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Transposon mutagenesis in Pseudomonas fluorescens reveals genes involved in blue pigment production and antioxidant protection.

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ABSTRACT

Pseudomonas fluorescens Ps_77 is a blue-pigmenting strain able to cause food product discoloration, causing relevant economic losses especially in the dairy industry. Unlike non-pigmenting *P. fluorescens,* blue pigmenting strains previously were shown to carry a genomic region that includes homologs of *trpABCDF* genes, pointing at a possible role of the tryptophan biosynthetic pathway in production of the pigment. Here, we employ random mutagenesis to first identify the genes involved in blue-pigment production in *P. fluorescens* Ps_77 and second to investigate the biological function of the blue pigment. Genetic analyses based on the mapping of the random insertions allowed the identification of eight genes involved in pigment production, including the second copy of *trpB* (*trpB_1*) gene. Phenotypic characterization of Ps_77 white mutants demonstrated that the blue pigment increases oxidative-stress resistance. Indeed, while Ps_77 was growing at a normal rate in presence of 5 mM of H_2O_2, white mutants were completely inhibited. The antioxidative protection is not available for non-producing bacteria in co-culture with Ps_77.

1. Introduction

Pseudomonas fluorescens is a well-known food spoiler and, although it is not commonly considered a human pathogen, it is an important species in public hygiene and food industry (Scales et al., 2014). The interest in food spoiling *P. fluorescens* has increased since 2010, when some packages of mozzarella were seized after a blue discolouration was noticed on the external surface of the cheese (RASFF, Annual Report, 2010). The strains responsible for the discolouration were identified as belonging to a specific genetic cluster of the *P. fluorescens* group (Andreani et al., 2014). This cluster, called the "blue branch", was demonstrated to contain all the blue-pigmenting strains as well as some unpigmented strains. Recent characterization of other strains producing the blue discoloration confirmed their monophyletic origin (Chierici et al., 2016).

The chemical nature of the blue pigment has been investigated, but no clear answer has been obtained yet. Based on a MALDI-TOF Mass spectrometry analysis, Andreani et al. (2015) suggested that the blue pigment may be an indigo-derivative. This result was also supported by a genomic investigation that revealed the exclusive presence of homologs of genes involved in tryptophan production in the genome of "blue branch" strains (Andreani et al., 2015); indeed, tryptophan has been reported to be involved in indole and indigo production in other bacterial species (Berry et al., 2002). The second copies of *trp* genes are included in a cluster of sixteen genes exclusive to the blue strains, identified in contig 4 of the genome of Ps_77 and thus called c4_BAR (Contig 4 Blue Accessory Region; Andreani et al., 2015).

The complete biosynthetic pathway, as well as the biological function of pigment production remain an open question. Several bacterial pigments, including *Pseudomonas* pigments, have a function as siderophore (Cornelis, 2010). Indeed, a previous study suggested a role for the blue pigment in iron metabolism (Andreani et al., 2015). However, some bacterial pigments have a role in oxidative stress resistance: for example, melanin produced by *Psedomonas aeruginosa* confers resistance to oxidative stress (Rodríguez-Rojas et al., 2009) and this was also reported for the indigo pigment produced by *Pseudomonas* sp. HAV-1 (Dua et al., 2014) and for the phenazine pigment in *Pseudomonas chlororaphis* GP72 (Xie et al., 2013).

In this study, we employ transposon mutagenesis (Goryshin et al., 2000) and characterize the white mutants to investigate the biosynthesis and the biological function of the blue pigment in strain Ps_77.

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2. Materials and methods

2.1. Transposon mutagenesis and identification of Tn5-flanking sequences of selected mutants

Detailed genomic and phenotypic information of Ps_77 are already available (Andreani et al., 2015, LCYB00000000; SAMN03085510; GCF_001542705.1). The wild-type strain and transposon-induced mutants were cultured and maintained in Luria Bertani Broth (LB; Sigma-Aldrich) or in Tryptic Soy Broth (TSB, Conda) and stored at −80 °C in LB Broth with 50% v/v glycerol (Sigma-Aldrich). Where appropriate, 50 ng/μL kanamycin sulfate (Sigma-Aldrich) was supplemented to the medium.

