost the capability of blue pigment production suggesting the hypothesis that it could be linked to mobile genetic elements or to NRPS (non ribosomial peptide synthetases). When present, blue discoloration occurs always in late growth phase, and glucose as carbon source seems to be necessary for it. With the actual data is still not possible to determinate the function and the real formula of this blue pigment.

PFGE analysis shows that some blue producing strains are recurring in different years, and this could be coincident with the aptitude of biofilm production by this species, that could make these particular strains resident in their environment. Moreover the profile similarity between “blue” and “not blue” producing strains strengthen the idea that genes encoding its synthesis could be in mobile DNA elements. This hypothesis is not confirmed by MLST profiles, as the blue strains of this study are closely related,

clustering in the same phylogenetic group, with other blue producing strains isolated from different food matrix in different places and different years, relating the blue production to the presence of a specific region in the core genome (Andreani N. et al. 2014). These information are still incomplete without the understanding of the role of this blue phenotype and further studies are needed.

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APPENDIX 1

Bacterial isolates used for this work

strain code	alias	16S rRNA ID	source of isolation	place	year	Note
UMB234	PF4	*P. fragi*	pasteurized milk	IT	1985	
UMB235	PF6	*P. fragi*	pasteurized milk	IT	1983	
UMB236	PF20	*P.grimontii*	Salad	IT	1992	
UMB237	PF24	*P. meridiana*	Salad	IT	1992	
UMB238	PS1	*P. fragi*	crescenza cheese	IT	1994	
UMB243	PS9	*P. fluorescens*	spinach	IT	2012	
UMB244	PS10	*P. fluorescens*	spinach	IT	2012	
UMB245	PS12	*Pseudomonas spp.*	spinach	IT	2012	
UMB247	PSM2	*P. libanensis*	mozzarella cheese	IT	2013	blue
UMB248	PSLG1	*P. libanensis*	mozzarella cheese	IT	2013	blue
UMB249	PSLG2	*P. fluorescens*	mozzarella cheese	IT	2013	blue
UMB250	PS23	*P. tessidea*	hard cheese	IT	2012	
UMB251	PS77	*P. fluorescens*	hard cheese	IT	2012	
UMB252	PS16	*P. fluorescens*	hard cheese	IT	2012	
UMB253	M1	*P. cedrina*	mozzarella cheese	IT	2010	blue
UMB254	M2	*P. cedrina*	mozzarella cheese	IT	2010	blue
UMB255	M3	*P. fluorescens*	mozzarella cheese	IT	2010	blue
UMB256	M4	*P. fluorescens*	mozzarella cheese	IT	2010	blue
UMB257	M5	*P. cedrina*	mozzarella cheese	IT	2010	blue
UMB258	M6	*P. fluorescens*	mozzarella cheese	IT	2010	blue
UMB260	M8	*P. fluorescens*	mozzarella cheese	IT	2010	blue
UMB261	M9	*P. fluorescens*	mozzarella cheese	IT	2010	blue
UMB263	M21	*P. gessardii*	mozzarella	IT	2010	

			cheese			
UMB265	M22	*P. gessardii*	mozzarella cheese	IT	2010	
UMB266	M23	*P. gessardii*	mozzarella cheese	IT	2010	
UMB267	M24	*P. gessardii*	mozzarella cheese	IT	2010	
UMB268	M25	*P. fluorescens*	mozzarella cheese	IT	2010	
UMB269	M26	*P. fluorescens*	mozzarella cheese	IT	2010	
UMB271	M29	*P. fluorescens*	mozzarella cheese	IT	2010	
UMB272	M38	*P. fluorescens*	mozzarella cheese	IT	2010	
UMB275	M48	*P. fluorescens*	mozzarella cheese	IT	2010	
UMB276	M63	*P. fluorescens*	mozzarella cheese	IT	2010	
UMB277	M146	*P. synxantha*	mozzarella cheese	IT	2010	
UMB278	M147	*P. fluorescens*	mozzarella cheese	IT	2010	
UMB279	M260	*P. fragi*	mozzarella cheese	IT	2011	
UMB280	M261	*P. costantinii*	mozzarella cheese	IT	2011	
UMB281	M262	*P. fragi*	mozzarella cheese	IT	2011	
UMB282	M263	*P. poae*	mozzarella cheese	IT	2011	
UMB283	M264	*P. costantinii*	mozzarella cheese	IT	2011	
UMB284	M265	*P. fluorescens*	mozzarella cheese	IT	2011	
UMB287	M269	*P. gessardii*	mozzarella cheese	IT	2011	blue
UMB289	M271	*P. fluorescens*	mozzarella cheese	IT	2011	blue

UMB290	M272	*P. fluorescens*	mozzarella cheese	IT	2011	blue
UMB291	M273	*P. fluorescens*	mozzarella cheese	IT	2011	blue
UMB292	M274	*P. fluorescens*	mozzarella cheese	IT	2011	blue
UMB293	M275	*P. poae*	mozzarella cheese	IT	2011	blue
UMB294	M276	*P. fluorescens*	mozzarella cheese	IT	2011	blue
UMB295	M277	*P. fluorescens*	mozzarella cheese	IT	2011	blue
UMB296	M278	*P. fluorescens*	mozzarella cheese	IT	2011	
UMB297	PR3	*P. fragi*	provola cheese	IT	2011	
UMB298	PR5	*P. fluorescens*	provola cheese	IT	2011	
UMB299	PR7	*P. gessardii*	provola cheese	IT	2011	
UMB301	3A	*P. fluorescens*	goat milk	IT	2012	
UMB302	3B	*P. fluorescens*	goat milk	IT	2012	
UMB303	MLdG	*P. azotoformans*	mozzarella cheese	IT	2012	
176673/1		*P. fluorescens*	mozzarella cheese	DE	2010	blue
200188/1		*P. azotoformans*	mozzarella cheese	DE	2010	blue
200188/2		*P. azotoformans*	mozzarella cheese	DE	2010	blue
200188/6		*P. fluorescens*	mozzarella cheese	DE	2010	blue
200188/8		*P. libanensis*	mozzarella cheese	DE	2010	blue
UMB309	R1	*P. fluorescens*	ricotta cheese	IT	2014	blue
9BG		*P. azotoformans*	mozzarella cheese	DE	2010	blue
9AP		*P. azotoformans*	mozzarella cheese	DE	2010	blue

9BP		*P. azotoformans*	mozzarella cheese	DE	2010	blue
SBW25		*P. fluorescens*				
H		*P. fluorescens*				
A506		*P. fluorescens*				
PF_01		*P. fluorescens*				
ATCC 13525		*P. fluorescens*				
DSM 50415		*P. fluorescens*				
DSM 50108		*P. fluorescens*				

APPENDIX 2

Alignment of shared conserved region in blue pigment-producing P. fluorescens *strains UMB247, UMB248, 200188/6, PS22 and PS77 belonging to phage genes cluster*

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                                    20                                  40
                                    |                                   |
UMB248     TTTTAGGTTT  TAAAAGTGAT  TACTGGGGCA  AAGTTGAGCT  40
UMB247     TTTTAGGTTT  TAAAAGTGAT  TACTGGGGCA  AAGTTGAGCT  40
200188/6   TTTTAAGTTG  TAAACATAAT  AGGAGGTGCG  AAGTTGAGCT  40
PS22       TTTTAGGTTT  TAAAAGTGAT  TACTGGCGCA  AAGTTGAGCT  40
PS77       TC--------  ----------  -GCGGGAGCG  AAGTTGAGTT  21
Consensus  TTTTAGGTTT  TAAAAGTGAT  TACTGGGGCA  AAGTTGAGCT

                                    60                                  80
                                    |                                   |
UMB248     GTGAGCGAAC  GCTGCTCCAG  GTATCAAATG  CCGCAGCCCG  80
UMB247     GTGAGCGAAC  GCTGCTCCAG  GTATCAAATG  CCGCAGCCCG  80
200188/6   GAGAGCGAAC  GCTGCTCCAG  GTATCAAACG  CTGCTGCCCG  80
PS22       GTGAGCGAAC  GCTGCTCCAG  GTATCAAATG  CCGCAGCCCG  80
PS77       GAGAACGAAC  ACTACTCCAA  GTATCGAAAG  CAGCGATCCG  61
Consensus  GTGAGCGAAC  GCTGCTCCAG  GTATCAAATG  CCGCAGCCCG

                                   100                                 120
                                    |                                   |
UMB248     TAAGTAATAA  GTTGTTCCTG  GCGCCAAGCC  AGTGATCTGA  120
UMB247     TAAGTAATAA  GTTGTTCCTG  GCGCCAAGCC  AGTGATCTGA  120
200188/6   CAAGTAATAA  GTTGTTCCTG  GTGCCAAGCC  TGTGATCTGG  120
PS22       TAAGTAATAA  GTTGTTCCTG  GCGCCAAGCC  AGTGATCTGA  120
PS77       AAGGTAGTAC  GTTGTTCCGG  CGTTCAATCC  TGAGATCTGT  101
Consensus  TAAGTAATAA  GTTGTTCCTG  GCGCCAAGCC  AGTGATCTGA

                                   140                                 160
                                    |                                   |
UMB248     CCAGCCTGCG  AGGCGCCCTG  ATAACCAATC  GTGCCCGTGA  160
UMB247     CCAGCCTGCG  AGGCGCCCTG  ATAACCAATC  GTGCCCGTGA  160
200188/6   CCGGCCTCCG  AGGCGCCCTG  ATAACCGATG  GTGCCCGTGA  160
PS22       CCAGCCTGCG  AGGCACCCTG  ATAACCGATG  GTGCCCGTGA  160
PS77       CCGCTGGCAG  CACTTCCCTG  ATACCCCACA  TTGCCTGCGA  141
Consensus  CCAGCCTGCG  AGGCGCCCTG  ATAACCNATN  GTGCCCGTGA

                                   180                                 200
                                    |                                   |
UMB248     CCGTGGGATC  GAACCCGGCC  GTGGTTGCAT  ACACGAACAT  200
UMB247     CCGTGGGATC  GAACCCGGCC  GTGGTTGCAT  ACACGAACAT  200
200188/6   TCGTGGGATC  GAAGCCCGCC  GTGGTTGCAT  ACACGAACAT  200
PS22       TCGTGGGATC  GAACCCGGCC  GTGGTTGCAT  ACACGAACAT  200
PS77       CAGTTGGATC  GAATCCTAAA  TTTGTTGAGT  ACACGAAAAT  181
Consensus  CCGTGGGATC  GAACCCGGCC  GTGGTTGCAT  ACACGAACAT

                                   220                                 240
                                    |                                   |
UMB248     GTAGCCAGCC  TTGTCAGCTG  CGGCACTAGT  GTTACAACTG  240
UMB247     GTAGCCAGCC  TTGTCAGCTG  CGGCACTAGT  GTTACAACTG  240
200188/6   GTAGCCAGCC  TTGTCAGCTG  CGGCACTAGT  ATTACAACTG  240
PS22       GTAGCCAGCC  TTGTCAGCTG  CGGCACTAGT  GTTACAACTG  240
PS77       GTAACCAGCA  ACGTCAGGAG  CCACGCTGGG  AGCACAACTC  221
Consensus  GTAGCCAGCC  TTGTCAGCTG  CGGCACTAGT  GTTACAACTG

                                   260                                 280
                                    |                                   |
UMB248     ACATTCGCGG  TGGTGCCGCT  CACAGTCGCC  GCTGTGCCGC  280
UMB247     ACATTCGCGG  TGGTGCCGCT  CACAGTCGCC  GCTGTGCCGC  280
200188/6   ACATTCGCGG  TGGTCCCGCT  CACAGTCGCC  GCTGTACCGC  280
PS22       ACATTCGCGG  TGGTCCCGCT  CACAGTCGCC  GCTGTGCCGC  280
PS77       ACGTTGGCAA  CTGTCCCACT  TACCGTTGCA  GCCGATCCGG  261
Consensus  ACATTCGCGG  TGGTCCCGCT  CACAGTCGCC  GCTGTGCCGC

                                   300                                 320
                                    |                                   |
UMB248     TAACGGCCGG  CGGCGCGGTG  TTCACCACCA  CCAGAGCGGA  320
UMB247     TAACGGCCGG  CGGCGCGGTG  TTCACCACCA  CCAGAGCGGA  320
200188/6   TGACAGCCGG  CGGCGCGGTG  TTCACCACCA  CCAGAGCGGA  320
PS22       TGACGGCCGG  CGGCGCGGTG  TTCACCACCA  CCAGAGCGGA  320
PS77       TTACCGCTGG  TGGTGCAGTG  TTAACTACAA  CCAATGCAGC  301
Consensus  TNACGGCCGG  CGGCGCGGTG  TTCACCACCA  CCAGAGCGGA

                                   340                                 360
                                    |                                   |
UMB248     AACAGGGGCG  TTACCCGCTG  CGTTTCGCTC  AATGATTTCA  360
UMB247     AACAGGGGCG  TTACCCGCTG  CGTTTCGCTC  AATGATTTCA  360
200188/6   CACAGGGGCG  GTGCCGGCCG  TGTTGCGCTC  AATGACTTCA  360
PS22       AACAGGGGCG  TTACCCGCTG  CGTTTCGCTC  AATGATTTCA  360
PS77       TACAGGTGCG  CTTCCGGCGG  CGTTTCTCTC  AATGACCTCT  341
Consensus  AACAGGGGCG  TTACCCGCTG  CGTTTCGCTC  AATGATTTCA

                                   380                                 400
                                    |                                   |
UMB248     ATCCGGTAGC  TGCGTACCAG  CGGTCCATCG  ACCAGGGCAT  400
UMB247     ATCCGGTAGC  TGCGTACCAG  CGGTCCATCG  ACCAGGGCAT  400
200188/6   ATCCGGTAGC  TGCGTACCAG  CGGTCCATCG  ACCAGGGCAT  400
PS22       ATCCGGTAGC  TGCGTACCAG  CGGTCCATCG  ACCAGGGCAT  400
PS77       ACCCGGTAAC  TTCGAATCAA  AGCACCATCG  ACCAAGGCAT  381
Consensus  ATCCGGTAGC  TGCGTACCAG  CGGTCCATCG  ACCAGGGCAT

