Journal of Food Protection, Vol. 76, No. 3, 2013, Pages 500–504
doi:10.4315/0362-028X.JFP-12-312

Research Note

Molecular Characterization of *Pseudomonas fluorescens* Isolates Involved in the Italian "Blue Mozzarella" Event

C. NOGAROL,[1]* P. L. ACUTIS,[2] D. M. BIANCHI,[1] C. MAURELLA,[3] S. PELETTO,[2] S. GALLINA,[1] D. ADRIANO,[1] F. ZUCCON,[1] S. BORRELLO,[4] M. CARAMELLI,[5] AND L. DECASTELLI[1]

[1]*S.C. Controllo Alimenti e Igiene delle Produzioni,* [2]*S.S. Genetica e Immunobiochimica, and* [3]*S.S. Biostatistica Epidemiologia e Analisi del Rischio, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, via Bologna, 148-10154 Torino, Italy;* [4]*Ministero della Salute, Viale G. Ribotta, 5-00144 Roma, Italy; and* [5]*Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, via Bologna, 148-10154 Torino, Italy*

MS 12-312: Received 16 July 2012/Accepted 19 October 2012

ABSTRACT

Between June and September 2010, widespread Italian consumer reports of unusual blue spoilage on fresh dairy products were publicized, resulting in the so-called blue mozzarella event. An inordinately high number of samples from mozzarella and whey cheese products of Italian and German production subsequently tested positive for *Pseudomonas fluorescens*. The aim of this study was to verify whether a selected *P. fluorescens* strain was responsible for this apparently unusual event. Molecular characterization of 181 isolated *P. fluorescens* strains was conducted using a newly optimized pulsed-field gel electrophoresis protocol. Although a high number of pulsotypes was found (132), only four pulsotypes were associated with more than one production plant, and only one German isolate had the same pulsotype as was detected in two Italian plants. This is the only evidence of possible cross-contamination among cheeses from the two countries. The overall results did not support the spread of contamination from German to Italian plants or the presence of one environmental strain that spread in both countries.

Product contamination with psychrotrophic microorganisms is particularly worrisome for the dairy industry because dairy products are distributed at temperatures permissive for the growth of these organisms *(4)*. The microbes that may be categorized as psychrotrophic are ubiquitous in nature and can be isolated from soil, water, and vegetation. Across the entire cheese chain production, long storage times and low temperatures combine to create a selective advantage for psychrotolerant bacteria, especially members of the genus *Pseudomonas,* that can enter raw milk via biofilms in bulk milk tanks and contaminated water and soil *(3, 20)*. Under certain growth conditions, numerous species of *Pseudomonas* (e.g., *P. putida, P. fluorescens, P. syringae,* and *P. aeruginosa*) synthesize blue *(10)* or yellow-green, fluorescent, or water-soluble *(12)* pigments because they contain genes encoding enzymes that produce pigmented molecules *(13)*. Although these microbes can be inactivated by heat treatment (pasteurization or ultrahigh temperature), their heat-resistant enzymes persist during milk processing *(2)* and may be active in the stored product *(11)*.

One of the predominant organisms in refrigerated milk is *P. fluorescens,* and its presence in processed milk samples indicates that it can colonize the dairy processing environment and that it has a short generation time at refrigeration temperatures *(4)*. The occurrence in raw milk of heat-stable enzymes produced by *P. fluorescens* has been described *(15, 17)*.

Between June and September 2010, widespread Italian consumer reports of a blue pigment on fresh dairy products sounded an alarm throughout the dairy industry. Samples of mozzarella and whey cheese (both made with pasteurized milk) from Italian and German dairy plants were submitted to microbiological analyses. Traditional microbiological techniques for bacterial identification were used to isolate and identify *P. fluorescens*. The unusual spoilage of fresh cheese and the high number of contaminated samples from Italian and German production found in Italian food markets quickly caught the attention of the news media. Mounting nationwide news coverage created the so-called blue mozzarella event. Because of news media attention, the number of samples reported by veterinary control officials and consumers increased quickly, leading to the isolation of *P. fluorescens* from both spoiled and unspoiled samples. The high number of cheeses contaminated by the same microorganism and found in markets throughout Italy within a very short period of time fit the classical epidemiological criteria for defining an epidemic *(16)*. Possible explanations for this apparent epidemic could be either the unusual and massive spread of a *P. fluorescens* strain or the contamination of Italian products from the importation and utilization of German raw materials. The use of German raw materials was indicated at the beginning of the event through an epidemiological survey, which revealed the involvement of