Transposon mutagenesis of Ps_77 was carried out with EZ-Tn5™ Tnp Transposome™ (Epicentre, (an Illumina Company)©). Electrocompetent cells were prepared as reported by Choi et al. (2006). Cells were electroporated in an electroporation cuvette (ThermoScientific) with an Eporator (Eppendorf; 25 Uf, 200 Ω, 2.5 kV). Mutagenesis of Ps_77 was performed twice to enhance the number of mutants losing the ability of producing the blue pigment. Screening of kanamycin sulfate resistant (Kan^R) mutants was performed on Minimal Bacterial Medium Agar (MBM Agar, 7 g/L K_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L tri-sodium citrate, 0.1 g/L $MgSO_4$, 1 g/L $(NH_4)_2SO_4$, 2 g/L glucose and 15 g/L agar; Boles et al., 2004) with 50 ng/μL kanamycin sulfate as on this medium the production of the dark blue pigment is more evident than in complete media. Kan^R mutants were picked based on the pigmentation and streaked three times on MBM Agar with 50 ng/μL kanamycin sulfate at 28 °C to check the phenotype. As a reaction to Kovac's reagent (isoamyl alcohol, para-Dimethylaminobenzaldehyde, concentrated hydrochloric acid; Sigma-Aldrich) was reported for blue pigmenting strains (Andreani et al., 2015), phenotype was checked by striking suspected white colonies on one drop of this reagent. All selected Kan^R mutants were grown in 5 mL of LB Broth with 50 ng/μL kanamycin sulfate and incubated overnight at 28 °C. Genomic DNA of selected strains was extracted using FastDNA™ SPIN Kit (MP BIOMEDICALS) and sequenced as described in Karlyshev et al. (2000) and Walterson et al. (2014). The PCR products were sent to Macrogen Inc. (Amsterdam, the Netherlands) for direct Sanger sequencing with the upstream primer (KAN-2 FP-3) or the downstream primer (KAN-2 RP-3). All the sequences were checked for quality and edited with FinchTV 1.4.0 software (Geospiza). The sequences of the primers are reported in Table 1S.

2.2. Bioinformatic analyses of disrupted genes

Insertion sequences were queried against NCBI using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). HMMER was applied with the aim to highlight protein homology of the query against Reference proteomes or SwissProt (Finn et al., 2015). SIM (Local Similarity Program; Huang and Miller, 1991) was applied to align amino acidic sequences. Additionally, sequences were queried against Ps_77 (LCYB00000000) through the Bioedit Blast Tool using the draft genome as database (Tippmann, 2004). Identification of COG (Cluster of Orthologous) was performed using KOALA (KEGG Orthology and Links Annotation; Kanehisa et al., 2015). Expression of the genes identified with the mutagenesis was evaluated using transcriptomic data obtained in a previous work (Andreani et al., 2015; SRR1725678; SRR1725679; SRR1725680; SRR1725681; SRR1725682; SRR1725683; SRR1725684; SRR1725723). The orthologues of c4_BAR proteins were identified using STRING v10 (http://string-db.org/, Szklarczyk et al., 2015) and the reconstruction of the genomic region in each species was performed using Biocyc (https://biocyc.org).

2.3. Evaluation of the transcription units of the contig_4 blue accessory region

c4_BAR contains sixteen genes, of which the first fourteen, including *trp* genes, have the same orientation. The evaluation of the transcription units within the fourteen genes of the c4_BAR was evaluated through PCR analysis. Thirteen primer pairs were designed using as template the sequence of Ps_77 (LCYB00000000) between 3′ and 5' of each couple of consecutive genes. Primers are reported in Table 1S. RNA was extracted and retrotranscribed as described in Andreani et al. (2015). PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler in a final volume of 20 μL of amplification mix containing 1U of GoTaq polymerase (Promega, Madison, WI), 1× GoTaq Buffer, 1.5 mM MgCl2, 0.2 mM each deoxynucleotide triphosphate (dNTP), 250 mM each primer and 1 μL of cDNA as template. The PCR cycle was 94 °C for 5 min and 35 cycles each 94 °C for 30 s, 58 °C for 30 s and extension at 72 °C for 30 s. Amplified products were analysed by electrophoresis on 1.8% agarose-Tris-acetate-EDTA (TAE) gels, stained with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) and visualized on a UV transilluminator (Gel Doc XRTM, Biorad).

