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Research Note

Strain Diversity of *Pseudomonas fluorescens* Group with Potential Blue Pigment Phenotype Isolated from Dairy Products

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ABSTRACT

The blue discoloration in Mozzarella cheese comes from bacterial spoilage due to contamination with *Pseudomonas*. Fourteen *Pseudomonas fluorescens* strains from international collections and 55 new isolates of dominant bacterial populations from spoiled fresh cheese samples were examined to assess genotypic and phenotypic strain diversity. Isolates were identified by 16S rRNA gene sequencing and tested for the production of the blue pigment at various temperatures on Mascarpone agar and in Mozzarella preserving fluid (the salty water in which the cheese is conserved, which becomes enriched by cheese minerals and peptides during storage). Pulsed-field gel electrophoresis analysis after treatment with the endonuclease *Spe*I separated the isolates into 42 genotypes at a similarity level of 80%. Based on the pulsotype clustering, 12 representative strains producing the blue discoloration were chosen for the multilocus sequence typing targeting the *gyrB, glnS, ileS, nuoD, recA, rpoB,* and *rpoD* genes. Four new sequence typing profiles were discovered, and the concatenated sequences of the investigated loci grouped the tested strains into the so-called "blue branch" of the *P. fluorescens* phylogenetic tree, confirming the linkage between pigment production and a specific genomic cluster. Growth temperature affected pigment production; the blue discoloration appeared at 4 and 14°C but not at 30°C. Similarly, the carbon source influenced the phenomenon; the blue phenotype was generated in the presence of glucose but not in the presence of galactose, sodium succinate, sodium citrate, or sodium lactate.

Key words: Blue discoloration; Mozzarella cheese spoilage; Multilocus sequence typing; *Pseudomonas fluorescens*; Pulsed-field gel electrophoresis; Restriction endonuclease analysis

The *Pseudomonas fluorescens* group is a frequent contaminant of ready-to-eat foods. Because these bacteria are psychrotrophic, contamination is a particular problem in refrigerated products such as fresh milk and cheeses *(7, 11, 17),* raw meat and fish *(3, 9),* and fresh vegetables *(4),* where spoilage occurs through intrinsic enzymatic activities such as proteolysis, lipolysis, and pectinolysis *(13)*.

P. fluorescens is also responsible for a specific defect in Mozzarella, which has been defined as a blue discoloration *(8, 11, 12)* and has appeared sporadically in some production batches. The ability to produce the blue pigment seems to be strain specific, and a correlation between this ability and a specific phylogenetic cluster within the species has been postulated *(2)*. The chemical nature of the substance that produces the blue color in the spoiled cheese is still under debate; some authors have attributed the color to leucoindigoidine *(6)* and others to a derivative of indole *(1)*. Although the origin of blue-producing strains is not yet known, prevention of the phenomenon is linked to rigorous maintenance of the sanitation system and the microbiological quality of the processing water *(11)*.

In this work, a collection of *P. fluorescens* isolates from dairy samples was examined to assess a possible relationship between genotype and phenotype in terms of production of the blue pigment.

MATERIALS AND METHODS

Bacterial strain collection. Sixty-nine isolates belonging to the *P. fluorescens* group were studied. Three strains (*P. fluorescens* ATCC 13525, 50154, and 50108) were purchased from international collections, and eight isolates (200188/1, 200188/2, 200188/6, 200188/8, 176673/1, 9AG, 9BG, and 9AP) were provided by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (Brescia, Italy). *P. fluorescens* strains SBW25, H, and A506 were kindly supplied by the University of Exeter (Exeter, UK), Universidade do Minho (Braga, Portugal), and Oregon State University (Corvallis), respectively. The remaining 55 isolates were recovered between 2010 and 2014 from spoiled dairy products with a blue discoloration by isolation on *Pseudomonas* agar base with added cephalothin, sodium fusidate, and cetrimide (Merck, Darmstadt, Germany). Pure cultures were stored at −80°C in tryptic soy broth (TSB) with 20% (vol/vol) glycerol (Sigma-Aldrich, St. Louis, MO).