                                   420                                 440
                                    |                                   |
UMB248     CTGCCAACTG  GTAAGTGAAC  GTGGTGGCGG  TGGTGGCAAC  440
UMB247     CTGCCAACTG  GTAAGTGAAC  GTGGTGGCGG  TGGTGGCAAC  440
200188/6   CTGCCAACTG  GTAAGTGAAC  GTGGTGGCGG  TGGTGGCAAC  440
PS22       CTGCCAACTG  GTAAGTGAAC  GTGGTGGCGG  TGGTGGCAAC  440
PS77       CAGCGCGCTG  GTATGTGAAG  GTGGTGCCGG  TCGTAGGTAC  421
Consensus  CTGCCAACTG  GTAAGTGAAC  GTGGTGGCGG  TGGTGGCAAC
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                                460                                 480
UMB248  TTCACGCAAC  AAGGCGTTCG  TGGCTGCGTT  GCGGATTCTG 480
UMB247  TTCACGCAAC  AAGGCGTTCG  TGGCTGCGTT  GCGGATTCTG 480
200188/6 TTCACGCAAC  AAGGCGTTCG  TGGCTGCGTT  GCGGATTCTG 480
   PS22 TTCACGCAAC  AAGGCGTTCG  TGGCTGCGTT  GCGGATTCTG 480
   PS77 CTCTCGAAGC  AATGCATTGG  TGCCAGCATT  ACGGATCCTT 461
Consensus TTCACGCAAC  AAGGCGTTCG  TGGCTGCGTT  GCGGATTCTG
                                500                                 520
UMB248  ACCAGCCGAT  CTGCGGCGTG  TGCCCCAGCT  GCCCAACCAA 520
UMB247  ACCAGCCGAT  CTGCGGCGTG  TGCCCCAGCT  GCCCAACCAA 520
200188/6 ACCAGCCGAT  CTGCGGCGTG  TGCCCCAGCT  GCCCAACCAA 520
   PS22 ACCAGCCGAT  CTGCGGCGTG  TGCCCCAGCT  GCCCAACCAA 520
   PS77 ACCAAGCGAT  CTTCCGCATG  GGCACCTGCG  GACCAACTGA 501
Consensus ACCAGCCGAT  CTGCGGCGTG  TGCCCCAGCT  GCCCAACCAA
                                540                                 560
UMB248  CGGTGAAGTA  AGGTGCCTCG  AACGCGCCCA  CTAGCTGCAA 560
UMB247  CGGTGAAGTA  AGGTGCCTCG  AACGCGCCCA  CTAGCTGCAA 560
200188/6 CGGTGAAGTA  AGGTGCCTCG  AACGCGCCCA  CTAGCTGCAA 560
   PS22 CGGTGAAGTA  AGGTGCCTCG  AACGCGCCCA  CTAGCTGCAA 560
   PS77 CGGTGAAGTA  CGGGCCTTCG  AACGTTCCCA  CCAGTAACAA 541
Consensus CGGTGAAGTA  AGGTGCCTCG  AACGCGCCCA  CTAGCTGCAA
                                580                                 600
UMB248  TCCCTGGGCA  GCGCCTGGTA  CAACTCGCAC  TGGCGACAAT 600
UMB247  TCCCTGGGCA  GCGCCTGGTA  CAACTCGCAC  TGGCGACAAT 600
200188/6 TCCCTGGGCA  GCGCCTGGTA  CAACTCGCAC  TGGCGACAAT 600
   PS22 TCCCTGGGCA  GCGCCTGGTA  CAACTCGCAC  TGGCGACAAT 600
   PS77 ACTTTCGGCG  GGGCCAGGGA  CGACGCGCAC  GGGTGATAAT 581
Consensus TCCCTGGGCA  GCGCCTGGTA  CAACTCGCAC  TGGCGACAAT
                                620                                 640
UMB248  GTGATGCTGT  AGGGCGTCAC  ATCAGCCAAG  TCCTCCGACG 640
UMB247  GTGATGCTGT  AGGGCGTCAC  ATCAGCCAAG  TCCTCCGACG 640
200188/6 GTGATGCTGT  AGGGCGTCAC  ATCAGCCAAG  TCCTCCGACG 640
   PS22 GTGATGCTGT  AGGGCGTCAC  ATCAGCCAAG  TCCTCCGACG 640
   PS77 GTGATGCTGT  AAGCAGTTAC  ATCGGCAAGA  TCCTCTAAGG 621
Consensus GTGATGCTGT  AGGGCGTCAC  ATCAGCCAAG  TCCTCCGACG
                                660                                 680
UMB248  CCCGGCCAAA  CACGTTGAAC  GAGCGGAACT  TCACCCACAC 680
UMB247  CCCGGCCAAA  CACGTTGAAC  GAGCGGAACT  TCACCCACAC 680
200188/6 CCCGGCCAAA  CACGTTGAAC  GAGCGGAACT  TCACCCACAC 680
   PS22 CCCGGCCAAA  CACGTTGAAC  GAGCGGAACT  TCACCCACAC 680
   PS77 CCCGACCAAA  CACATTGAAA  GATCGAAACT  TGACCCAGGC 661
Consensus CCCGGCCAAA  CACGTTGAAC  GAGCGGAACT  TCACCCACAC
                                700                                 720
UMB248  CGTTTTGCCG  ATTTGGTCGG  TGGTATAGCT  GTACTTCCAG 720
UMB247  CGTTTTGCCG  ATTTGGTCGG  TGGTATAGCT  GTACTTCCAG 720
200188/6 CGTTTTGCCG  ATTTGGTCGG  TGGTATAGCT  GTACTTCCAG 720
   PS22 CGTTTTGCCG  ATTTGGTCGG  TGGTATAGCT  GTACTTCCAG 720
   PS77 CGTTTTTCCA  ACCTGATCCA  CTGCATACGA  ATACTTCCAG 701
Consensus CGTTTTGCCG  ATTTGGTCGG  TGGTATAGCT  GTACTTCCAG
                                740                                 760
UMB248  ATCGCGTCAT  CAAGCCGCAC  AAACTGGGCG  TCCACAGGGT 760
UMB247  ATCGCGTCAT  CAAGCCGCAC  AAACTGGGCG  TCCACAGGGT 760
200188/6 ATCGCGTCAT  CAAGCCGCAC  AAACTGGGCG  TCCACCGGAT 760
   PS22 ATCGCGTCAT  CAAGCCGCAC  AAACTGGGCG  TCCACAGGAT 760
   PS77 ATTGCGTCAT  CAAGCCGCAC  AAATGCGGCA  TCTACTGGAT 741
Consensus ATCGCGTCAT  CAAGCCGCAC  AAACTGGGCG  TCCACAGGAT
                                780                                 800
UMB248  GGCTGGAAAC  CGACGACCCC  AGGCGCCCAC  GACGCAGGTA 800
UMB247  GGCTGGAAAC  CGACGACCCC  AGGCGCCCAC  GACGCAGGTA 800
200188/6 GGTTGGAAAC  CGACGACCCC  AGGCGCCCAC  GACGCAGGTA 800
   PS22 GGTTGGAAAC  CGACGACCCC  AGGCGCCCAC  GACGCAGGTA 800
   PS77 GAGTAGATAC  CGCTGAACTC  AGCCGCCCCC  GCCGCAAGTA 781
Consensus GGNTGGAAAC  CGACGACCCC  AGGCGCCCAC  GACGCAGGTA
                                820                                 840
UMB248  CTGCAAATTG  TAGGCGCCCG  GCCCGGTGAG  GGAAGCATCT 840
UMB247  CTGCAAATTG  TAGGCGCCCG  GCCCGGTGAG  GGAAGCATCT 840
200188/6 CTGCAAATTG  TAGGCGCCCG  GCCCGGTGAG  GGAAGCATCT 840
   PS22 CTGCAAATTG  TAGGCGCCCG  GCCCGGTGAG  GGAAGCATCT 840
   PS77 CTGTAGGTTG  TAAGCCCCCG  GTCCAGTCAG  TTGTGCATCG 821
Consensus CTGCAAATTG  TAGGCGCCCG  GCCCGGTGAG  GGAAGCATCT
                                860                                 880
UMB248  CGATAGCTGA  TCAACTCACC  ATCAATCCAA  CACAATGTGG 880
UMB247  CGATAGCTGA  TCAACTCACC  ATCAATCCAA  CACAATGTGG 880
200188/6 CGATAGCTGA  TCAACTCACC  ATCAATCCAA  CACAATGTGG 880
   PS22 CGATAGCTGA  TCAACTCACC  ATCAATCCAA  CACAATGTGG 880
   PS77 CGGTAACTGA  TTAACTCACC  TTCAACCCAA  CACAGAGTGG 861
Consensus CGATAGCTGA  TCAACTCACC  ATCAATCCAA  CACAATGTGG
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                                    900                                 920
UMB248  CACCACTATC CGCCTCGGCA GTGGTCGCGG CAGTAAGTTG 920
UMB247  CACCACTATC CGCCTCGGCA GTGGTCGCGG CAGTAAGTTG 920
200188/6 CACCACTATC CGCCTCGGCA GTGGTCGCGG CAGTAAGTTG 920
   PS22 CACCACTATC CGCCTCGGCA GTGGTCGCGG CAGTAAGTTG 920
   PS77 CGCCGCTATC CGCCTCAGCC GTTGTTGCCG CTGTAAGCTC 901
Consensus CACCACTATC CGCCTCGGCA GTGGTCGCGG CAGTAAGTTG
                                    940                                 960
UMB248  ATCGGCAACT GACAGCTTTA CCGACAAGGT GTTGGTGATA 960
UMB247  ATCGGCAACT GACAGCTTTA CCGACAAGGT GTTGGTGATA 960
200188/6 ATCGGCAACT GACAGCTTTA CCGACAAGGT GTTGGTGATA 960
   PS22 ATCGGCAACT GACAGCTTTA CCGACAAGGT GTTGGTGATA 960
   PS77 AGCAGGTACC GAAAGTTTCA CTGATAAGGT ATTGACCGTA 941
Consensus ATCGGCAACT GACAGCTTTA CCGACAAGGT GTTGGTGATA
                                    980                                1.000
UMB248  TCCGGATCAC CTCCCGCCGG CAACGGCGCC GTGAGCCTGC 1000
UMB247  TCCGGATCAC CTCCCGCCGG CAACGGCGCC GTGAGCCTGC 1000
200188/6 TCCGGATCAA CTCCCGCCGG CAACGGCGCC GTGAGCCTGC 1000
   PS22 TCCGGATCAC CTCCCGCCGG CAACGGCGCC GTGAGCCTGC 1000
   PS77 TCTGGATCAC TACCGCTTTC CAGGGGCCCG GTGAGGCGGC 981
Consensus TCCGGATCAC CTCCCGCCGG CAACGGCGCC GTGAGCCTGC
                                   1.020                               1.040
UMB248  CAATGCGCGA GCGACCATAG ACGGTCTCAA CCATTCGATA 1040
UMB247  CAATGCGCGA GCGACCATAG ACGGTCTCAA CCATTCGATA 1040
200188/6 CAATGCGCGA GCGACCATAG ACGGTCTCAA CCATTCGATA 1040
   PS22 CAATGCGCGA GCGACCATAG ACGGTCTCAA CCATTCGATA 1040
   PS77 CAATTCGGGA TCGACCATAG ATGCTTTCAA CCATCCGGTA 1021
Consensus CAATGCGCGA GCGACCATAG ACGGTCTCAA CCATTCGATA
                                   1.060                               1.080
UMB248  GCTATCACCG TCGGCGCTGA TCCAGATATC ACAGCCGCCC 1080
UMB247  GCTATCACCG TCGGCGCTGA TCCAGATATC ACAGCCGCCC 1080
200188/6 GCTATCACCG TCGGCGCTGA TCCAGATATC ACAGCCGCCC 1080
   PS22 GCTATCACCG TCGGCGCTGA TCCAGATATC ACAGCCGCCC 1080
   PS77 ACTGTCACCG TCTGCACTGA TCCAGACCTC ACAACCACCC 1061
Consensus GCTATCACCG TCGGCGCTGA TCCAGATATC ACAGCCGCCC
                                   1.100                               1.120
UMB248  CACGCCTCAT CGGCCCCGGC GACCGCGCCC CAGATCTGCG 1120
UMB247  CACGCCTCAT CGGCCCCGGC GACCGCGCCC CAGATCTGCG 1120
200188/6 CACGCCTCAT CGGCCCCGGC GACCGCGCCC CAGATCTGCG 1120
   PS22 CACGCCTCAT CGGCCCCGGC GACCGCGCCC CAGATCTGCG 1120
   PS77 CAGGCGTCAC CAGCTCCGGC GACGGCACCC CAGACCTGTA 1101