2.4. Growth assays

With the aim to investigate the biological function of the blue pigment, the phenotypic characterization of Ps_77 and its transposon insertion mutants was performed. All strains were grown overnight in LB broth at 28 °C in continuous shaking to reach an approximate count of 10^8 CFU/mL. Cultures were diluted to 10^3 CFU/mL in the final medium used for the assay to remove traces of LB broth for all the following phenotypic tests. All the tests were performed in triplicate. Growth curves were obtained using a plate reader (Multiskan Series Microplate Readers, Thermo Fisher Scientific), incubating the strains in MBM in a 96-well plate in continuous shaking at 22 °C and reading the optical density (O.D.) at 600 nm. Growth rate was calculated using DMFit tool of ComBase, an online tool allowing the shape of microbial growth curve, based on the Gompertz and Baranyi modified equation (Baranyi and Tamplin, 2004; Mytilinaios et al., 2012). Even if the model was initially created for the investigation of growth data in log CFU format, recent investigations revealed its applicability also to optical density-based growth curve (Mytilinaios et al., 2012; Rickett et al., 2015).

To assay whether the wild-type Ps_77 and four white mutant strains were able to use different carbon sources, a Biolog GN2 Micro Plate was used for each strain. Strains were grown overnight at 22 °C in LB broth and a 5-fold dilution was pipetted in each of the 96-wells of the Biolog GN2 plate. Biolog GN2 Micro Plates were incubated at 22 °C and bacterial growth was evaluated after 24 h by measuring the O.D. at 600 nm with a plate reader. Growth of the wild-type and the selected mutants was evaluated as difference of the OD 600 nm of the strains in the control well (water).

Growth assays were carried out in King's medium B broth (KB; 20 g/L of proteose peptone, Difco, 1,5 g/L of K_2HPO_4, 1,5 g/L of $MgSO_4 \cdot 7H_20$, 10 mL/L of glycerol) with 100 μg/mL human apo-transferrin (Sigma), a natural iron chelator, and 20 mM sodium bicarbonate, necessary for iron chelation by apotransferrin (Inglis et al., 2012) to obtain an iron-free environment. The production of the siderophore pyoverdine by the mutants was investigate after growth on MBM medium plates visualizing the fluorescence with a UV lamp (365 nm).

To evaluate stress resistance, the growth of clones was measured in MBM at several concentration of hydrogen peroxide (H_2O_2 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mM), NaCl (2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5%), different pH values (2.0, 2.5, 3.0, 3.5, 4.0 and 4.5) and after incubation of 2 h at 55 °C and 4 °C. Growth curves were obtained using a plate reader (Multiskan Series Microplate Readers, Thermo Fisher Scientific), incubating the 96-well plate in continuous shaking at 22 °C and reading the O.D. at 600 nm. The effect of hydrogen peroxide was evaluated also in a complete medium (TSB).

All the statistical analysis was performed using Prism Graphpad software. The non-parametric Wilcoxson test was applied to compare the growth rates of the strains in all the tested conditions. The paired t-test was applied to compare the growth after 24 h.

2.5. Competition experiment

Ps_77 and M3 were grown separately in TSB until an OD600nm of 0.2 and then diluted 20-fold and mixed together (in a 1:1 ratio) in MBM at 2.5 and 5 mM concentration of H_2O_2. Competitions were set up in a 96-well microplate and incubated in continuous shaking at 22 °C. 100 μL of co-coltures 10-fold diluted in MBM broth were plated in MBM agar after 24 h of incubation. Blue and white colonies were counted after 4 days of incubation at 22 °C and paired t-test was applied to compare the results.

3. Results

3.1. Generation and genetic characterization of white Ps_77 mutants

Using the EZ-Tn5™ Tnp Transposome™, 1.35·10^5 single individual transposon insertion mutants were obtained from the two different electroporation events. The combined application of MBM Agar and Kovac's reagent allowed an easy discrimination of the phenotype. White transposon mutants were picked and re-assayed on MBM agar to confirm stable insertion of Tn-5 transposon in the genome of Ps_77. Ten transposon mutants showing a stable white phenotype were selected for further characterization.

The genomic region flanking the transposon insertion sites was sequenced for each selected strain and the complete list of gene insertions observed in the investigated mutants is reported in Table 1. The localization of transposon-insertions allowed the identification of genes putatively involved in blue-pigment biosynthesis. Among the ten non-pigmenting mutants, insertions were localized in eight different genes. BlastKOALA tool allowed the annotation of only four out of eight genes, as reported in Table 1. The expression of these eight genes was checked on transcriptomic data from a previous study (Andreani et al., 2015). All these genes were expressed in MBM broth at 22 °C.