Isolate identification. Fresh cells of each isolate were obtained by overnight culture in TSB at 30°C. After centrifugation

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at 4,000 × *g* for 10 min, cultures were washed twice in deionized water and resuspended in 400 μl of 1× TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). DNA extraction was performed using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). The isolates were identified by partial sequencing of the 16S rRNA gene using primers BSF-8/20 and BSR-1541/20 according to the protocol of Wilmotte et al. *(18)*. The amplified products were sequenced by an outside provider (Eurofins Genomics, Ebersberg, Germany), and the sequences were then compared with those in the GenBank database (http://www.ncbi.nlm.nih.gov).

Determination of blue phenotype. All 69 isolates were evaluated for the production of the blue pigment by streak plating onto Mascarpone agar (MA; 40% [wt/vol] Mascarpone cheese, 60% deionized water, and 1.5% agar) *(5)* and incubating at 30°C for 72 h. Isolates with a blue discoloration on MA were selected and inoculated at 1% (vol/vol) in 2 ml of Mozzarella preserving fluid (PF; water, salt, and cheese minerals) that had been centrifuged (9,000 × *g* for 30 min), the pH adjusted to 6.3, and filtered through a 0.45-μm-pore-size membrane. The growth tests were carried out in 24-well microplates incubated at three temperatures (4, 14, and 30°C) to confirm production of the pigment. Color development was monitored daily.

Strain typing by restriction enzyme analysis with PFGE. The genomes of all 69 isolates were analyzed by restriction endonuclease analysis with pulsed-field gel electrophoresis (REA-PFGE). Pure cultures were grown overnight on nutrient agar (Merck) at 30°C, and single colonies were dissolved in cell suspension buffer (0.1 M Tris HCl and 0.1 M EDTA, pH 8) to reach an optical density at 600 nm of 0.6 to 0.8. Then, 200 μl of the cell suspension was mixed with an equal amount of 2% (wt/vol) agarose gel melted in TE buffer (0.01 M Tris HCl and 0.01 M EDTA, pH 8) and kept at 55°C. Each plug was immersed in 5 ml of cell lysis buffer (0.05 M Tris HCl, 0.05 M EDTA [pH 8], 1% [wt/vol] sarcosyl, and 0.2 mg/ml proteinase K; Sigma-Aldrich) and incubated overnight at 37°C with shaking at 80 rpm. Lysis solution was then removed. The plugs were rinsed four times by adding 8.5 ml of TE buffer prewarmed to 50°C and incubating at 50°C for 10 min. The genome was digested with the *Spe*I restriction enzyme (ThermoScientific, Waltham, MA) in a solution of 20 U of *Spe*I, 1× Tango buffer, and 1× TE buffer at 37°C for 6 h. Digested plug fragments were placed in a 1% (wt/vol) pulsed field certified agarose gel in TBE buffer (0.09 M Tris HCl, 0.09 M boric acid, and 2 mM EDTA, pH 8). Run conditions for the CHEF Mapper (BioRad Laboratories, Hercules, CA) were 14°C, 6 volts, initial switch at 1 s, final switch at 25 s, and runtime of 22 h. This protocol is a slight modification from that of Martin et al. *(11)* and Nogarol et al. *(12)*. The gel was stained with ethidium bromide solution (1 μg/ml). Images were captured with a Gel DOC XR (BioRad) and analyzed with GelJ software *(10)* to align band profiles. A similarity tree was created by using the Dice similarity method with a 1% tolerance and the unweighted pair group method with arithmetic mean (UPGMA) linkage.

