A century of gray: A genomic locus found in 2 distinct *Pseudomonas* spp. is associated with historical and contemporary color defects in dairy products worldwide

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ABSTRACT

Some gram-negative bacteria, including *Pseudomonas* spp., can grow at refrigeration temperatures and cause flavor, odor, and texture defects in fluid milk. Historical and modern cases exist of gray and blue color defects in fluid milk due to *Pseudomonas*, and several recent reports have detailed fresh cheese spoilage associated with blue-pigment-forming *Pseudomonas*. Our goal was to investigate the genomes of pigmented *Pseudomonas* isolates responsible for historical and modern pigmented spoilage of dairy products in the United States to determine the genetic basis of pigment-forming phenotypes. We performed whole genome sequencing of 9 *Pseudomonas* isolates: 3 from recent incidents of gray-pigmented fluid milk (*Pseudomonas fluorescens* group), 1 from blue-pigmented cheese (*P. fluorescens* group), 2 from a historical blue milk spoilage incident (*Pseudomonas putida* group), and 3 with no evidence for blue or gray pigment formation (2 from *P. fluorescens* group and 1 from *Pseudomonas chlororaphis* group). All 6 isolates collected from products with a gray or blue pigment defect were confirmed to produce pigment using potato dextrose agar or pasteurized milk. A subset of 2 isolates was selected for inoculation into milk and onto the surface of a model cheese for subsequent color measurement. These isolates produced different colors on potato dextrose agar, but produced nearly identical color defects in milk and on model cheese. For the same subset of 2 isolates, the gray color defect in milk was produced only in containers with ample headspace and not in full containers, suggesting that oxygen is vital for pigment formation. This work also demonstrated

that a *Pseudomonas* isolate from cheese can produce a pigment defect in milk, and vice versa. Comparative genomics identified an accessory locus encoding tryptophan biosynthesis genes that was present in all isolates that produced gray or blue pigment under laboratory conditions and was only previously reported in 2 *P. fluorescens* isolates responsible for blue mozzarella in Italy. Because this locus was found in genetically distant isolates belonging to different *Pseudomonas* species groups, it may have been acquired via horizontal gene transfer. These data suggest that several past and present gray- or blue-pigmented dairy spoilage events share a common genetic etiology that transcends species-level identification and merits further investigation to determine mechanistic details and modes of prevention.

Key words: *Pseudomonas*, gray, blue, milk, cheese

INTRODUCTION

Visual spoilage in dairy products has a high potential for economic harm to manufacturers. The relationship between food companies and their consumers has been revolutionized by social media, because consumers now have a platform on which to broadcast images and videos of spoiled or otherwise defective products. In several cases, attention was drawn to large-scale food spoilage incidents via social media (Karp, 2010; Stones, 2013), but this phenomenon is not isolated to large-scale events. The threat to a food manufacturer or brand from a single spectacularly spoiled unit of product cannot be overlooked or underestimated, particularly because a nontrivial proportion of consumers interpret food spoilage as a safety concern. In annual surveys performed by the Food Marketing Institute from 1993 to 1997, 41 to 69% of consumers volunteered spoilage-related answers when asked about the greatest threats to the safety of the food they ate (Food Marketing Institute, 1997). In similar surveys conducted in 1999 and 2000, 20 and 19% of consumers volunteered spoiled or expired food as the single most important

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source of food poisoning (Food Marketing Institute, 2000). Although these surveys are somewhat old, they remain a reminder that significant dissonance may exist between manufacturer and consumer knowledge about the meaning of “spoilage,” a topic beyond the scope of this publication. It suggests that consumers may misinterpret the severity of spoilage events or the consumption of spoiled food, particularly when recalls occur. A 2017 survey reported that 13% of consumers said they would never purchase a specific food product again once they were aware that it had been recalled, although the survey question did not specify a cause for the recall (Food Marketing Institute, 2017).