Six mutants (M2, M3, M4, M6, M7 and M19) lost the blue pigment production due to the disruption of four genes (PFLuk1_0667, PFLuk1_0668, PFLuk1_0673 and PFLuk1_0674) located in c4_BAR. PFLuk1_00667 was annotated as Tryptophan synthase beta chain *trpB_1*. The PFLuk1_00668 locus was annotated as hypothetical protein and no characteristic domain was identified using Hmmer. PFLuk1_00673 and PFLuk1_00674 were annotated as UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase (Table 1). Low similarity to other species was revealed by a Blast analysis. Both proteins seem to have a characteristic domain, namely a DegT/DnrJ/EryC1/StrS aminotransferase domain. Excluding the DegT/DnrJ/EryC1/StrS aminotransferase domain, the two amino acidic sequences are quite different. PFLuk1_00673 and PFLuk1_00674 lengths differs by six amino acids and SIM analysis revealed a low similarity between the two proteins with 31.6% identity in 345 residues overlap and gap frequency of 2.0%.

Three sequences were located outside the c_4 BAR region. These genes were categorized as belonging to *Metabolic pathways/Amino acid metabolism* (*hisG, speA* and *trpB_1*), two of these are also involved in *Biosynthesis of secondary metabolites* (*hisG* and *trpB_1*). One gene (*gacS*) was classified as *Environmental information processing.*

PFLuk1_00503 was mapped during the investigation of M5 and encodes for an ATP-Phosphoribosyltransferase and its ortholog in *P. fluorescens* A506 is *hisG,* a gene coding a protein involved in the biosynthesis of amino acid histidine. Another gene involved in amino acid metabolism and in particular in polyamines biosynthesis (PFLuk1_05234) was identified mapping the mutation of M12. This locus encodes for the biosynthetic arginine decarboxylase, responsible of the synthesis of agmatine from arginine, a precursor of putrescine (Moore and Boyle, 1990). Another mutant (M15) was characterised by disruption of gene encoded by PFLuk_02002, namely a signal

Table 1
Transposon-induced mutants. Table 1 reports non-pigmenting mutants obtained through transposon mutagenesis, the strain name, the mapping method, the mapped gene, the contig of ps_77 in which the gene is located, the exclusivity of the gene in blue pigmenting strains, the eventual presence in *P. fluorescens* A506.

Mutant strain	Disrupted gene	Ps_77 Contig	c4_BAR***	Ortholog gene in *P. fluorescens* A506	BlastKOALA results
M2	PFLuk1_00668 hypothetical protein*	4	Y	–	–
M3	PFLuk1_00667 Tryptophan synthase beta chain trpB_1**	4	Y	–	*Metabolic patways/Amino acid metabolism/Biosynthesis of secondary metabolites*
M4	PFLuk1_00673 UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase**	4	Y	–	–
M5	PFLuk1_00503 ATP phosphoribosyltransferase hisG**	4	N	*hisG* (PflA506_0882)	*Metabolic patways/Amino acid metabolism/Biosynthesis of secondary metabolites*
M6	PFLuk1_00673 UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase**	4	Y	–	–
M7	PFLuk1_00668 hypothetical protein**	4	Y	–	–
M12	PFLuk1_05234 Biosynthetic arginine decarboxylase speA*	61	N	*speA* (PflA506_0605)	*Metabolic patways/Amino acid metabolism*
M15	PFLuk1_02002 Signal transduction histidine-protein kinase BarA_1*	12	N	*gacS* (PflA506_3159)	*Environmental information processing*
M19	PFLuk1_00674 UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase wbpE_2*	4	Y	–	–
M20	PFLuk1_05423 hypothetical protein**	61	N	*tfoX_C* (PflA506_4519)	–

* mapping method as Karlyshev et al., 2000; ** mapping method as Walterson et al., 2014; ***location of the disrupted gene in c4_BAR region Yes/No.