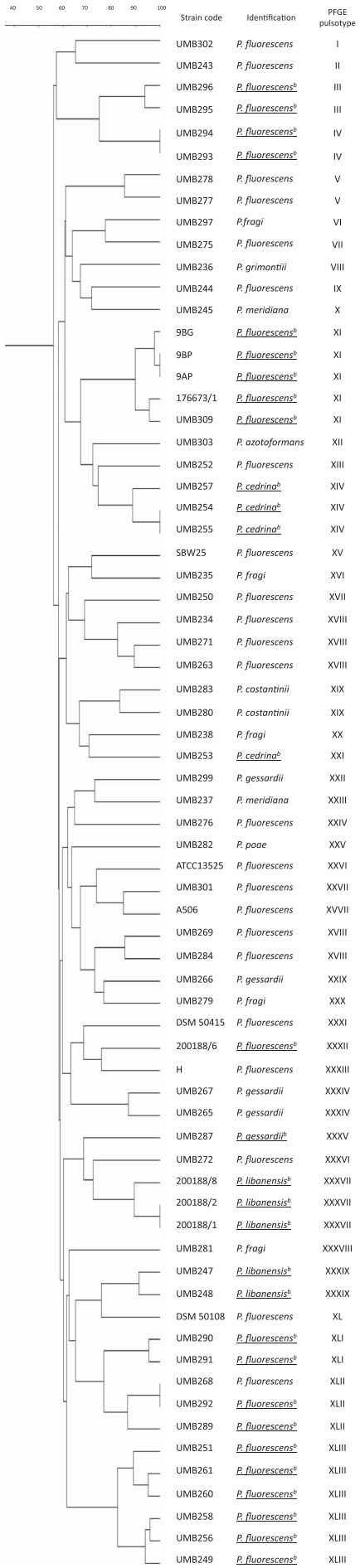
MLST analysis. A multilocus sequence typing (MLST) analysis was conducted with representative isolates according to the clustering results of the PFGE analysis and using the protocol of Andreani et al. *(2)*. Loci of seven housekeeping genes (*gyrB, glnS, ileS, nuoD, recA, rpoB,* and *rpoD*) were amplified in a 25-μl reaction mixture composed of 1× buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.25 μM each primer, 1 U of *Taq* (5 Prime, Hilden, Germany), and 10 ng of DNA template. The amplification was performed in a Mastercycler ep thermal cycler (Eppendorf, Hamburg, Germany) with the following 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; and a final extension step at 72°C for 7 min. The amplification products were visualized by electrophoresis on 1.8% (wt/vol) agarose gels, stained with ethidium bromide, and subsequently sequenced for both DNA strands. The sequences were trimmed and aligned using CLC software (Qiagen, Venlo, The Netherlands), concatenated following the alphabetical order of the loci, and aligned and compared with the sequenced strains in the MLST database (http://pubmlst.org/pfluorescens/), obtaining a phylogenetic tree based on the maximum likelihood algorithm with MEGA software *(16)*.

Effect of carbon source on production of blue pigment. The same isolates used for the MLST analysis were chosen to verify the effect of carbon source on the production of blue pigment. Fresh cells from an overnight culture in TSB at 30°C were inoculated at 1% (vol/vol) in 2 ml of M9 medium *(14)* separately supplemented with different carbon sources (D-glucose, D-galactose, sodium succinate, sodium citrate, and DL-lactic acid sodium salt) at 30 mM, adjusting the pH value to 6.3. Cultures were incubated at three different temperatures (4, 14, and 30°C), and color development was monitored daily.

RESULTS

Isolate identification. The 55 new isolates obtained from spoiled dairy products were identified by partial sequencing of the 16S rRNA gene. Results confirmed that they belonged to the *P. fluorescens* group. Most isolates (52.7%) were assigned to *P. fluorescens*, 9.1% to *Pseudomonas fragi,* 9.1% to *Pseudomonas gessardii*, 9.1% to *Pseudomonas libanensis*, 7.3% to *Pseudomonas cedrina*, 7.3% to *Pseudomonas costantinii* and *Pseudomonas meridiana,* and 1.8% to *Pseudomonas azotoformans*, *Pseudomonas grimontii,* and *Pseudomonas poae*. These results and those for the other strains evaluated in this work are shown in Figure 1.