Pigmented bacteria have a long history of causing visual spoilage in dairy foods. References to blue milk in German and French agricultural and scientific literature date back over 200 yr (Borowski, 1788; Parmentier and Deyeux, 1799). In 1884, the first inoculations of blue-pigmented bacteria into sterilized milk were performed, and the bacteria produced a blue pigment in raw milk, a gray pigment in neutral pH sterilized milk, and a blue-gray pigment in sterilized acidified milk (Hueppe, 1884). Blue milk from the United States was first described by Duckwall (1905), who isolated the causative strain, *Bacillus cyanogenus*, from a milk sample in Pennsylvania. Upon inoculation into sterilized milk, this isolate produced no acid or coagulation, but colored the milk “slate-gray, which turns blue with acids.” A 1914 report detailed an “outbreak” of blue milk in Iowa and included a detailed profile of the implicated organism, *Bacillus cyanogenes* (Hammer). Hammer described a gram-negative, motile, rod-shaped bacterium that produced a gray pigment in sterilized milk and a blue pigment in sterilized milk when cocultured with a lactic acid bacterium. Of note, although modern day *Bacillus* are exclusively gram-positive sporeformers, the generic name “*Bacillus*” was used more broadly in the past. These historical isolates no longer fall within the contemporary *Bacillus* genus.

Few reports exist of pigment defects in fluid milk in the last 100 years, which may be attributed to the widespread adoption of pasteurization for milk beginning in the early 20th century (Boor et al., 2017). A case of blue milk attributed to *Pseudomonas fluorescens* was reported in Germany in 2006 (Seiler, 2006), and a case of gray milk attributed to *Pseudomonas azotoformans* was reported by our group in 2017 (Evanowski et al., 2017). In both of these instances, pigment formation appeared to be dependent on the presence of oxygen.

There are also historical and contemporary reports of bacterial blue pigment in cheese. In the late 19th century, *Bacillus cyaneo-fuscus* contamination was blamed for blue Edam cheese in the Netherlands (Beyerinck, 1892), and blue cheese caused by *Bacillus cyanogenus*,

the same organism that caused blue milk, was also reported (Conn, 1892). *Pseudomonas fluorescens* biovar IV caused blue-pigmented spoilage of queso fresco cheese from a processor in the United States in 2006 (Martin et al., 2011), and in 2010, 70,000 units of mozzarella cheese produced at a facility in Germany and sold in Italy turned blue and were confiscated by Italian officials (Greenhalgh, 2010). Again, *P. fluorescens* was responsible, and indigoidine was suggested as the pigment causing the defect (Cantoni and Bersani, 2010; Sechi et al., 2011; Cenci-Goga et al., 2014). Multilocus sequence typing identified a “blue branch” of strains in the *P. fluorescens* phylogeny (Andreani et al., 2015b; Chierici et al., 2016). Leucoindigoidine was found to be a chemical marker of blue spoilage potential (Caputo et al., 2015), and a high-molecular-weight indigo derivative, rather than indigoidine, was suggested as the identity of the pigment (Andreani et al., 2015a).

Some recent investigations of the *Pseudomonas* responsible for blue color defect formation have used next-generation sequencing [i.e., whole-genome sequencing (WGS) and RNA-seq] to provide hypotheses for the metabolic pathways leading to blue pigment formation in cheese and for the possible identity of the pigment. Comparative genomics performed on whole genome sequences of 4 *Pseudomonas* strains, 2 of which produced blue pigment in cheese, revealed that the pigmented strains possessed additional accessory copies of genes involved in the tryptophan biosynthesis pathway, whereas nonpigmented strains had only 1 copy of each gene (Andreani et al., 2015a). An RNA-seq analysis performed on the same set of isolates showed an upregulation of iron uptake (e.g., pyoverdine), oxidoreductase, and transcriptional regulation genes in the pigmented strains that was not present in the unpigmented strains. Transposon-induced pigment-deficient mutants of Ps_77, a blue-pigment-producing strain of *P. fluorescens* isolated from beef, confirmed the involvement of the accessory copy of the tryptophan biosynthesis gene *trpB* in production of the blue phenotype (Andreani, 2016). Transposon-induced mutations in genes putatively involved in oxidative stress response, biofilm formation, and cell signaling were also able to eliminate the production of the blue pigment.