Consensus CACGCCTCAT CGGCCCCGGC GACCGCGCCC CAGATCTGCG
                                   1.140                               1.160
UMB248  TTTCACCGGG CAACAGCAGG CTTTCTGGTG GATTGAAGAT 1160
UMB247  TTTCACCGGG CAACAGCAGG CTTTCTGGTG GATTGAAGAT 1160
200188/6 TTTCACCGGG CAACAGCAGG CTTTCTGGTG GATTGAAGAT 1160
   PS22 TTTCACCGGG CAACAGCAGG CTTTCTGGTG GATTGAAGAT 1160
   PS77 ATTCACCCGG CAACAGCAGG CTTTCAGGCG GGTTAAAGAT 1141
Consensus TTTCACCGGG CAACAGCAGG CTTTCTGGTG GATTGAAGAT
                                   1.180                               1.200
UMB248  AATCGGCGCC AACACAGGCC CCGGCGCTGC GTTCTGATTG 1200
UMB247  AATCGGCGCC AACACAGGCC CCGGCGCTGC GTTCTGATTG 1200
200188/6 AATCGGCGCC AACACAGGCC CCGGCGCCGC GTTCTGATTG 1200
   PS22 AATCGGCGCC AACACAGGCC CCGGCGCCGC GTTCTGATTG 1200
   PS77 GATAGGTGCA AGCACTGGCC CGGGCGAAGC ATTCTGGTTG 1181
Consensus AATCGGCGCC AACACAGGCC CCGGCGCNGC GTTCTGATTG
                                   1.220                               1.240
UMB248  CCCTGGTATC CCGTCTTGCT CTGTACCGGA TAATTCGGTG 1240
UMB247  CCCTGGTATC CCGTCTTGCT CTGTACCGGA TAATTCGGTG 1240
200188/6 CCTTGGTATC CCGTCTTGCT CTGTACCGGA TAATTCGGTG 1240
   PS22 CCCTGGTATC CCGTCTTGCT CTGTACCGGA TAATTCGGTG 1240
   PS77 CCCTGGTAAC CGGTCTTCGA CTGCACCGGG TAGTTCGGCG 1221
Consensus CCCTGGTATC CCGTCTTGCT CTGTACCGGA TAATTCGGTG
                                   1.260                               1.280
UMB248  CACTGCCTGT GCCCAGGAGC GCATCCTCGG CCACGACCGC 1280
UMB247  CACTGCCTGT GCCCAGGAGC GCATCCTCGG CCACGACCGC 1280
200188/6 CACTGCCTGT ACCCAGGAGC GCATCCTCGG CCACGACCGC 1280
   PS22 CACTGCCTGT GCCCAGGAGC GCATCCTCGG CCACGACCGC 1280
   PS77 CGCTGCCCAC TCCCAGTAAC GCGTCCTCTG CAACAATTGC 1261
Consensus CACTGCCTGT GCCCAGGAGC GCATCCTCGG CCACGACCGC
                                   1.300                               1.320
UMB248  CAGCTTGCCG TCTTCATCCT CTTCGACCGA GATCAATCGG 1320
UMB247  CAGCTTGCCG TCTTCATCCT CTTCGACCGA GATCAATCGG 1320
200188/6 CAGCTTGCCG TCTTCATCCT CTTCGACCGA GATCAATCGG 1320
   PS22 CAGCTTGCCG TCTTCATCCT CTTCGACCGA GATCAATCGG 1320
   PS77 CAACTTGCCC TCTTCATCCT CTTCCACTGA GATTAACCGG 1301
Consensus CAGCTTGCCG TCTTCATCCT CTTCGACCGA GATCAATCGG
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                                1.340                                1.360
UMB248  ACCAGGCGGC GGTTAAGCTT CAACGCCGGT TCTGTGATCG 1360
UMB247  ACCAGGCGGC GGTTAAGCTT CAACGCCGGT TCTGTGATCG 1360
200188/6 ACCAGGCGGC GGTTAAGCTT CAACGCCGGC TCTGTGATCG 1360
PS22    ACCAGGCGGC GGTTAAGCTT CAACGCCGGC TCTGTGATCG 1360
PS77    ACCAGACGCC GGTCAAGCTT TAACGCTGGC TCAGTAACCG 1341
Consensus ACCAGGCGGC GGTTAAGCTT CAACGCCGGC TCTGTGATCG
                                1.380                                1.400
UMB248  TGACCAGGTC CATTGGTTCC AGCAGCACAT GTTGCCAGCC 1400
UMB247  TGACCAGGTC CATTGGTTCC AGCAGCACAT GTTGCCAGCC 1400
200188/6 TGACCAGGTC CATTGGTTCC AGCAGCACAT GTTGCCAGCC 1400
PS22    TGACCAGGTC CATTGGTTCC AGCAGCACAT GTTGCCAGCC 1400
PS77    TCACCAAGTC CATAGGCTCT AGCAAAACAT GCTGCCACCC 1381
Consensus TGACCAGGTC CATTGGTTCC AGCAGCACAT GTTGCCAGCC
                                1.420                                1.440
UMB248  GAGGGAAAAC TGGTACTCGT TACGGATGTA CAGCTTGCGC 1440
UMB247  GAGGGAAAAC TGGTACTCGT TACGGATGTA CAGCTTGCGC 1440
200188/6 GAGGGAAAAC TGGTACTCGT TGCGGATGTA CAGCTTGCGC 1440
PS22    GAGAGAAAAC TGGTACTCGT TGCGGATATA CAGCTTGCGC 1440
PS77    CAATGAAAAC CGGTACTCAT TGCGGATGTA GAGTTTTCGC 1421
Consensus GAGGGAAAAC TGGTACTCGT TGCGGATGTA CAGCTTGCGC
                                1.460                                1.480
UMB248  TGCACCAACA GCTGCGCCGA GTGCGACGCA ATGGCCGTAT 1480
UMB247  TGCACCAACA GCTGCGCCGA GTGCGACGCA ATGGCCGTAT 1480
200188/6 TGCACCAGCA ACTGCGCCGA GTGCGACGCA ATGGCCGTAT 1480
PS22    TGTACCAGCA ACTGAGCCGA GTGTGAAGCG ATGGCAGCGC 1480
PS77    TGCACCAATA GTTGAGCCGC GTGAGAAGCA ATCGTTGCGT 1461
Consensus TGCACCAACA GCTGCGCCGA GTGCGACGCA ATGGCCGTAT
                                1.500                                1.520
UMB248  CGCAGATCTC GTACGCCTTG ATGGTGTCCA TCGGCTTGGA 1520
UMB247  CGCAGATCTC GTACGCCTTG ATGGTGTCCA TCGGCTTGGA 1520
200188/6 CGCAGATCTC GTACGCCTTG ATGGTGTCCA TAGGCTTGGA 1520
PS22    TGCAAATCTC GTACGCCTTG ACGGTGTCCA TCGGCTTTGA 1520
PS77    CACAGATCTC GTAAGCTTTA ATAGTGTCCA TTGGCCTGGA 1501
Consensus CGCAGATCTC GTACGCCTTG ATGGTGTCCA TCGGCTTGGA
                                1.540                                1.560
UMB248  CCCGAATTGC TCGATGGCTG CCTGATCCGG TGCGCGCACC 1560
UMB247  CCCGAATTGC TCGATGGCTG CCTGATCCGG TGCGCGCACC 1560
200188/6 CCCGAATTGC TCGATGGCTG CCTGATCCGG TGCGCGCACC 1560
PS22    CCCGAACTGC TCTATGGCCG CCTGATCAGG CGCGCGCACC 1560
PS77    GCCGAACTGC TCAATGGCTG ACTGATCTAC TCCGCGTACC 1541
Consensus CCCGAATTGC TCGATGGCTG CCTGATCCGG TGCGCGCACC
                                1.580                                1.600
UMB248  ACGTCCGTGT TGTACTCATG ATCGCGATCG AGGATCTCCA 1600
UMB247  ACGTCCGTGT TGTACTCATG ATCGCGATCG AGGATCTCCA 1600
200188/6 ACGTCGGTGT TGTACTCATG ATCGCGGTCG AGGATCTCCA 1600
PS22    ACATCAGTGT TGTACTCGTG GTCGCGATCC AGAATCTCTA 1600
PS77    ACGTCGGTAT TGTATTCATG GTTTCGGTCA AGGATCTCCA 1581
Consensus ACGTCNGTGT TGTACTCATG ATCGCGATCG AGGATCTCCA
                                1.620                                1.640
UMB248  GCGACACCTC GTTGTAGCTG TCGGCCTGGC TTTTTATCTT 1640
UMB247  GCGACACCTC GTTGTAGCTG TCGGCCTGGC TTTTTATCTT 1640
200188/6 GTGACACCTC GTTGTAGCTG TCGGCCTGGC TTTTGATCTT 1640
PS22    GTGAAACTTC GTTGTAGCTG TCGGCTTGGC TCTTGATCTT 1640
PS77    GCGACACTTC GTTATAGCTG TCGGCCTGGC TCTTAATCTT 1621
Consensus GCGACACCTC GTTGTAGCTG TCGGCCTGGC TTTTNATCTT
                                1.660                                1.680
UMB248  CAGCTGTACC GGTGGCTCGC CCTCTTCCGC CAAAAAATCA 1680
UMB247  CAGCTGTACC GGTGGCTCGC CCTCTTCCGC CAAAAAATCA 1680
200188/6 CAGCTGTACC GGTGGCTCGC CCTCTTCCGC CAAAAAATCA 1680
PS22    CAGCTGAATC GGCGGTTCGC CCTCTTCCGC TAAAAAGTCG 1680
PS77    GAGCTGAACC GGTGGCGTAC CCTCCTCAGC CAAGAAGTCA 1661
Consensus CAGCTGTACC GGTGGCTCGC CCTCTTCCGC CAAAAAATCA
                                1.700                                1.720
UMB248  TCATCCGTGA GGTGCGCCAC GGGCGTGACA TTCGGGTACC 1720
UMB247  TCATCCGTGA GGTGCGCCAC GGGCGTGACA TTCGGGTACC 1720
200188/6 TCATCCGTGA GGTGCGCCAC GGGCGTGACA TTCGGGTACC 1720
PS22    TCATCTGTGA GGTGCGCTAC GGGCGTGACA TTCGGGTACC 1720
PS77    TCGTCGGTCA GATGGGCAAC TGCGGTGATG TTGGGGTACC 1701
Consensus TCATCCGTGA GGTGCGCCAC GGGCGTGACA TTCGGGTACC
                                1.740                                1.760
UMB248  AGGTCACGCC GTTGCCGGTG ACCACCTGAT CACCAAATGG 1760
UMB247  AGGTCACGCC GTTGCCGGTG ACCACCTGAT CACCAAATGG 1760
200188/6 AAGTCACACC GTTGCCGGTG ACCACCTGAT CACCGAATGG 1760
PS22    AGGTAACACC GTTTCCCGTG ACGACCTGAT CACCAAATGG 1760
PS77    AGGTGACACC GTTACCGGTC ACAACCTGGT CACCAAAGGG 1741
Consensus AGGTCACACC GTTGCCGGTG ACCACCTGAT CACCAAATGG
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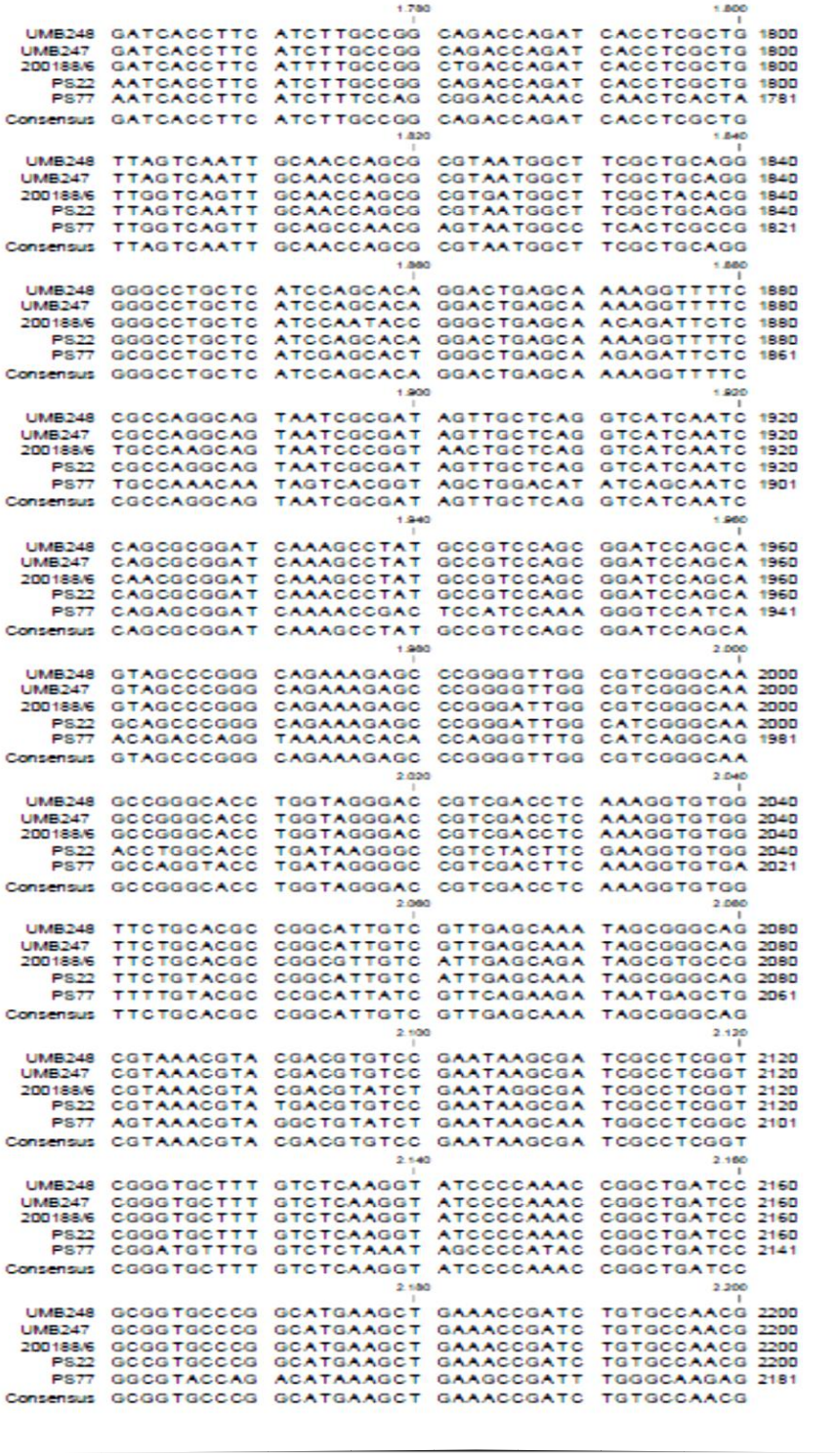
1.780
1.800
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UMB247 GATCACCTTC ATCTTGCCGG CAGACCAGAT CACCTCGCTG 1800
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PS22 AATCACCTTC ATCTTGCCGG CAGACCAGAT CACCTCGCTG 1800
PS77 AATCACCTTC ATCTTTCCAG CGGACCAAAC CAACTCACTA 1781
Consensus GATCACCTTC ATCTTGCCGG CAGACCAGAT CACCTCGCTG
1.820
1.840
UMB248 TTAGTCAATT GCAACCAGCG CGTAATGGCT TCGCTGCAGG 1840
UMB247 TTAGTCAATT GCAACCAGCG CGTAATGGCT TCGCTGCAGG 1840
200188/6 TTGGTCAGTT GCAACCAGCG CGTGATGGCT TCGCTACACG 1840
PS22 TTAGTCAATT GCAACCAGCG CGTAATGGCT TCGCTGCAGG 1840
PS77 TTGGTCAGTT GCAGCCAACG AGTAATGGCC TCACTCGCCG 1821
Consensus TTAGTCAATT GCAACCAGCG CGTAATGGCT TCGCTGCAGG
1.860
1.880
UMB248 GGGCCTGCTC ATCCAGCACA GGACTGAGCA AAAGGTTTTC 1880
UMB247 GGGCCTGCTC ATCCAGCACA GGACTGAGCA AAAGGTTTTC 1880
200188/6 GGGCCTGCTC ATCCAATACC GGGCTGAGCA ACAGATTCTC 1880