Determination of blue phenotype. A blue or dark blue pigment was produced by 30 isolates at 30°C on MA plates (Table 1), whereas the remaining 39 isolates produced variable coloration from yellow to dark green or black. None of the strains obtained from the international or academic collections produced the blue pigment. The blue color was observed after 72 h of incubation and was diffusible in the MA medium, consistent with the findings of Cantoni et al. *(5)*. The results observed from the growth in the Mozzarella PF are summarized in Table 1. Color development was noticed after 7 days of incubation at 30 and 14°C and after 10 days of incubation at 4°C. Production of blue pigment differed between isolates grown in PF and those grown on MA. At 30°C, the isolates developed a yellow instead of blue color (Table 1). At 14°C, the blue pigment was produced after 72 h but by only six isolates (UMB247, UMB248, UMB253, UMB254, UMB288, and 200188/6), and the color changed to yellow-green during the following days of incubation. At 4°C, most strains produced a brilliant blue or dark blue pigment after 10 days. Some isolates



(UMB249, UMB258, UMB260, UMB292, 176673/1, and 9AP) produced the blue color in MA but not in PF (Table 1).

Strain typing by restriction enzyme analysis with PFGE. The similarity tree obtained by the comparison of the genomic patterns generated by *Spe*I digestion is shown in Figure 1. A threshold value of 80% similarity was chosen to distinguish genotypes, according to the repeatability of the restriction pattern generated by independent replicate analyses of the reference strain *P. fluorescens* ATCC 13525^T (data not shown). This cutoff value is in agreement with that reported by Nogarol et al. *(12)*. Forty-three genomic profiles corresponding to different genotypes were recognized among the 69 isolates, which were defined as pulsotypes. Strains producing blue pigment belonged to 12 different pulsotypes (III, IV, XI, XIV, XXI, XXXII, XXXV, XXXVII, XXXIX, XLI, XLII, and XLIII) (Table 1) and were placed in different clusters of the UPGMA tree (Fig. 1). Isolates grouped in pulsotypes XIV, XXI, and XLIII were recovered from the same Mozzarella sample (except for isolate UMB248), and isolates in pulsotypes III, IV, XXXV, and XLII were collected from another Mozzarella sample. In these two cases, the contamination was evidently polymicrobial, characterized by the coexistence of different strains of the same species. Conversely, five isolates producing blue pigment (UMB249, UMB256, UMB258, UMB260, and UMB261), which were recovered in different years, had the same pulsotype (XLIII); other isolates (9BG, 9BP, 9AP, 176673/1, and UMB309) grouped in a single pulsotype (XI) were also collected at different times and places. These cases support the hypothesis that resilient strains *P. fluorescens* persist in dairy factory environments *(12)*.

MLST analysis. From each of the 12 pulsotypes that included isolates producing blue pigment, one representative strain 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), tracking down the corresponding alleles and ST profiles (Table 2). Six of 12 strains (200188/6, 200188/8, 9BG, UMB253, UMB254, UMB260) showed 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 12 selected blue-producing strains have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with accession numbers from KU512209 to KU512285.

The comparison with the sequences of strains investigated by Andreani et al. *(2)* revealed that the strains with the

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FIGURE 1. *Dendrogram constructed by UPGMA cluster analysis of the restriction profiles obtained by PFGE of the 69 isolates and their specie attribution. The scale bar shows percent similarity. Blue-producing isolates are underlined and marked with* [b].