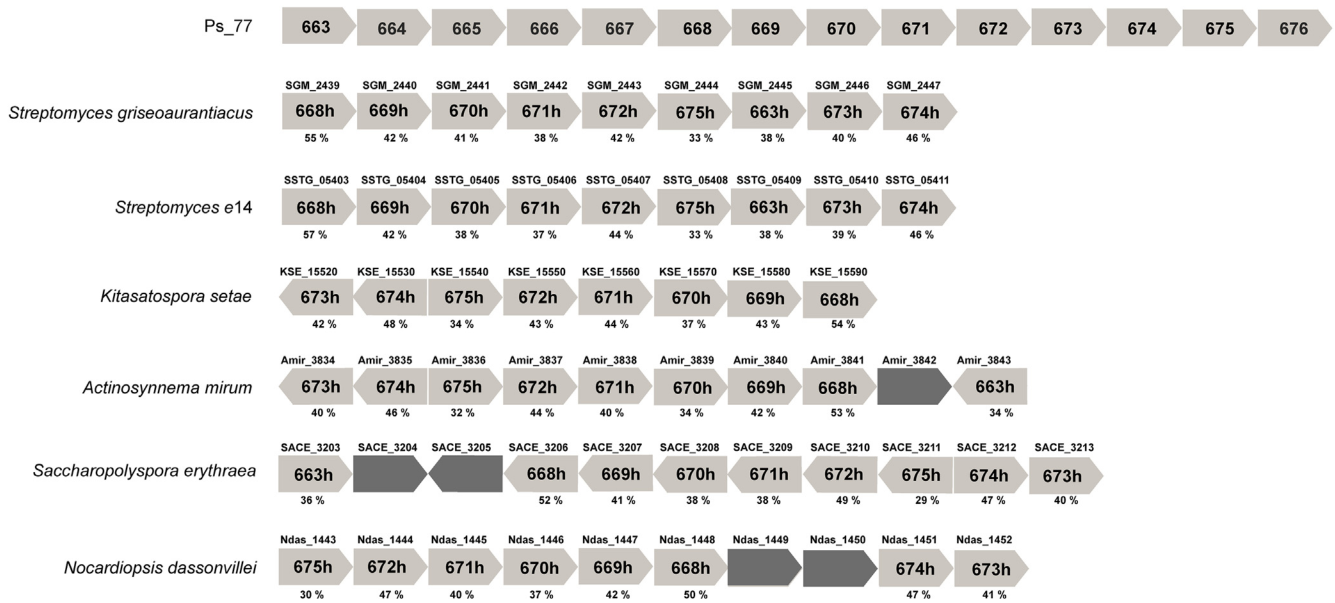

Fig. 1. Gene cluster structure of the c_4 BAR gene homologs in Actinobacteria: *S. griseoaurantiacus* M045 (*Streptomycetaceae*); *Streptomyces* e14 (*Streptomycetaceae*); *K. setae* KM6054 (*Streptomycetaceae*); *A. mirum* DSM43827 (*Pseudonocardiaceae*); *S. erythraea* NRRL2338 (*Pseudonocardiaceae*); *N. dassonvillei* DSM43111 (*Nocardiopsaceae*). Above each gene, the locus_tag is reported.

transduction histidine-protein kinase BarA, annotated as *gacS* in *Pseudomonas* species. *gacS* belongs to the GacS/GacA two-component system, involved in the signal/transduction pathway. Finally, M20 was characterised by gene disruption of PFLuk1_05423, annotated as Hypothetical protein by Prokka. Hmmer analysis showed a low level of similarity of this protein with *tfoX_C* encoding for a competence protein. No particular phenotype has been reported in literature as a result of gene deletion, apart from the loss of competence, or the ability to take up free DNA from the environment (Smeets et al., 2006).

3.2. Genetic and functional organisation of c_4 BAR region

Most of the genes included in the c_4 BAR region, with the exclusion of *trp* genes, are part of a conserved cluster, with a similar genetic organisation observed in some species of Actinobacteria, as revealed by HMMER and STRING investigations. The structure of the cluster in the different species is illustrated in Fig. 1. To define the transcription units of c4_BAR genes in Ps_77, the cDNA was amplified using primers located in all the fourteen genes and a unique large operon was identified.

3.3. Growth and phenotypic characterization of Ps_77 and white transposon mutants

Strains M5, M12, M15 and M20 were excluded from the phenotypic evaluation as the involvement of the four genes in other important pathways could influence the interpretation of results. The focus was addressed on four mutants of the c_4 BAR genes, excluding M6 and M7 as analogous to M4 and M2, respectively.

To test whether the blue pigment could be related to iron metabolism homeostasis, growth rate was measured for the wild-type strain Ps_77 and all four white mutants in MBM, KB broth and iron limited KB at 22 °C, as well as in BIOLOG GN2 plates. The wild type Ps_77 and the white mutants had statistically indistinguishable growths in all the media tested ($p > 0.05$ for all the pair comparisons, Table 2S, Fig. 1S). The wild-type strain produced the blue pigment in the three condition tested and the pigment production was unequivocally visible after 48 h of incubation.

The white mutant colonies appeared fluorescent as the wild-type strain at UV lamp exposure demonstrating that the lack of blue pigment does not influence pyoverdine production.