PS22 GGGCCTGCTC ATCCAGCACA GGACTGAGCA AAAGGTTTTC 1880
PS77 GCGCCTGCTC ATCGAGCACT GGGCTGAGCA AGAGATTCTC 1861
Consensus GGGCCTGCTC ATCCAGCACA GGACTGAGCA AAAGGTTTTC
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1.920
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200188/6 TGCCAAGCAG TAATCCCGGT AACTGCTCAG GTCATCAATC 1920
PS22 CGCCAGGCAG TAATCGCGAT AGTTGCTCAG GTCATCAATC 1920
PS77 TGCCAAACAA TAGTCACGGT AGCTGGACAT ATCAGCAATC 1901
Consensus CGCCAGGCAG TAATCGCGAT AGTTGCTCAG GTCATCAATC
1.940
1.960
UMB248 CAGCGCGGAT CAAAGCCTAT GCCGTCCAGC GGATCCAGCA 1960
UMB247 CAGCGCGGAT CAAAGCCTAT GCCGTCCAGC GGATCCAGCA 1960
200188/6 CAACGCGGAT CAAAGCCTAT GCCGTCCAGC GGATCCAGCA 1960
PS22 CAGCGCGGAT CAAACCCTAT GCCGTCCAGC GGATCCAGCA 1960
PS77 CAGAGCGGAT CAAAACCGAC TCCATCCAAA GGGTCCATCA 1941
Consensus CAGCGCGGAT CAAAGCCTAT GCCGTCCAGC GGATCCAGCA
1.980
2.000
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200188/6 GTAGCCCGGG CAGAAAGAGC CCGGGATTGG CGTCGGGCAA 2000
PS22 GCAGCCCGGG CAGAAAGAGC CCGGGATTGG CATCGGGCAA 2000
PS77 ACAGACCAGG TAAAAACACA CCAGGGTTTG CATCAGGCAG 1981
Consensus GTAGCCCGGG CAGAAAGAGC CCGGGGTTGG CGTCGGGCAA
2.020
2.040
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200188/6 GCCGGGCACC TGGTAGGGAC CGTCGACCTC AAAGGTGTGG 2040
PS22 ACCTGGCACC TGATAAGGGC CGTCTACTTC GAAGGTGTGG 2040
PS77 GCCAGGTACC TGATAGGGGC CGTCGACTTC AAAGGTGTGA 2021
Consensus GCCGGGCACC TGGTAGGGAC CGTCGACCTC AAAGGTGTGG
2.060
2.080
UMB248 TTCTGCACGC CGGCATTGTC GTTGAGCAAA TAGCGGGCAG 2080
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200188/6 TTCTGCACGC CGGCGTTGTC ATTGAGCAGA TAGCGTGCCG 2080
PS22 TTCTGTACGC CGGCATTGTC ATTGAGCAAA TAGCGGGCAG 2080
PS77 TTTTGTACGC CCGCATTATC GTTCAGAAGA TAATGAGCTG 2061
Consensus TTCTGCACGC CGGCATTGTC GTTGAGCAAA TAGCGGGCAG
2.100
2.120
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200188/6 CGTAAACGTA CGACGTATCT GAATAGGCGA TCGCCTCGGT 2120
PS22 CGTAAACGTA TGACGTGTCC GAATAAGCGA TCGCCTCGGT 2120
PS77 AGTAAACGTA GGCTGTATCT GAATAAGCAA TGGCCTCGGC 2101
Consensus CGTAAACGTA CGACGTGTCC GAATAAGCGA TCGCCTCGGT
2.140
2.160
UMB248 CGGGTGCTTT GTCTCAAGGT ATCCCCAAAC CGGCTGATCC 2160
UMB247 CGGGTGCTTT GTCTCAAGGT ATCCCCAAAC CGGCTGATCC 2160
200188/6 CGGGTGCTTT GTCTCAAGGT ATCCCCAAAC CGGCTGATCC 2160
PS22 CGGGTGCTTT GTCTCAAGGT ATCCCCAAAC CGGCTGATCC 2160
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2.180
2.200
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PS22 GCCGTGCCCG GCATGAAGCT GAAACCGATC TGTGCCAACG 2200
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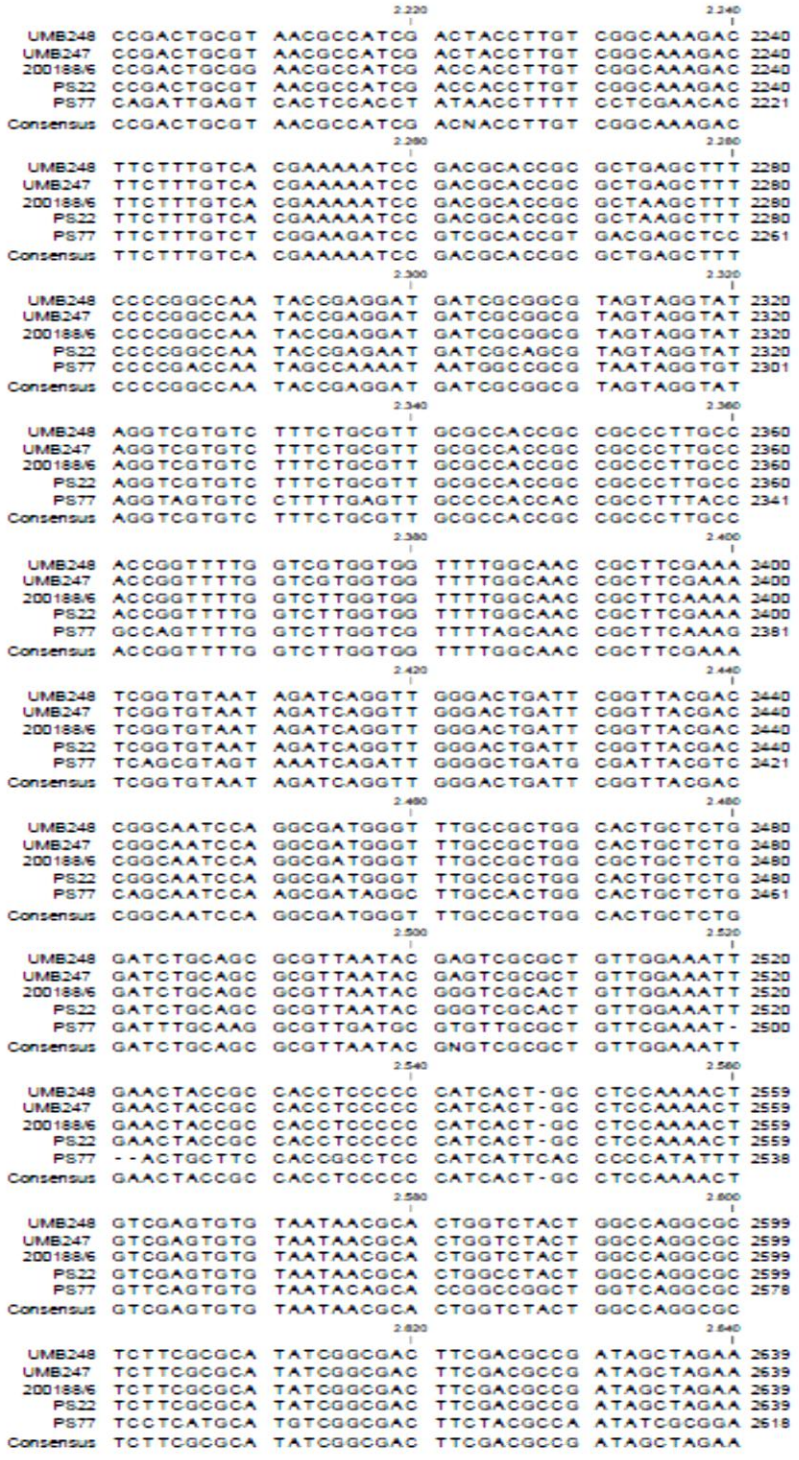
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PS77 CAGATTGAGT CACTCCACCT ATAACCTTTT CCTCGAACAC 2221
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2,280
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UMB247 TTCTTTGTCA CGAAAAATCC GACGCACCGC GCTGAGCTTT 2280
200188/6 TTCTTTGTCA CGAAAAATCC GACGCACCGC GCTAAGCTTT 2280
PS22 TTCTTTGTCA CGAAAAATCC GACGCACCGC GCTAAGCTTT 2280
PS77 TTCTTTGTCT CGGAAGATCC GTCGCACCGT GACGAGCTCC 2261
Consensus TTCTTTGTCA CGAAAAATCC GACGCACCGC GCTGAGCTTT
2,300
2,320
UMB248 CCCCGGCCAA TACCGAGGAT GATCGCGGCG TAGTAGGTAT 2320
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PS22 CCCCGGCCAA TACCGAGAAT GATCGCAGCG TAGTAGGTAT 2320
PS77 CCCCGACCAA TAGCCAAAAT AATGGCCGCG TAATAGGTGT 2301
Consensus CCCCGGCCAA TACCGAGGAT GATCGCGGCG TAGTAGGTAT
2,340
2,360
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200188/6 AGGTCGTGTC TTTCTGCGTT GCGCCACCGC CGCCCTTGCC 2360
PS22 AGGTCGTGTC TTTCTGCGTT GCGCCACCGC CGCCCTTGCC 2360
PS77 AGGTAGTGTC CTTTTGAGTT GCCCCACCAC CGCCTTTACC 2341
Consensus AGGTCGTGTC TTTCTGCGTT GCGCCACCGC CGCCCTTGCC
2,380
2,400
UMB248 ACCGGTTTTG GTCGTGGTGG TTTTGGCAAC CGCTTCGAAA 2400
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PS22 ACCGGTTTTG GTCTTGGTGG TTTTGGCAAC CGCTTCGAAA 2400
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2,420
2,440
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Consensus TCGGTGTAAT AGATCAGGTT GGGACTGATT CGGTTACGAC
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2,480
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PS22 CGGCAATCCA GGCGATGGGT TTGCCGCTGG CACTGCTCTG 2480
PS77 CAGCAATCCA AGCGATAGGC TTGCCACTGG CACTGCTCTG 2461
Consensus CGGCAATCCA GGCGATGGGT TTGCCGCTGG CACTGCTCTG
2,500
2,520
UMB248 GATCTGCAGC GCGTTAATAC GAGTCGCGCT GTTGGAAATT 2520
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PS22 GATCTGCAGC GCGTTAATAC GGGTCGCACT GTTGGAAATT 2520
PS77 GATTTGCAAG GCGTTGATGC GTGTTGCGCT GTTCGAAAT- 2500
Consensus GATCTGCAGC GCGTTAATAC GNGTCGCGCT GTTGGAAATT
2,540
2,560
UMB248 GAACTACCGC CACCTCCCCC CATCACT-GC CTCCAAAACT 2559
UMB247 GAACTACCGC CACCTCCCCC CATCACT-GC CTCCAAAACT 2559
200188/6 GAACTACCGC CACCTCCCCC CATCACT-GC CTCCAAAACT 2559
PS22 GAACTACCGC CACCTCCCCC CATCACT-GC CTCCAAAACT 2559
PS77 --ACTGCTTC CACCGCCTCC CATCATTCAC CCCCATATTT 2538
Consensus GAACTACCGC CACCTCCCCC CATCACT-GC CTCCAAAACT
2,580
2,600
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PS22 GTCGAGTGTG TAATAACGCA CTGGCCTACT GGCCAGGCGC 2599
PS77 GTTCAGTGTG TAATACAGCA CCGGCCGGCT GGTCAGGCGC 2578
Consensus GTCGAGTGTG TAATAACGCA CTGGTCTACT GGCCAGGCGC
2,620
2,640
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200188/6 TCTTCGCGCA TATCGGCGAC TTCGACGCCG ATAGCTAGAA 2639
PS22 TCTTCGCGCA TATCGGCGAC TTCGACGCCG ATAGCTAGAA 2639
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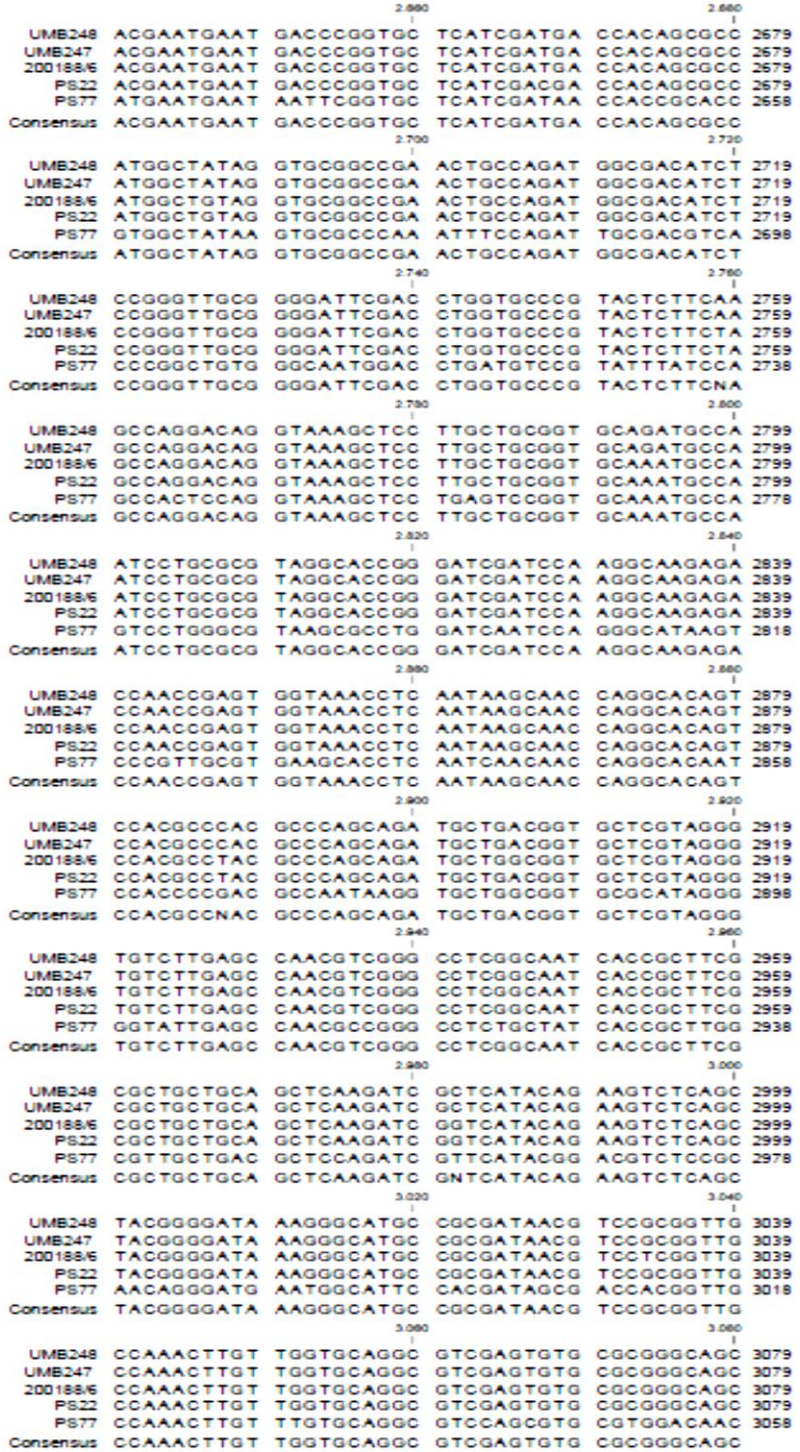
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2.680
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PS22 ACGAATGAAT GACCCGGTGC TCATCGACGA CCACAGCGCC 2679
PS77 ATGAATGAAT AATTCGGTGC TCATCGATAA CCACCGCACC 2658
Consensus ACGAATGAAT GACCCGGTGC TCATCGATGA CCACAGCGCC
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2.720
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PS22 ATGGCTGTAG GTGCGGCCGA ACTGCCAGAT GGCGACATCT 2719
PS77 GTGGCTATAA GTGCGCCCAA ATTTCCAGAT TGCGACGTCA 2698
Consensus ATGGCTATAG GTGCGGCCGA ACTGCCAGAT GGCGACATCT
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2.760
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PS22 CCGGGTTGCG GGGATTCGAC CTGGTGCCCG TACTCTTCTA 2759
PS77 CCCGGCTGTG GGCAATGGAC CTGATGTCCG TATTTATCCA 2738
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2.800
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200188/6 GCCAGGACAG GTAAAGCTCC TTGCTGCGGT GCAAATGCCA 2799
PS22 GCCAGGACAG GTAAAGCTCC TTGCTGCGGT GCAAATGCCA 2799
PS77 GCCACTCCAG GTAAAGCTCC TGAGTCCGGT GCAAATGCCA 2778
Consensus GCCAGGACAG GTAAAGCTCC TTGCTGCGGT GCAAATGCCA
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2.840
UMB248 ATCCTGCGCG TAGGCACCGG GATCGATCCA AGGCAAGAGA 2839
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PS22 ATCCTGCGCG TAGGCACCGG GATCGATCCA AGGCAAGAGA 2839
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Consensus ATCCTGCGCG TAGGCACCGG GATCGATCCA AGGCAAGAGA
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2.880
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2.920
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PS22 TGTCTTGAGC CAACGTCGGG CCTCGGCAAT CACCGCTTCG 2959
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PS22 CGCTGCTGCA GCTCAAGATC GGTCATACAG AAGTCTCAGC 2999
PS77 CGTTGCTGAC GCTCCAGATC GTTCATACGG ACGTCTCCGC 2978
Consensus CGCTGCTGCA GCTCAAGATC GNTCATACAG AAGTCTCAGC
3.020
3.040
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PS22 TACGGGGATA AAGGGCATGC CGCGATAACG TCCGCGGTTG 3039
PS77 AACAGGGATG AATGGCATTC CACGATAGCG ACCACGGTTG 3018
Consensus TACGGGGATA AAGGGCATGC CGCGATAACG TCCGCGGTTG
3.060
3.080
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200188/6 CCAAACTTGT TGGTGCAGGC GTCGAGTGTG CGCGGGCAGC 3079
PS22 CCAAACTTGT TGGTGCAGGC GTCGAGTGTG CGCGGGCAGC 3079
PS77 CCAAACTTGT TTGTGCAGGC GTCCAGCGTG CGTGGACAAC 3058
Consensus CCAAACTTGT TGGTGCAGGC GTCGAGTGTG CGCGGGCAGC