TABLE 1. *List of the 30 blue-producing isolates investigated in this work with their isolation details, PFGE profile, and their color development in Mascarpone agar medium (MA) and preserving fluid (PF) of Mozzarella cheese*

Strain code	Source	Place	Year	PFGE pulsotype	MA	PF		
					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	Colorless	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	Colorless	Yellow
UMB258	Mozzarella cheese	Italy	2010	XLII	Blue	Colorless	Blue	Yellow
UMB260	Mozzarella cheese	Italy	2010	XLII	Blue	Colorless	Blue	Yellow
UMB261	Mozzarella cheese	Italy	2011	XLII	Dark blue	Blue	Blue	Yellow
UMB268	Mozzarella cheese	Italy	2010	XLII	Blue	Blue	Colorless	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	Colorless	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	Colorless	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	Colorless	Yellow	Yellow
9BP	Mozzarella cheese	Germany	2010	XI	Dark blue	Blue	Blue	Yellow

same allelic profile were frequently isolated from the same geographic region. In particular, all the strains belonging to the allelic profile 25 have been isolated in North East Italy, while those attributed to the allelic profile 29 were collected from Germany. The concatenated sequences obtained from the 12 strains selected in this work were aligned with those available in the *P. fluorescens* MLST database in order to build a phylogenetic tree (Fig. 2): all our new isolates were assembled within the cluster formed by the former blue-producing strains. This outcome corroborates the hypothesis of a "blue branch" in *P. fluorescens* species, indicating that all the isolates that produce the discoloration share a common evolutionary development, as already suggested by Andreani et al. *(2)*.

TABLE 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*

Strain code	*glnS*	*gyrB*	*ileS*	*nuoD*	*recA*	*rpoB*	*rpoD*	ST (MLST)
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

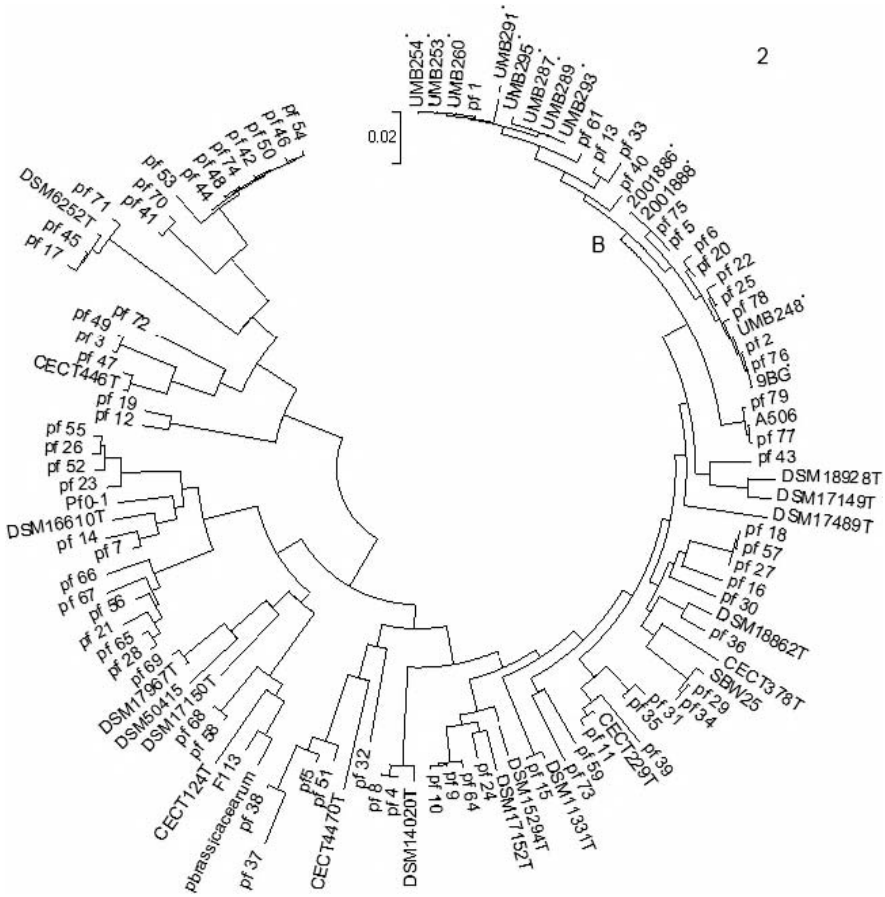

FIGURE 2. *Maximum likelihood tree obtained from the comparison of concatenated sequences by MLST analysis from all the investigated loci (http://pubmlst.org/pfluorescens/). The "blue branch" is indicated by letter B.*