3.4. Stress resistance of Ps_77 and white mutants

The capability of the blue pigment to confer resistance to different types of stress as osmotic (salt), oxidative, thermal and high/low pH was tested comparing the growth of wild-type and the four white mutants in presence of the stress agents (H_2O_2, NaCl, high/low pH and incubation at 55 °C-4 °C). The results indicated that all stressors produced the same effect on the wild-type as on the unpigmented mutants (Fig. 2S), except for oxidative stress (Fig. 2). The growth of the four mutants in presence of hydrogen peroxide was slightly reduced at 2.5 mM of H_2O_2 and completely inhibited at 5 mM resulting at both concentrations significantly different form the growth of the wild-type (Wilcoxson test, $p < 0.05$). Fig. 2 reports the lower ratio of growth of the four mutants *versus* the wild-type at two concentrations (2.5 and 5 mM) of hydrogen peroxide after 24 h of incubation. Similar effect of resistance to hydrogen peroxide by the blue wild-type strain occurred in complete medium (TSB, Fig. 2A) and minimal medium (MBM, Fig. 2B).

As blue pigment is released extra-cellularly, a co-colture of Ps_77 and M3 strains was set up in MBM liquid medium added with hydrogen peroxide at 2.5 and 5 mM to evaluate whether pigment mediating antioxidative protection have the ability to protect non-producing bacteria. The growth of the white mutant was significantly lower than the wild type (paired t-test $p < 0.05$, Fig. 3) indicating a not diffusible antioxidant effect of the blue pigment in the culture.

4. Discussion

The present work aimed to investigate the biosynthetic pathway of the blue pigment, as well as its biological function in one well-studied *P. fluorescens* strain through the creation of a library of non-pigmenting strains and their genotypic and phenotypic characterization. A high number of mutants of *P. fluorescens* Ps_77 was created through the application of EZ-Tn5™ Tnp Transposome™ kit. However, only ten showed a stable insertion, as showed by the phenotype observed on MBM agar and after testing with Kovac's reagent. The negative reaction of Kovac's reagent of the white mutants confirms that the blue pigment or its precursor is an indole-derivative. It has been in fact demonstrated that this reagent can produce different coloured substances when reacting with different indole derivatives (Ehmann, 1977).

The transposon mutagenesis identified eight genes involved in blue

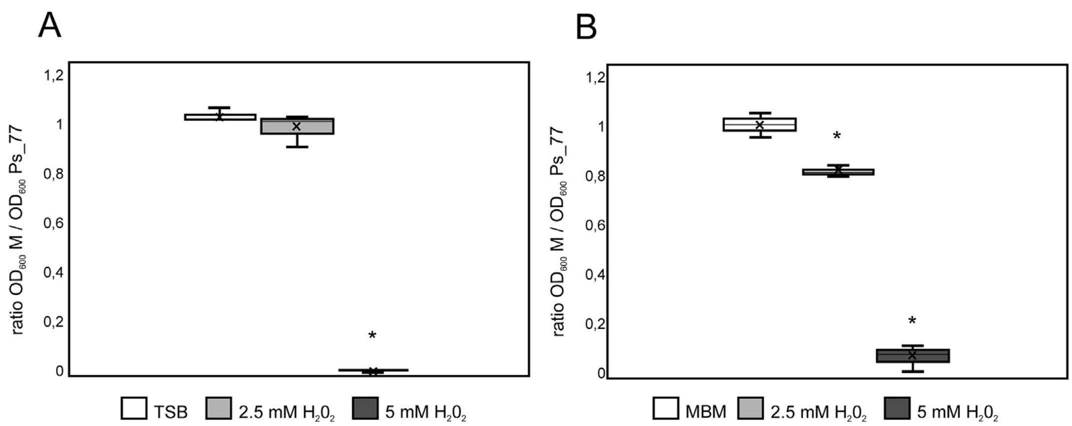

Fig. 2. Ratio of the growth of mutants (M2, M3, M4, M19) and Ps_77 in TSB (A) and MBM (B) added with H_2O_2 at 2.5 and 5 mM after 24 h (*$p < 0.05$).