* Author for correspondence. Tel: +39 (0)11 2686233; Fax: +39 (0)11 2473450; E-mail: chiara.nogarol@izsto.it.

TABLE 1. *Number of PFGE pulsotypes obtained for each dairy production plant*

Plant code	No. of isolates	No. of pulsotypes
IT1	39	21
IT2	2	2
IT3	2	2
IT4	2	2
IT5	6	5
IT6	4	4
IT7	12	10
IT8	2	2
IT9	4	1
IT10	2	2
IT11	2	2
IT12	1	1
IT13	1	1
IT14	1	1
IT15	2	2
IT16	14	12
IT17	1	1
IT18	1	1
IT19	1	1
IT20	2	2
IT21	1	1
IT22	3	3
IT23	4	4
IT24	2	2
IT25	2	1
IT26	1	1
IT27	1	1
IT28	1	1
IT29	1	1
G1	38	26
G2	14	7
G?	6	6

two German dairy plants in which the same problem was found *(6)*.

The aim of this study was to assess the genetic relatedness of the isolates obtained from these cheeses and formulate possible explanations for the widespread contamination. We optimized a new pulsed-field gel electrophoresis (PFGE) protocol for the molecular characterization of *P. fluorescens* species. PFGE is considered the ''gold standard'' technique for evaluating molecular correlations between isolates from specimens collected in a defined time frame or from a defined geographic area to determine whether the isolates may have been derived from a common source *(18)*. Very few molecular studies on *P. fluorescens* have been conducted. Most studies of this pathogen have focused on nosocomial infections *(7, 19)*, and very little is known about food-contaminating strains.

MATERIALS AND METHODS

***P. fluorescens* strain collection.** Between June and September 2010, 181 *P. fluorescens* isolated at microbiological laboratories in Italy were collected. *P. fluorescens* strains were isolated from dairy products according to the ISO/TS 11059:2009 *(9)*. In all, 97% (176) isolates were obtained from mozzarella cheese samples; the remaining 3% (5 isolates) were obtained from whey cheese (2 isolates), stracchino cheese (1 isolate), primo sale cheese (1 isolate), and caciocavallo cheese (1 isolate). *P. fluorescens* was isolated in both spoiled and unspoiled samples of fresh cheeses: 90 isolates (49.8%) were from chromatically altered cheeses (82 isolates from blue cheese and 8 isolates from pink cheese), and 91 isolates (50.2%) were from unaltered samples. A total of 123 isolates from the products of 29 Italian dairy processing plants were analyzed. The other 58 isolates from products sold in Italy were identified as coming from two German processing plants.

A collection of all the *P. fluorescens* arriving at our laboratory was created, stored at −20°C in a cryobank, and analyzed together when the collection was complete.

PFGE protocol. All 181 collected isolates were characterized under the following protocol. All preliminary phases were conducted with the reference strain of *P. fluorescens* (NCTC 10038) to optimize a suitable protocol. The bacterial colonies were grown overnight on Columbia blood agar (Microbiol, Cagliari, Italy) at 37°C. A single colony was dissolved in citrate-buffered saline (100 mM Tris-HCl, 1 mM EDTA [pH 8]) to obtain a bacterial suspension that would guarantee a balance in enzymatic restriction activity. The cell suspension was adjusted to 0.4 and 0.5 optical density units at 600 nm with a GeneQuant *pro* UV/vis spectrophotometer (Biochrom Ltd., Cambridge, UK). Aliquots (200 μl) of 2% agarose gel (pulsed-field certified agarose, Bio-Rad Laboratories, Hercules, CA) in TBE buffer (44.5 mM Tris base, 44.5 boric acid, 1 mM EDTA [pH 8]) were prepared in 1.5-ml microcentrifuge tubes and kept in a dry heat block at 60°C. An equal volume (200 μl) of cell suspension was immediately added to the agarose tubes, and the agarose-cell mixture was gently mixed by pipetting two or three times.