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                                    3,100                                3,120
                                      |                                    |
UMB248  CGGGGTAAAT  AAGGAACTGA  TCACCCACTT  GCAGCTCTGC 3119
UMB247  CGGGGTAAAT  AAGGAACTGA  TCACCCACTT  GCAGCTCTGC 3119
200188/6 CGGGGTAAAT  AAGGAACTGA  TCGCCCACTT  GCAGCTCTGC 3119
   PS22 CGGGGTAAAT  AAGGAACTGA  TCGCCCACTT  GCAGCTCTGC 3119
   PS77 CTGGGTAAAT  CAGGAACTGG  TCACCGGCTT  GCGGTAGTGC 3098
Consensus CGGGGTAAAT  AAGGAACTGA  TCACCCACTT  GCAGCTCTGC
                                    3,140                                3,160
                                      |                                    |
UMB248  CGGTAGCCCG  AGTATCAGAG  TGACGGCACC  GTCGGCCGTT 3159
UMB247  CGGTAGCCCG  AGTATCAGAG  TGACGGCACC  GTCGGCCGTT 3159
200188/6 CGGCAGGCCG  AGAATCAGAG  TGACGGCACC  GTCGGCCGTT 3159
   PS22 CGGCAGGCCG  AGAATCAGAG  TGACGGCACC  GTCGGCCGTT 3159
   PS77 AGGTAACCCG  AGGATCAGAC  TGATGGCGCC  ATCCCCTGCT 3138
Consensus CGGTAGCCCG  AGNATCAGAG  TGACGGCACC  GTCGGCCGTT
                                    3,180                                3,200
                                      |                                    |
UMB248  TGGCGACGAA  CAGTGCGCGA  AACACCAGCA  TTCCCGCCGT 3199
UMB247  TGGCGACGAA  CAGTGCGCGA  AACACCAGCA  TTCCCGCCGT 3199
200188/6 TGGCGACGAA  CAGTGCGCGA  AACACCAGCA  TTACCGCCGT 3199
   PS22 TGGCGACGAA  CAGTGCGCGA  AACACCAGCA  TTACCGCCGT 3199
   PS77 TGTCGCCGTA  CCGTACGCGA  CACGCCGGCA  TTACCGCCGT 3178
Consensus TGGCGACGAA  CAGTGCGCGA  AACACCAGCA  TTACCGCCGT
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                                      |                                    |
UMB248  TCACAAAGCG  AATCACACCC  TGATCAAACC  ACCCATTTTG 3239
UMB247  TCACAAAGCG  AATCACACCC  TGATCAAACC  ACCCATTTTG 3239
200188/6 TCACAAAGCG  GATTACGCCC  TGATCAAACC  ACCCATTTTG 3239
   PS22 TCACAAAGCG  GATCACGCCC  TGATCAAACC  ACCCATTTTG 3239
   PS77 TCACAAAGCG  AATCACGCCC  TGGTCAAACC  ATCCGTGCTC 3218
Consensus TCACAAAGCG  AATCACGCCC  TGATCAAACC  ACCCATTTTG
                                    3,260                                3,280
                                      |                                    |
UMB248  CGCGCCGATG  TTGGTGCGAA  TACGAAGGCC  GCTCGTGGAC 3279
UMB247  CGCGCCGATG  TTGGTGCGAA  TACGAAGGCC  GCTCGTGGAC 3279
200188/6 CGCGCCGATG  TTGGTGCGAA  TACGAAGGCC  GCTCGTGGAC 3279
   PS22 CGCGCCGATG  TTGGTGCGAA  TACGAAGGCC  GCTCGTGGAC 3279
   PS77 CGCCGTTACA  TCGGTGCGGA  TACTCAACGA  TGTAGTGCCA 3258
Consensus CGCGCCGATG  TTGGTGCGAA  TACGAAGGCC  GCTCGTGGAC
                                    3,300                                3,320
                                      |                                    |
UMB248  TCCAGCACCG  AACCCGCAGT  TTCGAACAAC  GAGCGATTCA 3319
UMB247  TCCAGCACCG  AACCCGCAGT  TTCGAACAAC  GAGCGATTCA 3319
200188/6 TCCAGCACCG  AACCCGCAGT  TTCGAACAAT  GAGCGATTCA 3319
   PS22 TCCAGCACCG  AACCCGCAGT  TTCGAACAAC  GAGCGATTCA 3319
   PS77 GCAAGCACAT  GCCCCGCCGT  CTCAAACAGG  GCACGGTTAA 3298
Consensus TCCAGCACCG  AACCCGCAGT  TTCGAACAAC  GAGCGATTCA