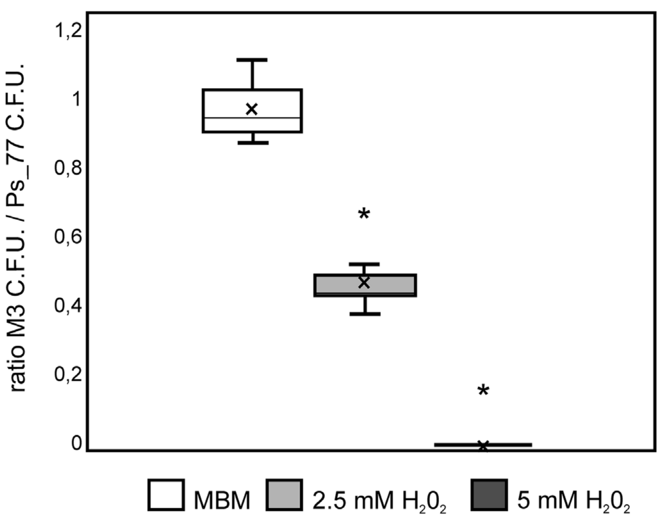

Fig. 3. Ratio of the bacterial cell counts of M3 mutant and Ps_77 in MBM added with H_2O_2 at 2.5 and 5 mM (*$p < 0.05$).

pigment production in Ps_77 and among these four are included in c_4 BAR region. This result confirms previous hypothesis that indicated the involvement of the second copies of *trp* genes in the blue pigment biosynthesis through an indole/indigo biosynthesis pathway (Andreani et al., 2015). Carriage of multiple *trpB* gene copies has been reported in several bacterial and archaeal species (Xie et al., 2001; Merkl, 2007; Busch et al., 2014) and are frequently involved in biosynthesis of molecules with different functions, despite the high-energy cost required for this pathway. It has been demonstrated that usually the second copy possesses substrate specificity, not assembled with TrpA and seems to have other functions, such as indole salvage (Busch et al., 2014; Hiyama et al., 2014). In *Pseudomonas aeruginosa* the first enzyme of the tryptophan biosynthesis pathway (Anthranilate synthase coded by *trpE* and *trpG* genes) is duplicated and the second copy is involved in the quorum sensing signalling but not in tryptophan biosynthesis (Palmer et al., 2013). Moreover, indole and the indole-derivatives, synthetized from tryptophan are interspecies and interkingdom signalling molecules involved in important roles as bacterial pathogenesis and eukaryotic immunity (Lee et al., 2015).

Only a *trpB_1* mutation was identified as conferring a white phenotype in Ps_77. The second copies of other *trp* genes (*trpA, trpC, trpD, trpF*) were not identified to be disrupted in the white mutants. This result can have several explanations. The mutation in the other *trp* genes might be complemented by the first copy of the aforementioned genes or only TrpB might be the only one directly involved in the pigment production. A third possible explanation might be that the mutants isolated were not enough to identify all the genes involved in pigment production. The other three white mutants with the disrupted gene located c_4 BAR are a hypothetical protein (PFLuk1_0668) and two aminotransferases (PFLuk1_0673 and PFLuk1_0674). DegT/DnrJ/EryC1/StrS aminotransferases have been widely reported as having a regulatory and protein kinase (sensor) function (Murphy et al., 1993; Madduri and Hutchinson, 1995). In *Bacillus stearothermophilus*, *degT* has a proper regulatory function, being involved in the transfer of environmental stimuli (Takagi et al., 1990). Aminotransferases of this family have been identified as involved in aminotrasfers that lead to amino sugars implicated in the formation of LPS and aminoglycosides in *Porphyromonas gingivalis* (Shoji et al., 2002). On the other hand, Chen et al. (2000) speculate that the lower blue pigment production in *P. gingivalis degT*⁻ mutants might be due to the impossibility of the mutant strain to secrete the extracellular pigment through the production of vesicles. Excluding the DegT/DnrJ/EryC1/StrS domain, the two proteins (PFLuk1_0673 and PFLuk1_0674) are quite different. This result induces to suppose the two loci have different functions, even if sharing the same domain and might be involved in the secretion of the blue pigment or of a precursor of the blue pigment.

In species belonging to three different families of Actinobacteria (Streptomicetaceae, Pseudonocardiaceae and Nocardiopsaceae), the three genes PFLuk1_0668, PFLuk1_0673 and PFLuk1_0674 are included, with their neighbours in c_4 BAR (excluding *trp* genes), in a large gene cluster. The function of these proteins is unknown. Several of these Actinobacteria species were shown to be producers of a rich array of active metabolites (Li et al., 2013; Girard et al., 2014). The c_4 BAR identified in Ps_77 comprised the nine Actinobacteria homologue genes assembled with the five *trp* genes. The analysis of the transcripts in Ps_77 demonstrates that all the fourteen genes are co-expressed and are included in a single large operon supporting the hypothesis that these genes are coding the enzymes for the biosynthesis of the pigment and related function as transport and secretion.