A volume of about 100 μl was dispensed into reusable plug molds (Bio-Rad Laboratories), which were kept at 4°C for 10 min. The agarose plugs were digested into 2 ml of lysis solution (50 mM Tris-HCl, 50 mM EDTA [pH 8], 1% Sarcosyl) with 0.1 mg/ml proteinase K for 2 h at 55°C in a shaking water bath. The lysis suspension was carefully removed from the plugs by rinsing twice with 5 ml of sterile water and three times with 5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8]). The rinsing steps were performed at 55°C in a shaking water bath for 10 min each. After the final step, 1 ml of fresh TE buffer was added, and each plug was kept at 4°C until used.

Restriction endonuclease digestion was conducted on each plug with *Spe*I, a rare-cutting enzyme, according to previous studies *(7, 14)*. A digestion mix obtained with a final concentration of 1× MultiCore buffer (Promega, Madison, WI) was added to 4 units per reaction of restriction enzyme. Sterile water was used to reach the final volume (100 μl). The plug digestion mix was incubated overnight at 37°C according to the manufacturer's instructions. A 1% pulsed-field certified agarose gel in TBE buffer was prepared by carefully pouring the gel into the casting platform, avoiding any bubbles. Each well was loaded with half of the restricted plug, and all wells were sealed with agarose. The gel was placed in an electrophoresis chamber and covered with 2,000 ml of 0.5× TBE buffer. PFGE was performed using a CHEF Mapper (BioRad Laboratories) at 14°C and 6 V/cm^2 for 22 h. As described by Rainey et al. *(14)*, the initial and final switch times were 1 and 25 s, respectively. The gel was stained with 0.1 μl/ml GelGreen nucleic acid stain (Biotium, Hayward, CA) for 40 min, visualized under UV light, and photographed with a GelDoc XR (Bio-Rad Laboratories).

Evaluation of gels. The DNA band profiles were analyzed with BioNumerics software (version 6.1, Applied Maths, Sint-