Our goal in this study was to investigate the gray- and blue-pigment-producing strains of *Pseudomonas* from our own culture collection, as well as publicly available blue-pigmented strains, to aid in the global effort to understand the mechanisms of gray and blue pigment production and ultimately work to prevent their formation in dairy products. To this end, we performed WGS and phenotypic evaluations on 3 recently obtained isolates of *Pseudomonas* from pasteurized milk with a gray defect, 1 recently obtained isolate from blue-pigmented

fresh cheese, 3 recently obtained isolates unassociated with these defects, and 2 isolates responsible for the historic production of blue and gray pigments in milk.

MATERIALS AND METHODS

Isolate Selection and Growth Conditions

We used *Pseudomonas* strains with and without pigmented phenotypes for comparative genomics. We selected 7 representative *Pseudomonas* strains that had been identified previously by partial sequencing of their 16S rDNA from the Milk Quality Improvement Program isolate collection (Table 1). These included 4 strains that had previously shown evidence of causing blue or gray color defects in dairy products and 3 that did not. The 3 isolates with no prior evidence for blue or gray pigment formation originated from dairy products or dairy processing environments: R5-0199 was selected specifically for its close partial 16S rDNA sequence match with the 4 recently obtained *P. fluorescens* group isolates associated with color defects. Additional details on the history and characterization of these isolates can be accessed via Food Microbe Tracker (<http://www.foodmicrobetracker.com>; Vangay et al., 2013).

Additionally, 2 *Pseudomonas* isolates were obtained from the USDA Agricultural Research Service Northern Regional Research Laboratory Culture Collection (<https://nrrl.ncaur.usda.gov/>). These 2 isolates, B-251 and B-252, were given laboratory IDs of FSL R10-3386 and FSL R10-3389, respectively (Table 1). Both isolates are reported to originate from unpasteurized blue milk obtained in Iowa in October 1913 (Hammer, 1914; Haynes, 1961). The lyophilized cultures were rehydrated in 2 mL of brain heart infusion (BHI) broth (BD Diagnostics, Sparks, MD), which was immediately inoculated onto BHI agar plates (BD Diagnostics). The plates were incubated at 28°C for 24 h. A single colony from each of these plates was inoculated onto a new BHI agar plate, which was incubated at 28°C for 24 h. An isolated colony from each of these plates was inoculated into 5 mL of BHI broth, and the broth was then incubated at 32°C for 18 h before freezing at −80°C in BHI broth supplemented with 15% glycerol for long-term storage.

For the milk and cheese inoculation experiments and for the WGS experiments described in the subsections that follow, the isolates were recovered from stocks of BHI broth with 15% glycerol stored at −80°C. The stocks were removed from storage, and a loopful of each was streaked onto BHI agar plates before the plates were incubated at 32°C for 48 h. A colony of each isolate was selected and inoculated into 5 mL of BHI broth, and the broth was then incubated without

Table 1. *Pseudomonas* isolates used in this study for phenotyping, genotyping, or both

Isolate	Taxonomic group	Phenotype	Isolate source	Isolation date	Isolation location	Reference
FSL E2-8864	<i>P. fluorescens</i> group	Gray pigment in fluid milk	Pasteurized fluid milk	July 2018	United States	This publication
FSL R10-2514	<i>P. fluorescens</i> group	Gray pigment in fluid milk	Pasteurized packaged 2% fat milk	June 2017	United States	This publication
FSL E2-0548	<i>P. fluorescens</i> group	Gray pigment in fluid milk; fluorescent and blue pigments in cheese	Pasteurized packaged whole milk	August 2015	United States	Evanowski et al., 2017
FSL W5-0203	<i>P. fluorescens</i> group	Gray pigment in fluid milk; fluorescent and blue pigments in cheese	In-process queso fresco cheese curds	September 2006	United States	Martin et al., 2011; Trmčić et al., 2015
FSL R10-3386 (NRRL B-251)	<i>P. putida</i> group	Gray pigment in sterilized fluid milk	Unpasteurized milk with blue discoloration	October 1913	Iowa, United States	Hammer, 1914; Haynes, 1961
FSL R10-3389 (NRRL B-252)	<i>P. putida</i> group	Gray pigment in sterilized fluid milk	Unpasteurized milk with blue discoloration	October 1913	Iowa, United States	Hammer, 1914; Haynes, 1961
FSL W5-0299	<i>P. fluorescens</i> group	Bright yellow pigment	Cheese vat agitator drain tube	October 2006	United States	This publication
FSL R5-0199	<i>P. fluorescens</i> group	No pigment	Pasteurized packaged 2% fat milk	July 2006	United States	Ranieri and Boor, 2009
FSL W7-0098	<i>P. chlororaphis</i> group	No pigment	Packaged pasteurized 2% fat milk	May 2008	United States	Van Tassel et al., 2012; Trmčić et al., 2015