Among the mutants disrupted in genes located outside c_4 BAR, two are involved in amino acid metabolism (*hisG* and *speA*). The effect of these mutations in pigment production it is not evident despite HisG and SpeA proteins are included in biosynthetic pathway that share intermediate compounds with *trp* metabolism. (i.e. Phosphoribosyl pyrophosphate; PRPP).

On the contrary, the implication of *gacS* product in blue pigment biosynthesis regulation it is not difficult to suppose. In fact GacS/GacA system has been reported to regulate several phenotypes in bacteria. In *Escherichia coli* and *Vibrio fischeri* the siderophore-mediated iron sequestration is regulated by GacS/GacA system (Sahu et al., 2003; Foxall et al., 2015). In *Pseudomonas fluorescens* FD6 (a biocontrol strain), it has been demonstrated that *gacS* knock-out strains cannot produce a wide range of secondary metabolites. In particular, biofilm formation and siderophore production were downregulated, as well as the protection activity against *Botrytis cinerea* HY2-1 (Chang et al., 2014).

To summarize, the blue pigment biosynthetic pathway is strictly related to *trp* genes, confirming the importance of the genomic region previously identified in c_4 BAR of Ps_77. For this reason, the phenotypic characterization of the mutants was focused on the four mutants with the disruption located in c_4 BAR. Specifically, the study of the four mutants could better clarify the function of the pigment better than the other mutants as *GacS*-, *hisG*- and *speA*-knock-out strains that are involved in other important pathways.

Wild type and mutant strains grow with a comparable growth rate in normal medium (MBM; KB), iron-depleted medium and different carbon sources. This suggests that the lacking of the pigment does not affect the growth capability or the utilisation of different carbon sources as well as the production of the pigment is not induced by the absence of iron in the medium, suggesting that the pigment is not a siderophore (Cornelis, 2010).

Several natural pigments produced by bacteria, fungi or microalgae have stress resistance properties (Tuli et al., 2015). No effect on osmotic, thermic and pH stress-resistance was recorded. However, Ps_77 demonstrated an increased resistance to oxidative stress induced by hydrogen peroxide. This result suggests that the blue pigment (or its parent compound) might act as an antioxidant agent or regulates an antioxidant response and is not involved in a general stress response. Moreover, the involvement of GacA/GacS system in oxidative resistance in *P. fluorescens* (Heeb et al., 2005) and the antioxidant activity of indole, indole derivatives and indigo (Dua et al., 2014; Lee et al., 2015) support this hypothesis.

The capability of surviving to oxidative stress is of great importance to survive in the environment. Several strains of *Pseudomonas fluorescens* are component of the rhizosphere, an environment in which the active metabolism of the root tip generates a high amount of reactive oxygen species. A study demonstrated that the success of root colonization in *P. putida* depends on its capability to resist to oxidative stress (Kim et al., 2000). Bacterial tryptophan metabolites interfere with immune responses in plants and animals and kynurenine pathway may allow immunomodulatory interplay between bacteria and host (Genestet et al., 2014; Lee et al., 2015; Bortolotti et al., 2016).

The competition experiment demonstrated that the resistance to hydrogen peroxide is limited to the blue producing strain and it cannot be shared with strains sharing the same environment. The diffusion of the blue pigment in the plate or liquid medium occurs despite their insolubility in water (Andreani et al., 2015). This means that the diffusion of the blue pigment in the medium has to be supposed complexed with other compounds. For this reason, it would be likely to assume that the blue pigment in this form cannot act as antioxidant agent. The antioxidant activity might be exerted by the blue pigment or its precursor inside the bacterial cell or linked to the plasmatic membrane.

5. Conclusions

The data obtained in the present study strongly suggest a role in resistance to oxidative stress of the blue pigment, despite its chemical structure was not completely elucidated. The pigment, that was confirmed to be an indole derivative, is produced and secreted in the environment. This capability successfully adapts the blue-producing strains to survive in the different environment from which *P. fluorescens* is frequently isolated as polluted environment, rhizosphere or frequently sanitized food industries. The elimination of blue producing strains of *P. fluorescens* from the production line remains an unresolved problem for dairy industries. The increased resistance to antioxidant agents might help explain the difficulty of eradication.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.028.

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