                                    3,340                                3,360
                                      |                                    |
UMB248  CACCGCAATC  GTCGCTATAA  ACAGTACGCA  GGCATCCTGG 3359
UMB247  CACCGCAATC  GTCGCTATAA  ACAGTACGCA  GGCATCCTGG 3359
200188/6 CACCGCAATC  GTCGCTATAA  ACAGTACGCA  GGCATCCTGG 3359
   PS22 CACCGCAATC  GTCGCTATAA  ACAGTACGCA  GGCATCCTGG 3359
   PS77 CACCACAATC  GGTGCTGTAC  ACGGTGCGTA  GGCAACCTGG 3338
Consensus CACCGCAATC  GTCGCTATAA  ACAGTACGCA  GGCATCCTGG
                                    3,380                                3,400
                                      |                                    |
UMB248  CTGATAGACC  CCTTTAGGCA  CCTTGGTATC  AAGCAGCTCC 3399
UMB247  CTGATAGACC  CCTTTAGGCA  CCTTGGTATC  AAGCAGCTCC 3399
200188/6 CTGATAGACC  CCTTTAGGCA  CCTTGGTATC  AAGCAGCTCC 3399
   PS22 CTGATAGACC  CCTTTAGGCA  CCTTGGTATC  AAGCAGCTCC 3399
   PS77 TTGAAAGACG  CCCTTGGGCA  CCTTGGTATC  CAGTAGCTCC 3378
Consensus CTGATAGACC  CCTTTAGGCA  CCTTGGTATC  AAGCAGCTCC
                                    3,420                                3,440
                                      |                                    |
UMB248  ATCGGCGACT  TGACCGAAAA  CGTCGCCTGC  TCGCGGTCGG 3439
UMB247  ATCGGCGACT  TGACCGAAAA  CGTCGCCTGC  TCGCGGTCGG 3439
200188/6 ATCGGCGATT  TGACCGAAAA  CGTCGCCTGC  TCGCGGTCGG 3439
   PS22 ATCGGCGATT  TGACCGAAAA  CGTCGCCTGC  TCGCGGTCGG 3439
   PS77 ATAGGGGACT  TAACAGCAAA  AGTTGCTTGT  TCGCGATCTG 3418
Consensus ATCGGCGACT  TGACCGAAAA  CGTCGCCTGC  TCGCGGTCGG
                                    3,460                                3,480
                                      |                                    |
UMB248  CAGGATCTAC  CTCGGCCACT  CGCCCAATAA  AGCGCAATAC 3479
UMB247  CAGGATCTAC  CTCGGCCACT  CGCCCAATAA  AGCGCAATAC 3479
200188/6 CAGGATCCAC  TTCGGCCACT  CGCCCAATGA  AGCGCAGTAC 3479
   PS22 CAGGATCCAC  TTCGGCCACT  CGCCCAATGA  AGCGCAGTAC 3479
   PS77 CGGGATCGAC  CTCGGCAACA  CGGCCGATGA  AGCGTGTAAC 3458
Consensus CAGGATCNAC  CTCGGCCACT  CGCCCAATGA  AGCGCANTAC
                                    3,500                                3,520
                                      |                                    |
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UMB247  AGTGCCGATC  ACAGGCGCAG  TCCAATCGGG  CATGAACGCC 3519
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   PS77 CGTCCCGACG  ACCGCCGCCC  CCCAATCAGG  CATGAACGCC 3498
Consensus AGTGCCGATC  ACAGGCGCAG  TCCAATCGGG  CATGAACGCC
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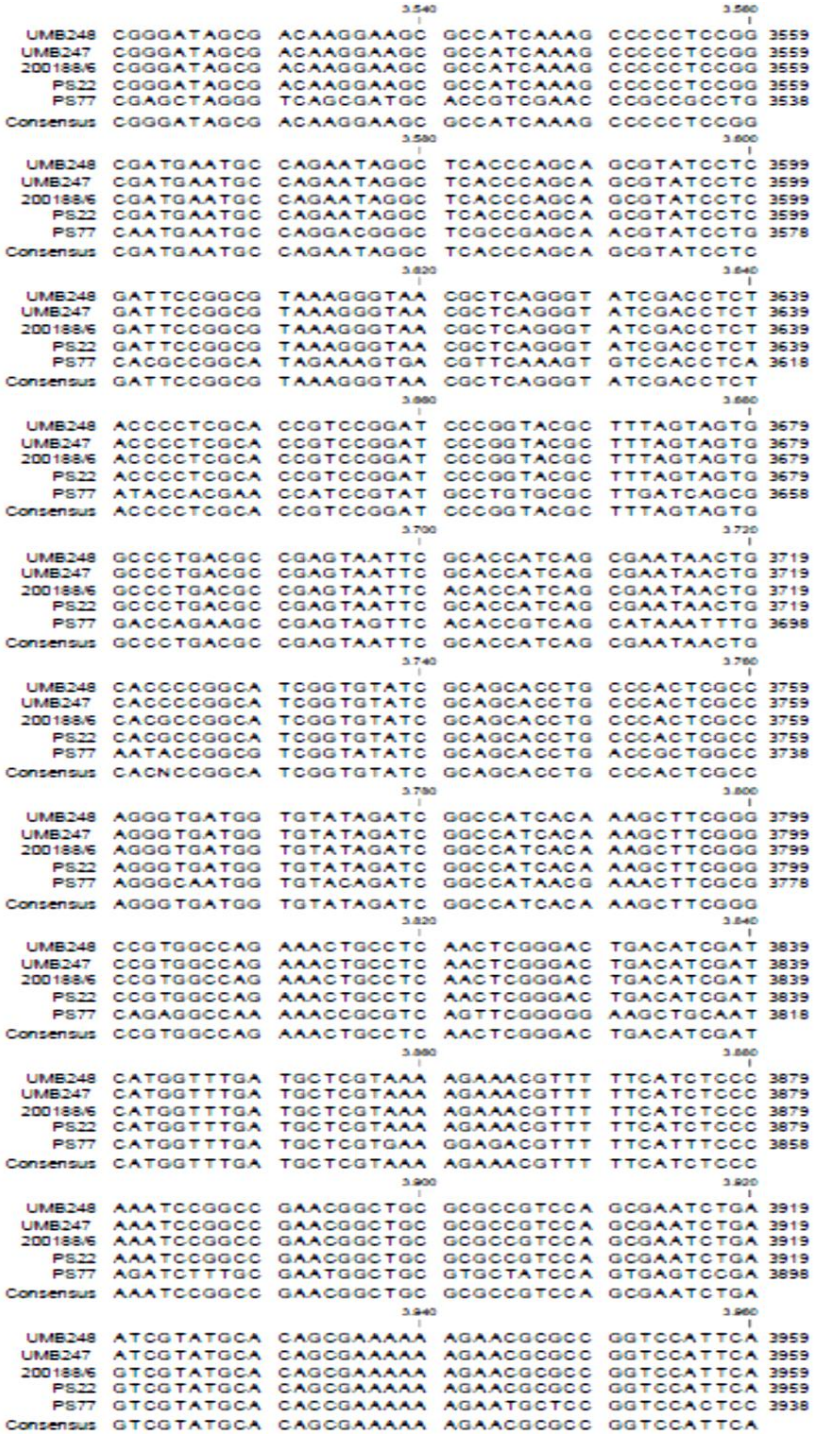
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PS77 CGAGCTAGGG TCAGCGATGC ACCGTCGAAC CCGCCGCCTG 3538
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3,580
3,600
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3,640
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200188/6 ACCCCTCGCA CCGTCCGGAT CCCGGTACGC TTTAGTAGTG 3679
PS22 ACCCCTCGCA CCGTCCGGAT CCCGGTACGC TTTAGTAGTG 3679
PS77 ATACCACGAA CCATCCGTAT GCCTGTGCGC TTGATCAGCG 3658
Consensus ACCCCTCGCA CCGTCCGGAT CCCGGTACGC TTTAGTAGTG