Influence of carbon source in blue pigment production. The 12 selected strains were further subjected to a phenotypic analysis to verify the influence of the carbon source for the formation of the blue pigment. Results obtained after 96 h at 30 and 14°C and after 2 weeks at 4°C are reported in Table 3. Cell growth was observed for all strains with each carbon source tested, but the blue coloration was produced only when glucose was added in the M9 medium at incubation temperatures of 4 and 14°C (Table 3). At 30°C a green color was developed instead of blue. For the other carbon sources, the strains were able to generate other discolorations, but not the blue one.

DISCUSSION

In relation to the events of blue discoloration in Mozzarella cheese that have occurred in Italy since 2010, the interest in *P. fluorescens*, responsible for this defect, as a contaminant of dairy plants and fresh dairy products has increased *(7, 11, 12, 15)*. The study on the development of the blue phenotype made in this work has revealed that the appearance of the phenomenon is affected by the incubation

TABLE 3. *Colors developed by blue-producing strains in M9 medium added with different carbon sources (30 mM) grown at different temperatures*

	D-Glucose			D-Galactose			Sodium citrate			DL-Sodium lactate			Sodium succinate		
Strain code	4°C	14°C	30°C	4°C	14°C	30°C	4°C	14°C	30°C	4°C	14°C	30°C	4°C	14°C	30°C
200188/6	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
200188/8	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
9BG	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
UMB248	Blue	Blue	Green	Yellow	Yellow	Green	Colorless	Colorless	Green	Colorless	White	Green	Colorless	Colorless	Green
UMB253	Blue	Blue	Green	Yellow	Grey	Green	Colorless	Grey	Green	Colorless	Grey	Green	Colorless	Grey	Green
UMB254	Blue	Blue	Green	Yellow	Grey	Yellow	Colorless	Grey	White	Colorless	Grey	White	Colorless	Grey	White
UMB259	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
UMB261	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
UMB289	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
UMB291	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
UMB293	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green
UMB295	Blue	Blue	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green	Grey	Grey	Green

temperature, as occurs only at 4 and 14°C but not at 30°C. Results of genome restriction analysis highlighted that populations of blue-producing strains may coexist with those that do not generate the discoloration and that the same pulsotype can be isolated years later in the same place, revealing the possibility that the same strain can persist in a dairy plant for a long time. Due to the high heterogeneity of *P. fluorescens* genome, the restriction endonuclease analysis plus PFGE protocol was not able to discriminate blue-producing strains by genome profile, as previously reported by Nogarol et al. *(12)*. On the other hand, the MLST analysis has strenghtened the hypothesis that blue-producing strains have a common ancestor, as suggested by Andreani et al. (*2*), even if it is not clear the reason for the development of this phenotype. In addition to the temperature factor, the type of the carbon source available in the PF is associated to the spoilage since the formation of the blue pigment has only happened when glucose is present in the medium. This sugar could be available into the preserving liquid of Mozzarella cheese coming from residual enzymatic activity of lactic acid bacteria on lactose. The phenomenon has not occurred in the presence of galactose or of organic acids, which were used as a carbon source by the investigated strains and are present in fresh dairy products.

The meaning of the phylogenetic split of this cluster from other strains of *P. fluorescens* is unknown; also the molecule that constitutes the blue pigment has not yet been identified with certainty *(1, 6),* and even its function has been recognized. However, it should be noted that these strains all originated from the dairy environment and that the phenomenon occurs in products subjected to chill storage. The precise identification of the pigment and of the genetic determinants coding for its synthesis may help to understand the role of this phenomenon and the way to prevent it.

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