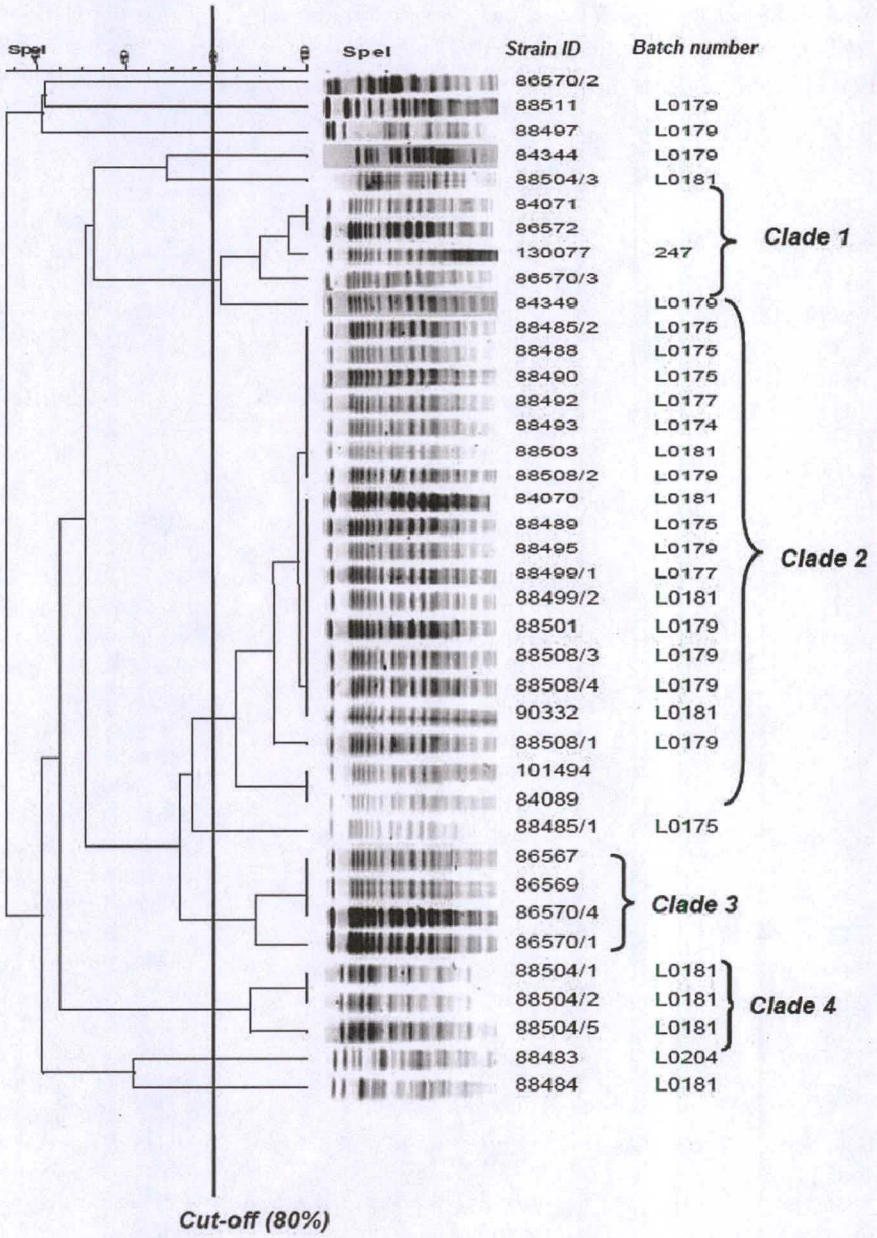

FIGURE 1. *Phylogenetic tree of isolates obtained from the IT1 plant.*

Martens-Latem, Belgium) using the Dice band-based method. Cluster analysis was done by the unweighted pair group method using arithmetic averages, with an optimization of 0.5% and a band tolerance of 1%. Because of the high discriminatory power of the PFGE protocol, the similarity cutoff value was set at 80%. According to other research *(1, 7, 18),* pulsotypes with similarities above this cutoff are considered genetically related. To analyze the collected data, all PFGE pulsotypes were assigned Roman numerals, from I to CXXXII; the Italian dairy plants were assigned an ''IT'' code, and the German plants were assigned a ''G'' code.

RESULTS

DNA band pattern analysis with BioNumerics software resulted in 132 pulsotypes; 113 of which included only one isolate. Each of the remaining 19 pulsotypes (I, IV, V, VI, X, XI, XVIII, XXII, XXIV, XXVII, XXIX, XXXII, XXXIV, XXXVI, XXXVIII, LI, LVI, LXV, and CXIX) included more than one isolate. Isolates belonging to the same pulsotype were derived from the same dairy plant, except for pulsotypes VI, XI, XVIII, and XXIV. Two dairy plants (IT1 and IT11) harbored pulsotypes VI and XI; pulsotype VI was also present at the IT9 plant. These three dairy plants were located in the same geographic area

TABLE 2. *Isolates from plant IT1 grouped according to batch number*[a]

Batch no.	No. of isolates	No. of pulsotypes
L0247	1	1
L0175	5	3
L0179	10	6
L0174	1	1
L0181	9	7
L0177	2	1
L024	1	1

[a] Ten of the 39 isolates were not included here because their batch number was unknown. The band pattern of these 10 isolates was evenly distributed among the other pulsotypes.

(northwestern Italy). Pulsotype XI was present also in one German plant (G1). Pulsotypes XVIII and XXIV were present in both German plants.