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3,720
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3,760
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3,840
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3,920
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PS22 GTCGTATGCA CAGCGAAAAA AGAACGCGCC GGTCCATTCA 3959
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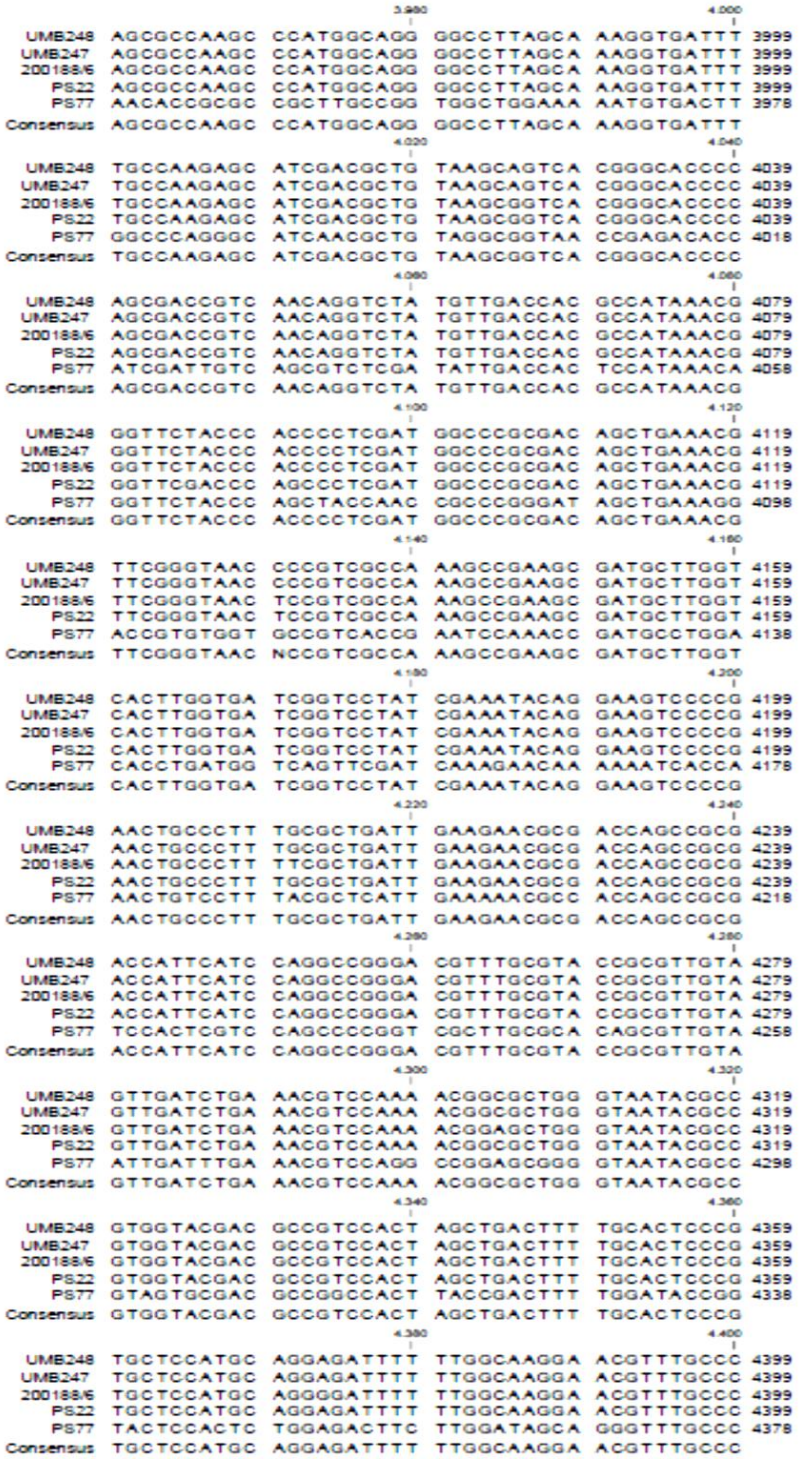
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PS22 GTTGATCTGA AACGTCCAAA ACGGCGCTGG GTAATACGCC 4319
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Consensus GTTGATCTGA AACGTCCAAA ACGGCGCTGG GTAATACGCC
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4,360
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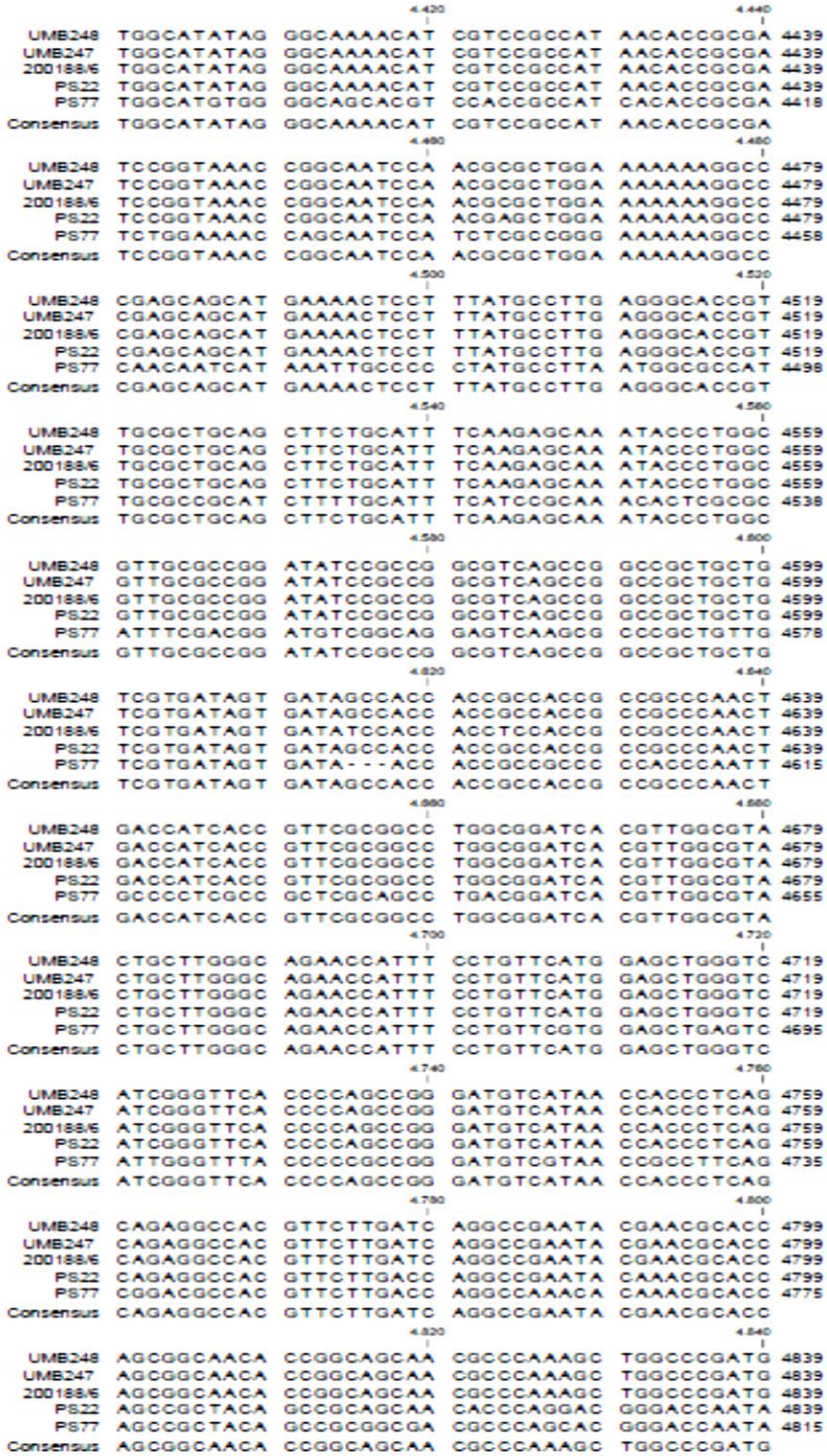
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PS22 TGCGCTGCAG CTTCTGCATT TCAAGAGCAA ATACCCTGGC 4559
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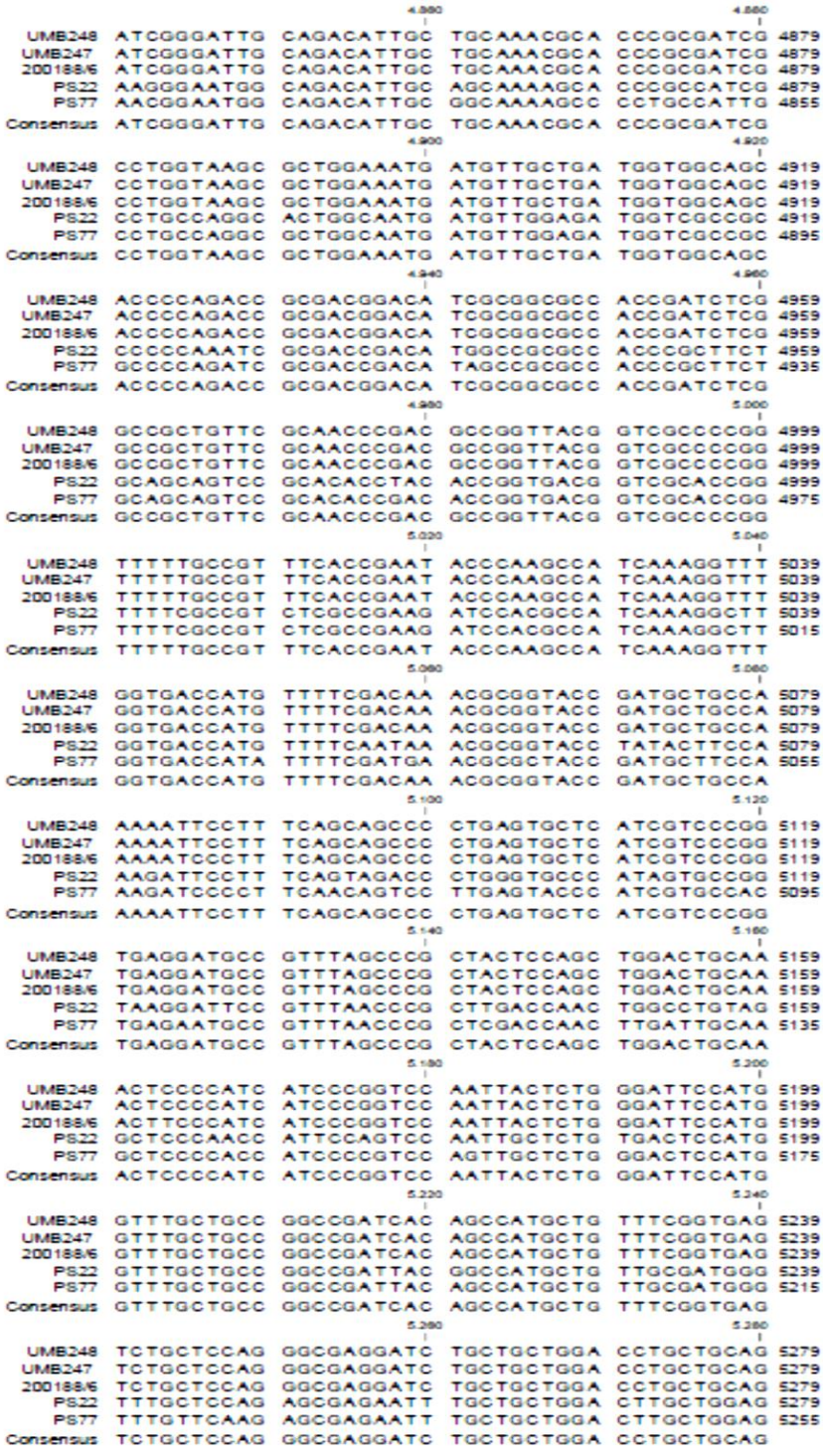
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PS77 GCAGCAGTCC GCACACCGAC ACCGGTGACG GTCGCACCGG 4975
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5,040
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5,160
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5,200
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5,240
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5,280
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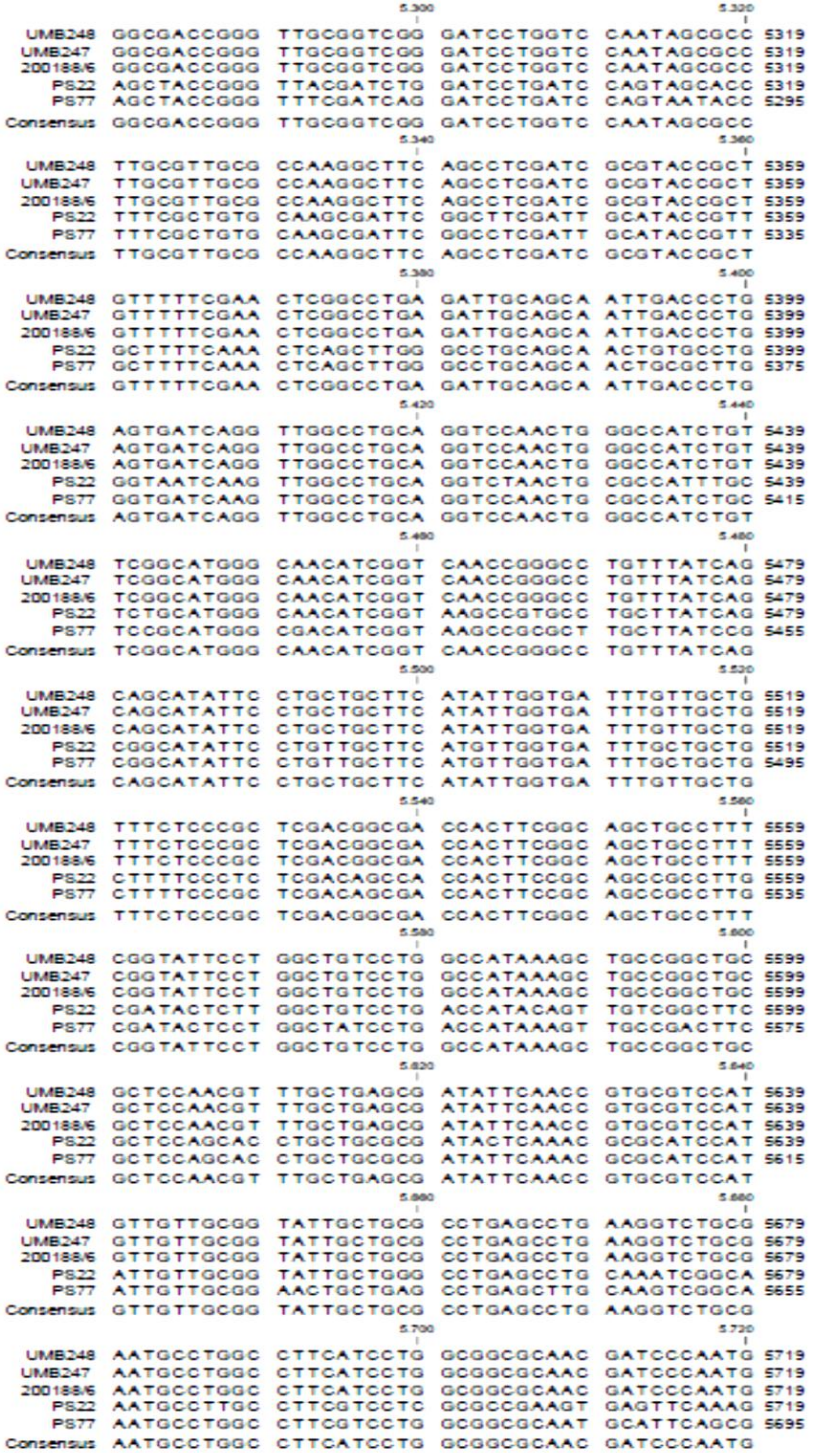
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5,340
5,360
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5,400
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5,480
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5,520
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5,560
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5,680
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5,700
5,720
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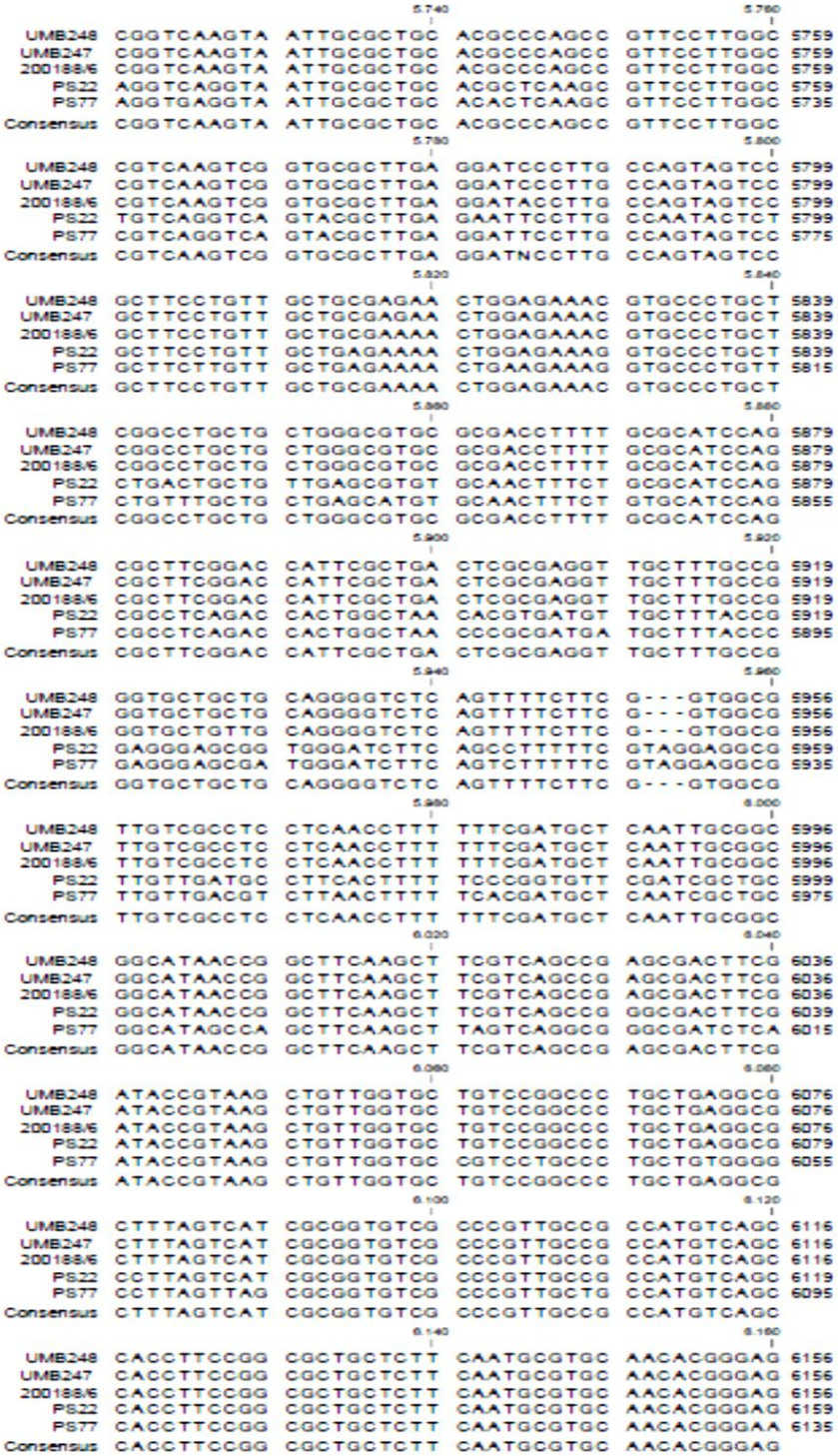
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5820 5840
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5940 5960
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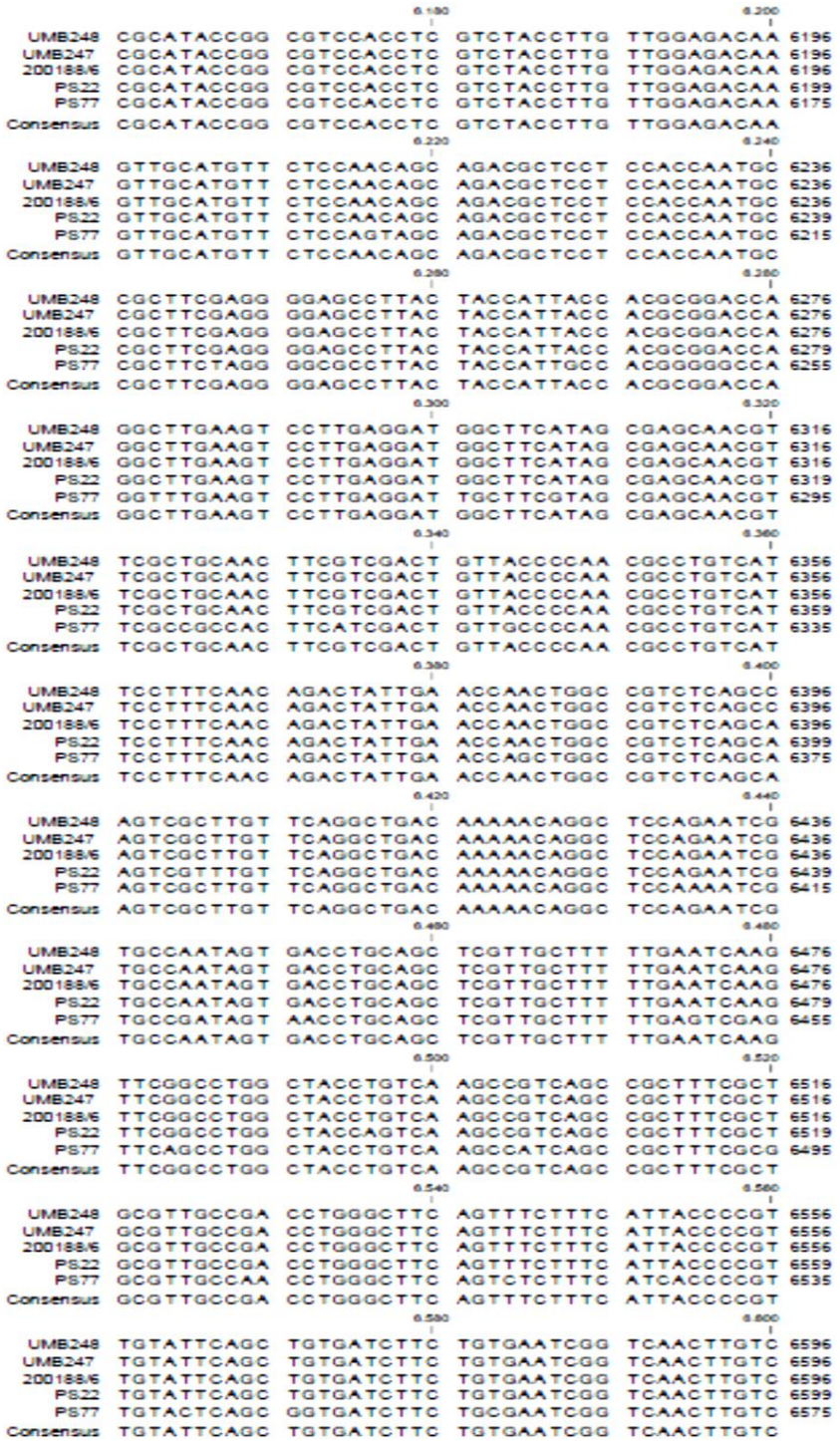
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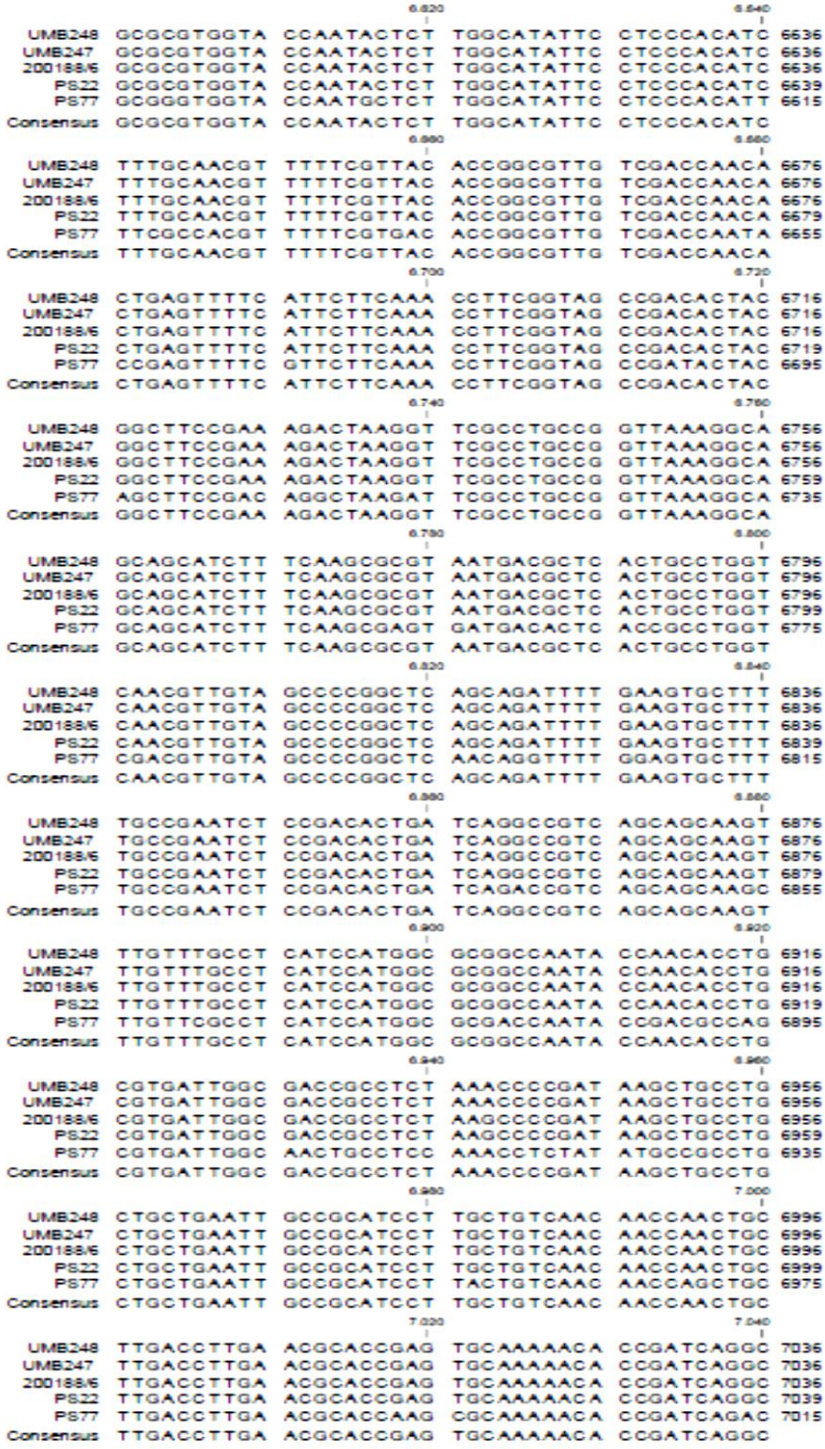
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6.800
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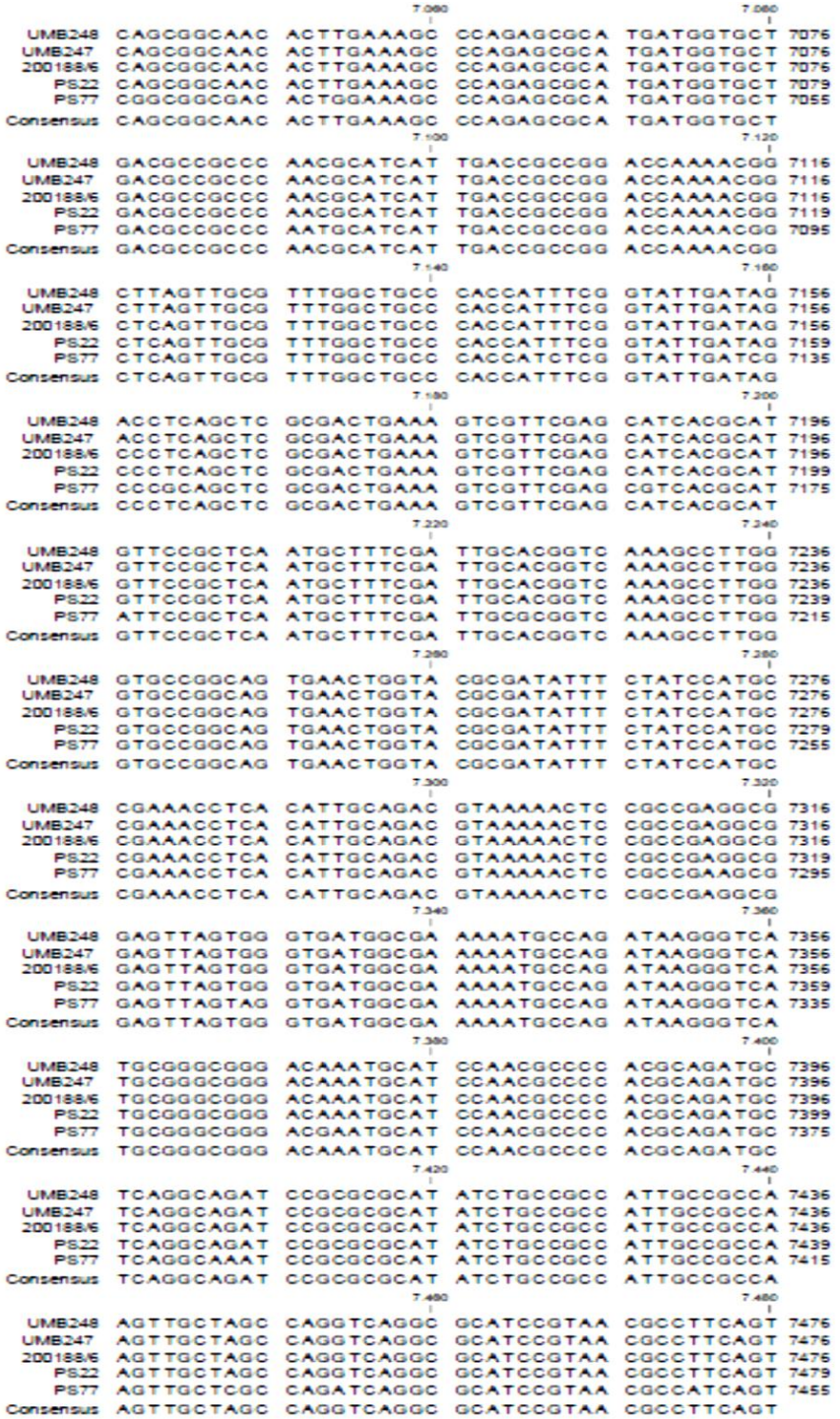
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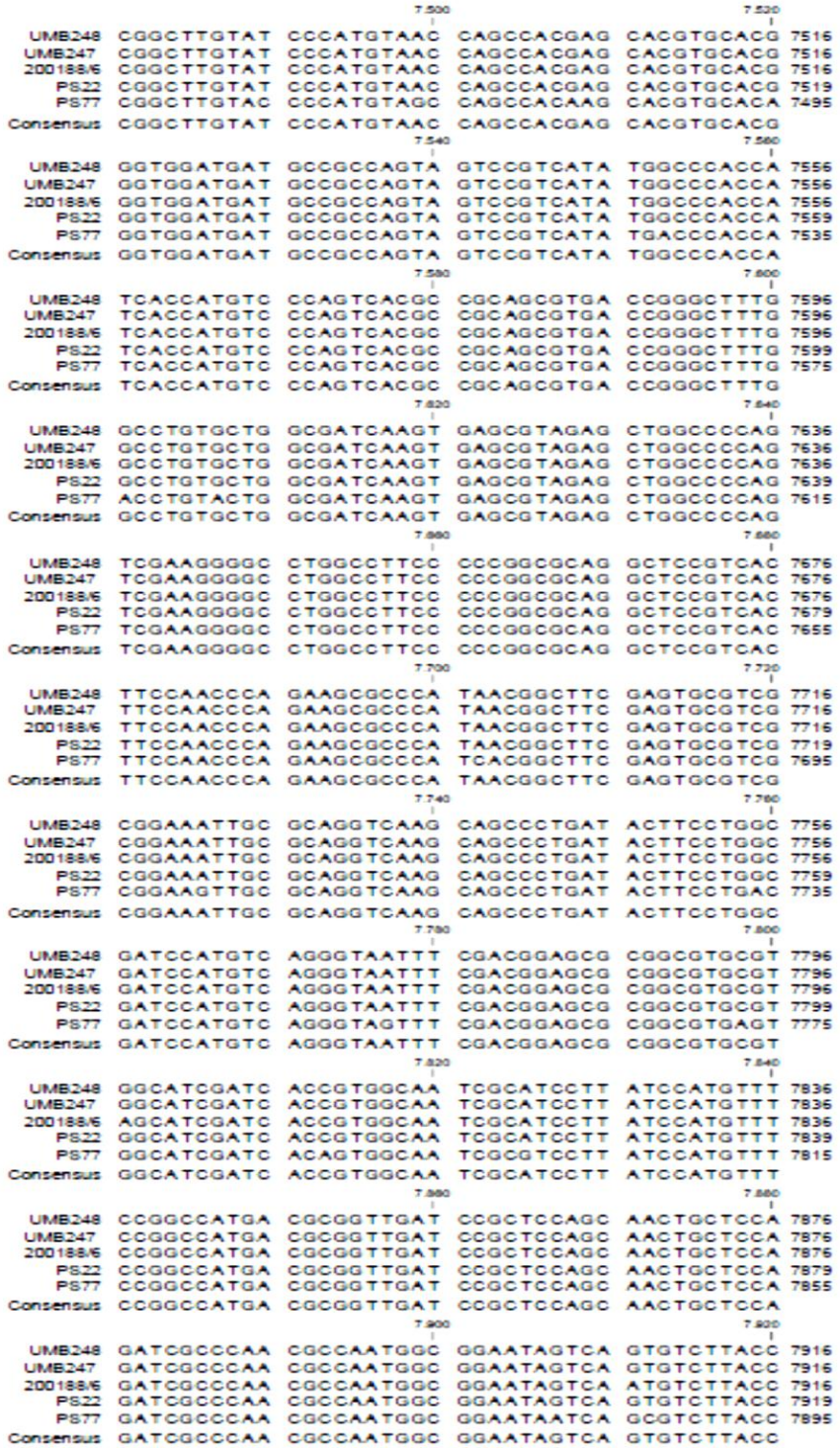
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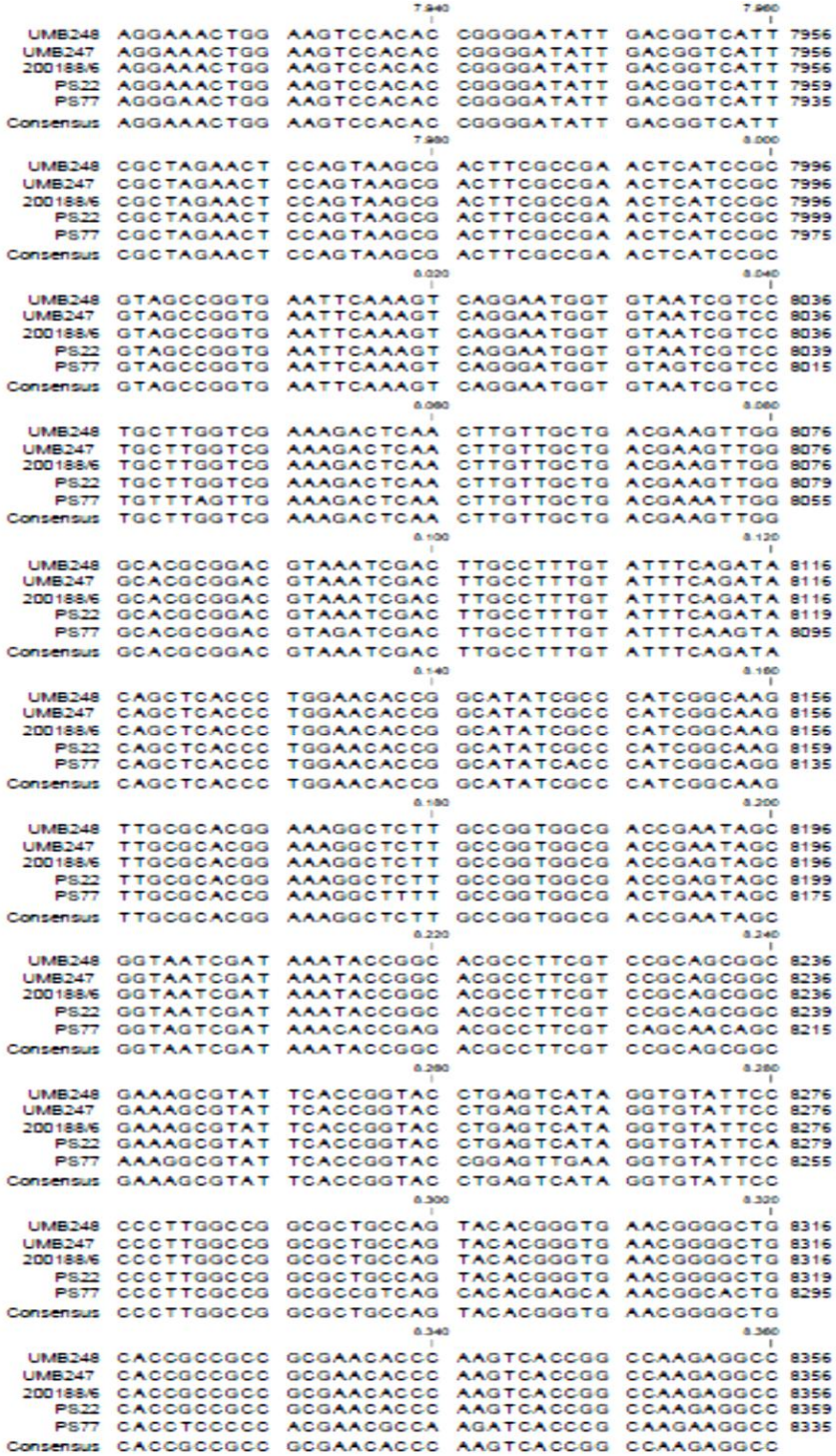
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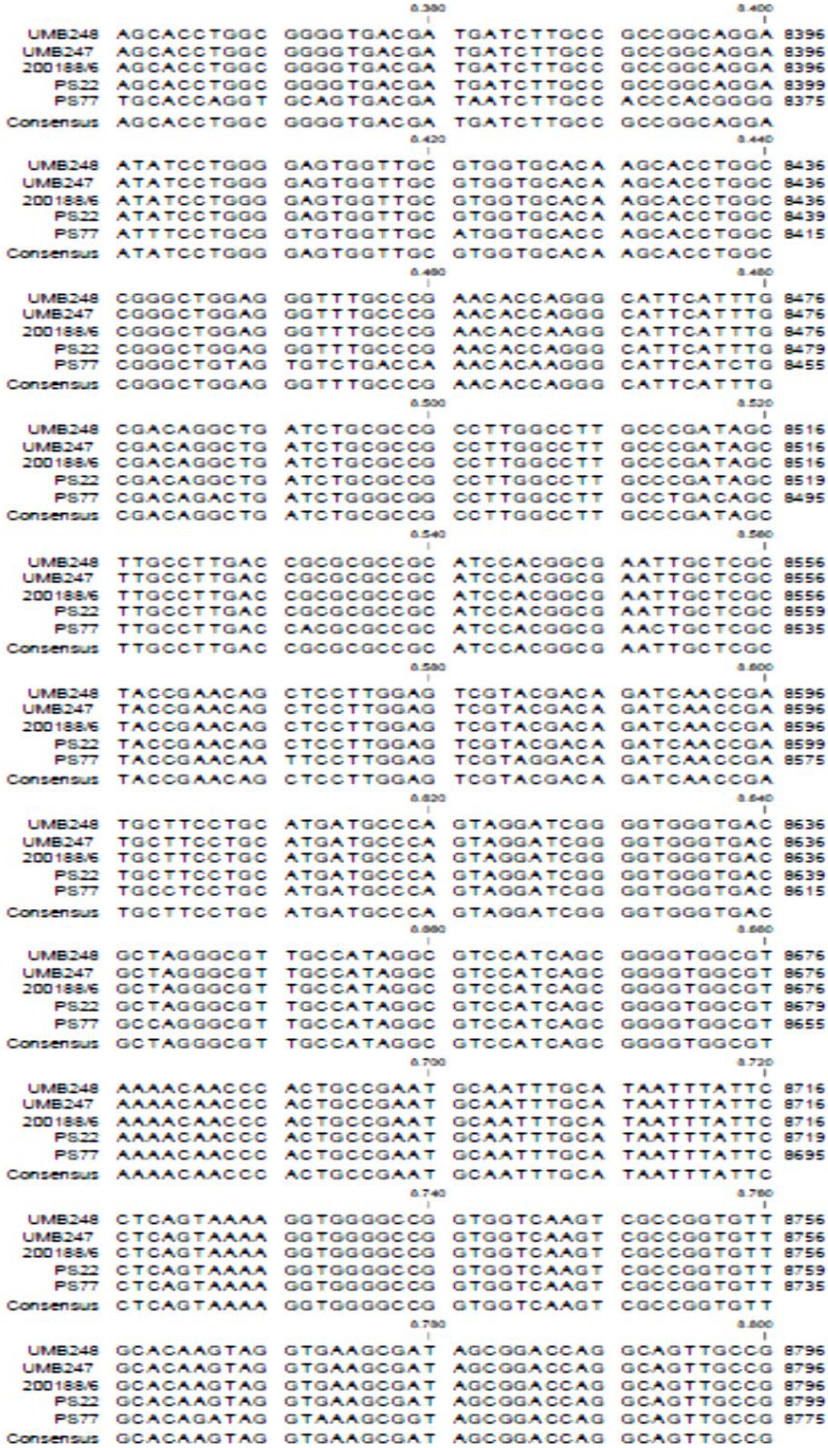
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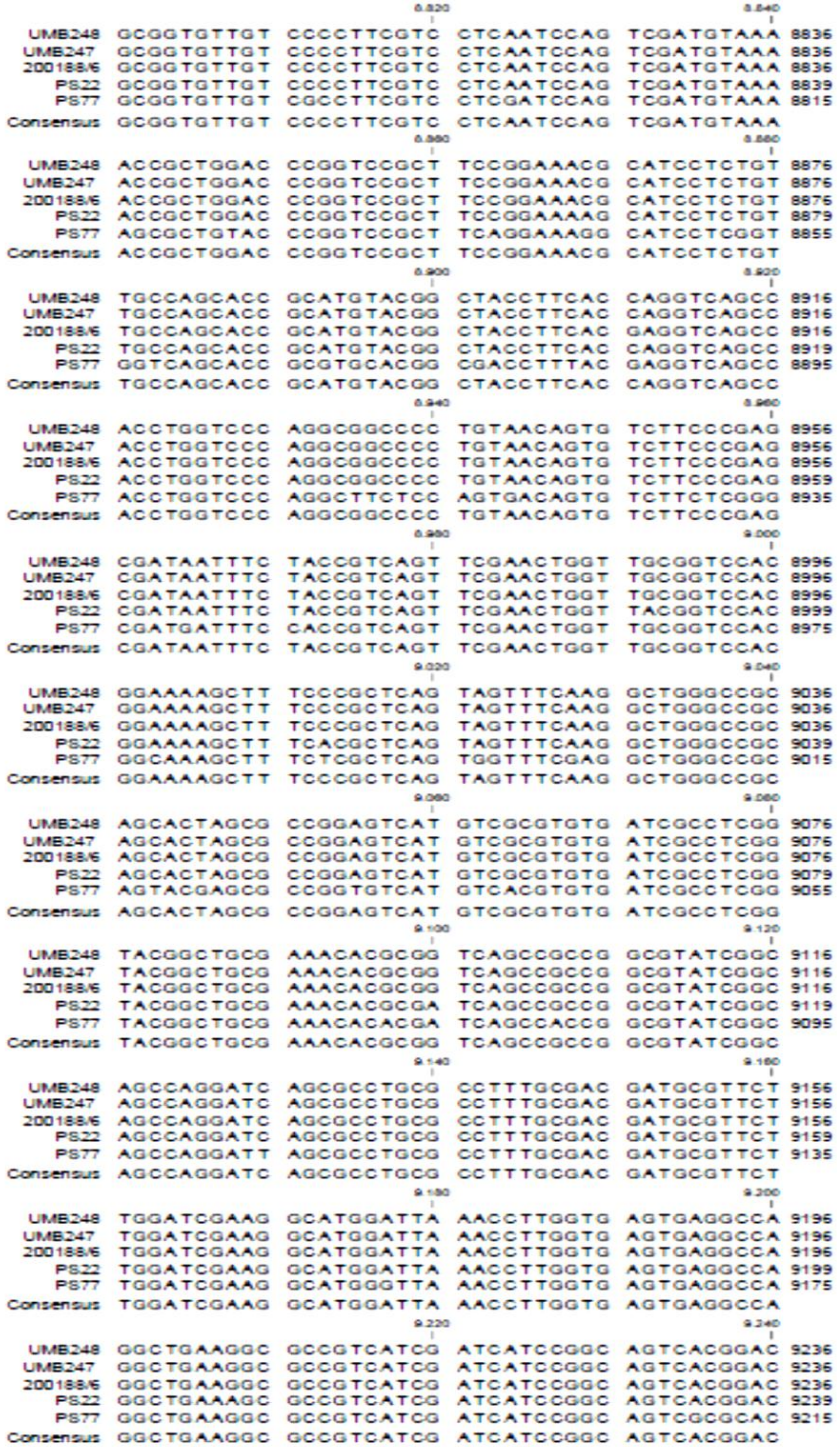
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8,880
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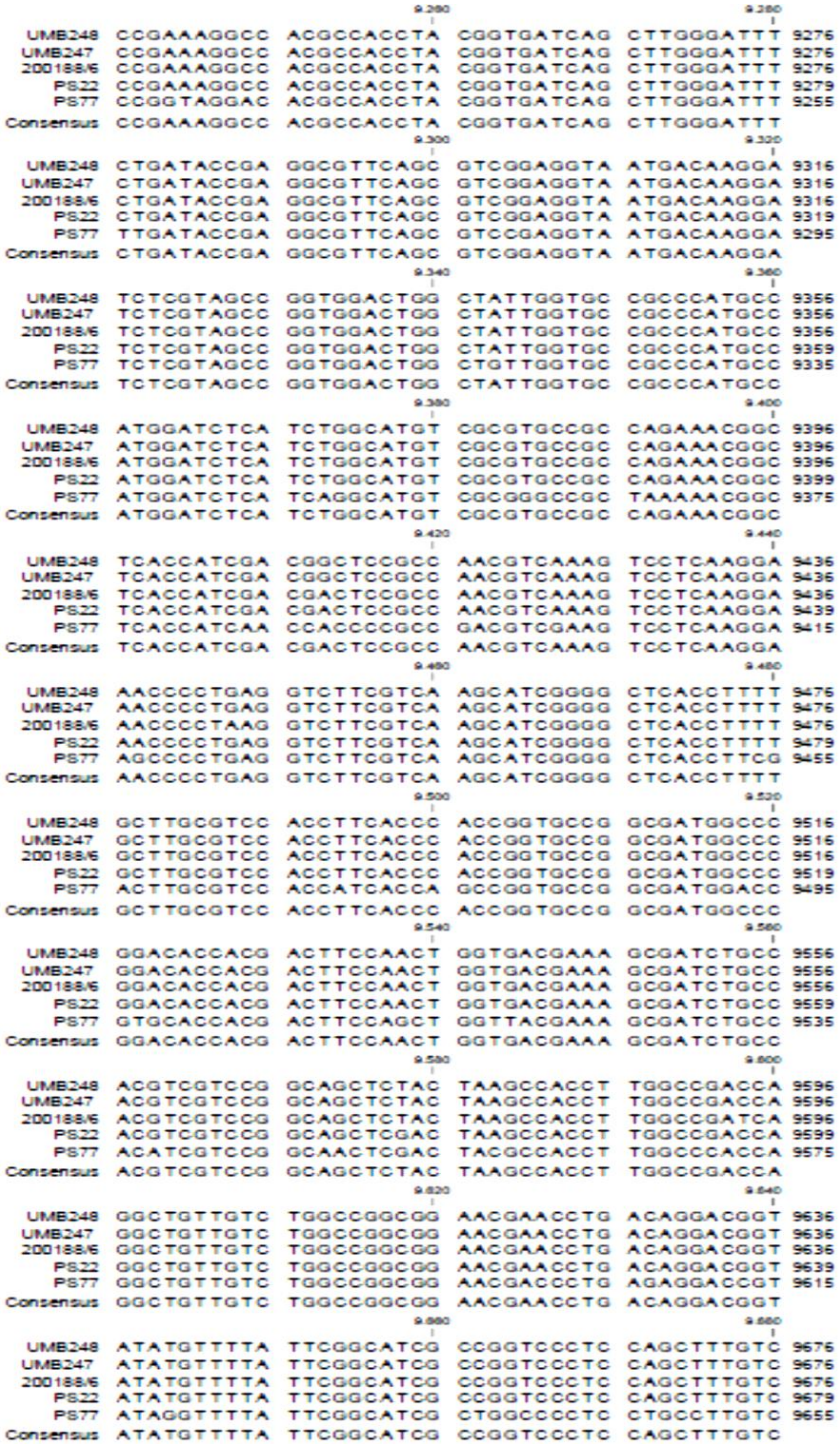
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Consensus GACCGGCTCA AGGCAGTGCT TTCGGGCGCT GGCCTGGGCG
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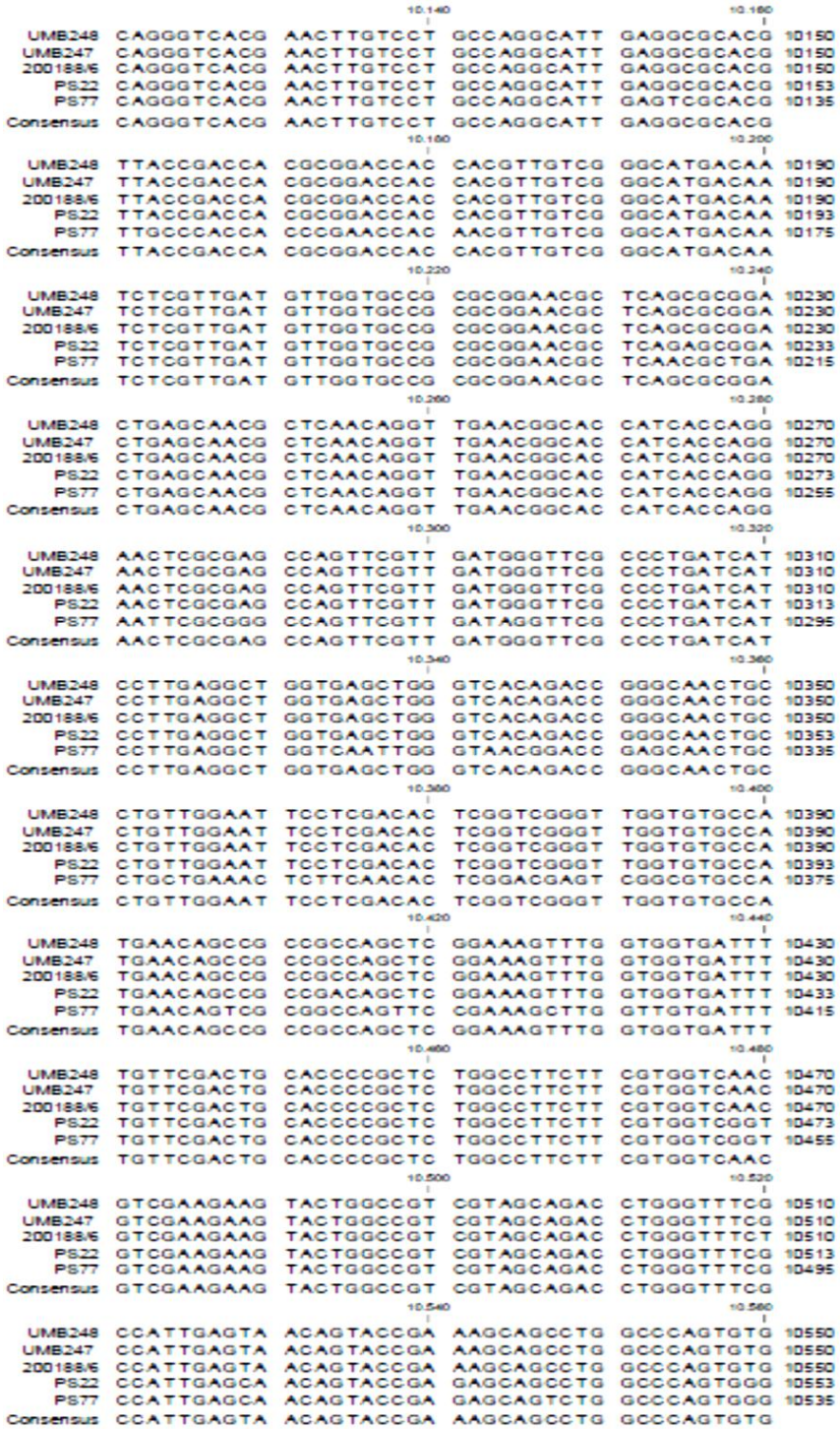
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10,320
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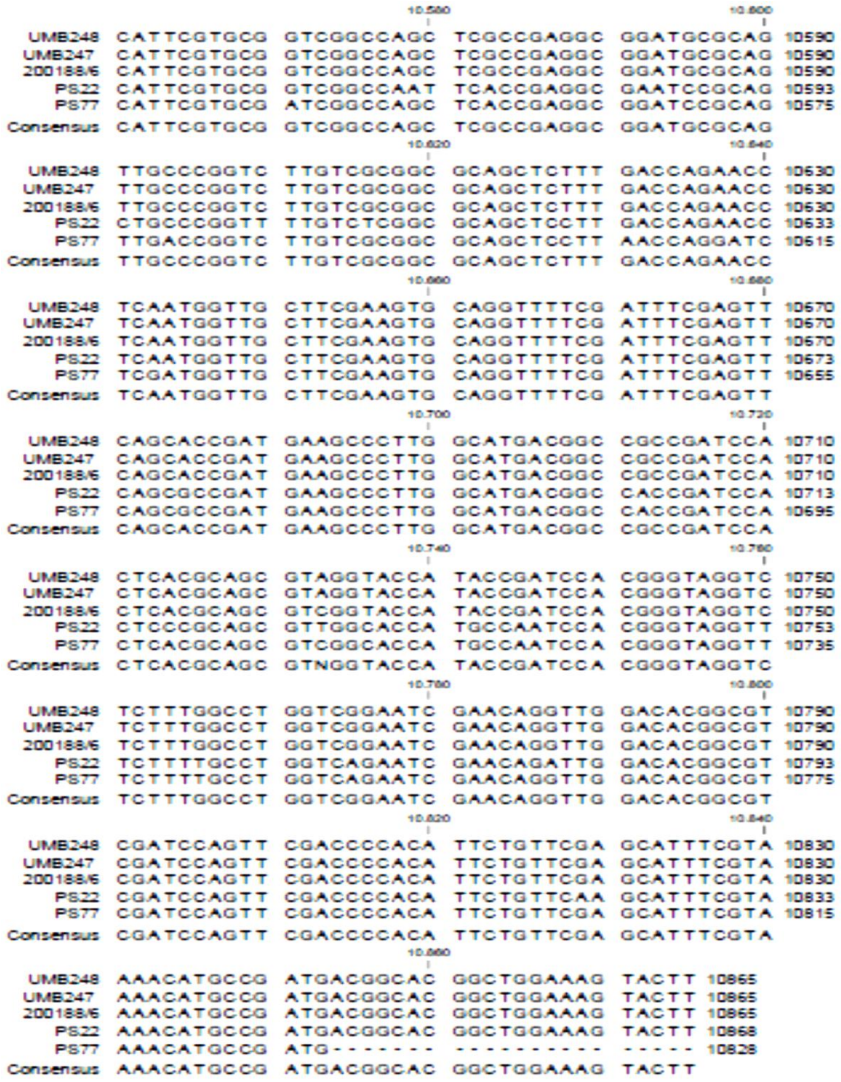
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10,600
UMB248 CATTCGTGCG GTCGGCCAGC TCGCCGAGGC GGATGCGCAG 10590
UMB247 CATTCGTGCG GTCGGCCAGC TCGCCGAGGC GGATGCGCAG 10590
200188/6 CATTCGTGCG GTCGGCCAGC TCGCCGAGGC GGATGCGCAG 10590
PS22 CATTCGTGCG GTCGGCCAAT TCACCGAGGC GAATCCGCAG 10593
PS77 CATTCGTGCG ATCGGCCAGC TCACCGAGGC GGATCCGCAG 10575
Consensus CATTCGTGCG GTCGGCCAGC TCGCCGAGGC GGATGCGCAG
10,620
10,640
UMB248 TTGCCCGGTC TTGTCGCGGC GCAGCTCTTT GACCAGAACC 10630
UMB247 TTGCCCGGTC TTGTCGCGGC GCAGCTCTTT GACCAGAACC 10630
200188/6 TTGCCCGGTC TTGTCGCGGC GCAGCTCTTT GACCAGAACC 10630
PS22 CTGCCCGGTT TTGTCTCGGC GCAGCTCCTT GACCAGAACC 10633
PS77 TTGACCGGTC TTGTCGCGGC GCAGCTCCTT AACCAGGATC 10615
Consensus TTGCCCGGTC TTGTCGCGGC GCAGCTCTTT GACCAGAACC
10,660
10,680
UMB248 TCAATGGTTG CTTCGAAGTG CAGGTTTTCG ATTTCGAGTT 10670
UMB247 TCAATGGTTG CTTCGAAGTG CAGGTTTTCG ATTTCGAGTT 10670
200188/6 TCAATGGTTG CTTCGAAGTG CAGGTTTTCG ATTTCGAGTT 10670
PS22 TCAATGGTTG CTTCGAAGTG CAGGTTTTCG ATTTCGAGTT 10673
PS77 TCGATGGTTG CTTCGAAGTG CAGGTTTTCG ATTTCGAGTT 10655
Consensus TCAATGGTTG CTTCGAAGTG CAGGTTTTCG ATTTCGAGTT
10,700
10,720
UMB248 CAGCACCGAT GAAGCCCTTG GCATGACGGC CGCCGATCCA 10710
UMB247 CAGCACCGAT GAAGCCCTTG GCATGACGGC CGCCGATCCA 10710
200188/6 CAGCACCGAT GAAGCCCTTG GCATGACGGC CGCCGATCCA 10710
PS22 CAGCGCCGAT GAAGCCCTTG GCATGACGGC CACCGATCCA 10713
PS77 CAGCGCCGAT GAAGCCCTTG GCATGACGGC CACCGATCCA 10695
Consensus CAGCACCGAT GAAGCCCTTG GCATGACGGC CGCCGATCCA
10,740
10,760
UMB248 CTCACGCAGC GTAGGTACCA TACCGATCCA CGGGTAGGTC 10750
UMB247 CTCACGCAGC GTAGGTACCA TACCGATCCA CGGGTAGGTC 10750
200188/6 CTCACGCAGC GTCGGTACCA TACCGATCCA CGGGTAGGTC 10750
PS22 CTCCCGCAGC GTTGGCACCA TGCCAATCCA CGGGTAGGTT 10753
PS77 CTCACGCAGC GTCGGCACCA TGCCAATCCA CGGGTAGGTT 10735
Consensus CTCACGCAGC GTNGGTACCA TACCGATCCA CGGGTAGGTC
10,780
10,800
UMB248 TCTTTGGCCT GGTCGGAATC GAACAGGTTG GACACGGCGT 10790
UMB247 TCTTTGGCCT GGTCGGAATC GAACAGGTTG GACACGGCGT 10790
200188/6 TCTTTGGCCT GGTCGGAATC GAACAGGTTG GACACGGCGT 10790
PS22 TCTTTTGCCT GGTCAGAATC GAACAGATTG GACACGGCGT 10793
PS77 TCTTTTGCCT GGTCAGAATC GAACAGGTTG GACACGGCGT 10775
Consensus TCTTTGGCCT GGTCGGAATC GAACAGGTTG GACACGGCGT
10,820
10,840
UMB248 CGATCCAGTT CGACCCCACA TTCTGTTCGA GCATTTCGTA 10830
UMB247 CGATCCAGTT CGACCCCACA TTCTGTTCGA GCATTTCGTA 10830
200188/6 CGATCCAGTT CGACCCCACA TTCTGTTCGA GCATTTCGTA 10830
PS22 CGATCCAGTT CGACCCCACA TTCTGTTCAA GCATTTCGTA 10833
PS77 CGATCCAGTT CGACCCCACA TTCTGTTCGA GCATTTCGTA 10815
Consensus CGATCCAGTT CGACCCCACA TTCTGTTCGA GCATTTCGTA
10,860
UMB248 AAACATGCCG ATGACGGCAC GGCTGGAAAG TACTT 10865
UMB247 AAACATGCCG ATGACGGCAC GGCTGGAAAG TACTT 10865
200188/6 AAACATGCCG ATGACGGCAC GGCTGGAAAG TACTT 10865
PS22 AAACATGCCG ATGACGGCAC GGCTGGAAAG TACTT 10868
PS77 AAACATGCCG ATG------- ---------- ----- 10828
Consensus AAACATGCCG ATGACGGCAC GGCTGGAAAG TACTT