The similarity cutoff resulted in 11 clades of genetically related pulsotypes: 4 involving products from one Italian plant (IT1), one from one German plant, one from two geographically nearby Italian plants (IT7 and IT14), and 5 involving both the German plants.

The number of isolates from the products tested for each plant and the corresponding number of pulsotypes are reported in Table 1. Multiple pulsotypes were present in products from a single plant. Because of the lack of complete information from the dispatching laboratories, we were unable to determine the production plant for six German isolates (''G?'' in Table 1), each of which had a different pulsotype.

We also performed restricted data analysis on PFGE pulsotypes obtained from the dairy plant with the most isolates (IT1). The 39 isolates from samples obtained from the IT1 plant were grouped into 21 pulsotypes; 17 of these contained only one isolate and 6 contained more than one isolate (from 2 to 9). Setting the cutoff value at 80% similarity yielded four clades of genetically related strains (Fig. 1). The 39 isolates from the IT1 plant were then grouped according to the production batch number (Table 2), which revealed that multiple pulsotypes were recovered from single batches.

Regarding the presence of the blue color defect, PFGE analysis did not allow identification of the specific isolates responsible, i.e., isolates that produced the blue color defect and isolates that did not had the same pulsotype.

DISCUSSION

The results of the present study did not support either hypothesis advanced to explain *P. fluorescens* contamination of the ''blue mozzarella'': the high number of pulsotypes (132) did not support either the hypothesis for cross-contamination from German to Italian plants or for the presence of one environmental strain that spread in both countries.

Only four pulsotypes (VI, XI, XVIII, and XXIV) were found in more than one plant. Pulsotype VI was present in three geographically close plants (two of which also harbored pulsotype XI), and pulsotypes XVIII and XXIV were present in the two German plants. A retrospective epidemiological survey (which was beyond the scope of this study) could potentially determine possible common contamination sources at these plants, e.g., milk (from common livestock sources) or water supplies. Only one German isolate had a pulsotype (XI) in common with two Italian plants (IT1 and IT11), providing the only evidence for cross-contamination among cheeses in the two countries. Further investigation of the raw material exchange among these plants could provide further information; the import of contaminated raw material is the most plausible explanation for this cross-contamination. Introduction of contaminated raw milk into the production process can lead to contamination of working surfaces, the water used for processing, and/or the plant environment, which can result in postpasteurization contamination of the final cheese product *(4, 5, 8)*.

The results allow us to speculate that in dairy processing plants contamination from a variety of internal and external sources occurs as the same time. Each plant in this study harbored numerous *P. fluorescens* pulsotypes. However, of the 132 isolates, 113 had unique pulsotypes, i.e., these isolates came from different unrelated sources. In other words, the higher the number of samples collected from a given plant, the higher the number of pulsotypes isolated (Table 1).

Although the IT1 plant contained the highest number of pulsotypes (because it provided highest number of samples), the majority of pulsotypes were genetically unrelated, which suggests multiple sources of contamination of the final products, such as bulk milk and/or processing environments (e.g., water supply and working surfaces). Similar evidence emerged from the analysis of different batches from the same plant (plant IT1, Table 2), in each of which multiple unrelated pulsotypes were recovered.

Clades of genetically related pulsotypes were found in plant IT1 (Fig. 1), in the German plants, and in other two geographically close Italian plants, probably derived from common progenitors persisting in the plant environment.

No genetic differences between *P. fluorescens* isolates that produced the blue color and those that did not were identified by PFGE analysis, probably because PFGE does not target the genes encoding pigment-producing enzymes. The results of this study indicate that the ''blue mozzarella'' event was not caused by a single strain of *P. fluorescens* that spread massively and unusually; instead, it was caused by a common phenomenon probably highlighted by increased awareness and subsequent improvements in microbiological controls and analyses of fresh dairy products.

ACKNOWLEDGMENTS

This work was supported by the laboratories of the Italian Network of Istituti Zooprofilattici Sperimentali, which provided us with the P. fluorescens isolates. Special thanks are due to Drs. Marina Nadia Losio and Barbara Bertasi (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy) for their technical support.

REFERENCES

1. Barrett, T. J., P. Gerner-Smidt, and B. Swaminathan. 2006. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. *Foodborne Pathog. Dis.* 3:20–31.
2. Chen, L., R. M. Daniel, and T. Coolbear. 2003. Detection and impact of protease and lipase activities in milk and milk powders. *Int. Dairy J.* 13:255–275.
3. Cousin, M. A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. *J. Food Prot.* 45:172–207.
4. Dogan, B., and K. J. Boor. 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. *Appl. Environ. Microbiol.* 69:130–138.
5. Eneroth, A., A. Christiansson, J. Brendehaug, and G. Molin. 1998. Critical contamination sites in the production line of pasteurised milk, with reference to the psychrotrophic spoilage flora. *Int. Dairy J.* 8: 829–834.
6. European Commission. 2010. The rapid alert system for food and feed (RASFF). Annual report. 2010. Available at: http://ec.europa.eu/food/food/rapidalert/docs/rasff_annual_report_2010_en.pdf. Accessed 15 October 2012.
7. Gershman, M. D., D. J. Kennedy, J. Noble-Wang, C. Kim, J. Gullion, M. Kacica, B. Jensen, N. Pascoe, L. Saiman, J. McHale, M. Wilkins,

D. Schoonmaker-Boop, J. Clayton, M. Arduino, and A. Srinivasan. 2008. Multistate outbreak of *Pseudomonas fluorescens* bloodstream infection after exposure to contaminated heparinised saline flush prepared by a compounding pharmacy. *Clin. Infect. Dis.* 47:1372–1379.

8. Gruetmacher, T. J., and R. L. Bradley, Jr. 1999. Identification and control of processing variables that affect the quality and safety of fluid milk. *J. Food Prot.* 62:625–631.
9. International Organization for Standardization. 2009. Milk and milk products—method for the enumeration of *Pseudomonas* spp. ISO/TS 11059:2009 (IDF/RM 225: 2009). International Organization for Standardization, Geneva.
10. Krieg, N. 1984. Bergey's manual of systematic bacteriology, 1st ed., vol. I, sect. 4, p. 164–168. Williams & Wilkins, Baltimore.
11. Mayerhof, H. J., R. T. Marshall, C. H. White, and M. Lu. 1973. Characterization of a heat stable protease of *Pseudomonas fluorescens* P26. *Appl. Microbiol.* 25:44–48.
12. Meyer, J. M., and M. A. Abdallah. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J. Gen. Microbiol.* 107:319–328.
13. Palleroni, N. J., R. W. Ballard, E. Ralston, and M. Dondoroff. 1972. Deoxyribonucleic acid homologies in the genus *Pseudomonas*. *J. Bacteriol.* 23:333–339.
14. Rainey, P. B., M. J. Bailey, and I. P. Thompson. 1994. Phenotypic and genotypic diversity of fluorescent pseudomonads isolated from field-grown sugar beet. *Microbiology* 140:2315–2331.
15. Rajmohan, S., C. E. R. Dodd, and W. M. Waites. 2002. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. *J. Appl. Microbiol.* 93:205–213.
16. Riley, L. W. 2004. Molecular epidemiology of infectious diseases. principles and practices. ASM Press, Washington, DC.
17. Schokker, E. P., and M. A. J. S. Van Boekel. 1999. Kinetics of thermal inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F: influence of pH, calcium and protein. *J. Agric. Food Chem.* 47:1681–1686.
18. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33:2233–2239.
19. Terletskiy, V., G. Kuhn, P. Francioli, and D. S. Blanc. 2007. Application and evaluation of double digest selective label (DDSL) typing technique for *Pseudomonas aeruginosa* hospital isolates. *J. Microbiol. Methods* 72:283–287.
20. Wiedmann, M. D., D. Weilmeier, S. Dineen, R. Ralyea, and K. J. Boor. 2000. Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. *Appl. Environ. Microbiol.* 66:2085–2095.