shaking at 32°C for 18 to 24 h to produce a bacterial suspension of approximately 8 log cfu/mL.

Initial Color Defect Screening

To initially characterize pigment production for the 6 isolates that had previously been reported to produce blue or gray pigmentation, of our 9 total *Pseudomonas* isolates, we recovered isolates FSL E2-0548, FSL E2-8864, FSL R10-2514, FSL R10-3386, FSL R10-3389, and FSL W5-0203 from frozen glycerol stocks as described above, and a single colony from the BHI plate was streaked onto 2 sets of potato dextrose agar (PDA; BD Diagnostics) plates. The first set of plates was incubated at 6°C for 12 d, and the second set was incubated at 21°C for 5 d. Every 24 h during incubation, the plates were removed from their respective incubators and photographed using an EOS Rebel T3 DSLR camera (Canon, Tokyo, Japan) with the following settings: f/5.6, 1/60 s exposure, ISO-800, 55 mm focal length, compulsory flash, and fluorescent white balance.

Based on the results of the screening on PDA, we performed further screening of the 6 isolates selected above, along with FSL R5-0199, an unpigmented strain, in pasteurized milk. Pasteurized, homogenized 2% fat milk in 473-mL (16 US fluid ounce) containers was purchased from a local supermarket, and half of the milk (approximately 230 mL) was aseptically removed from each container. We prepared 18-h bacterial suspensions of the selected isolates as described previously, and a 1-mL aliquot of each was centrifuged at $18,000 \times g$ for 1 min. The supernatants were removed, and the pellets were resuspended in 1 mL of pasteurized milk. Each 1-mL bacterial suspension was then added to a prepared half-full container of milk. The containers were sealed, gently inverted to mix their contents, and incubated at 21°C for 96 h. After 48, 72, and 96 h, photographs were taken using the same camera settings as described above for the PDA photographs, but without the camera flash.

Milk and Cheese Color Defect Assessment

Based on the results of the preliminary screenings performed on PDA and in milk, we selected a subset of 2 pigment-producing isolates (FSL E2-0548 and FSL W5-0203) and 1 negative control isolate (FSL W5-0299) for further analysis of pigment formation in milk and on cheese. We specifically selected FSL E2-0548 and FSL W5-0203 because they originated from different matrices (fluid milk and cheese), and because they exhibited different color phenotypes on PDA at 6°C. Selection of these 2 isolates also allowed us to test the hypothesis that the same isolates can cause

color defects in both cheese and fluid milk. We selected FSL W5-0299 as the negative control because it did not exhibit blue or gray pigmentation on cheese, but yellow (N. H. Martin, unpublished data). Milk samples for color defect assessment were produced as described by Evanowski et al. (2017). Briefly, a 1-mL aliquot of an overnight bacterial suspension of each isolate was pelleted by centrifugation and resuspended and serially diluted in PBS (Weber Scientific, Hamilton, NJ). To inoculate, 500 μ L of each appropriate dilution was added to 2 half-filled 946-mL (1 US quart) high-density polyethylene containers of pasteurized, homogenized 2% fat milk (Cornell University Dairy, Ithaca, NY) and 1 mL of each appropriate dilution was added to 2 completely filled 946-mL high-density polyethylene containers of pasteurized, homogenized 2% fat milk. This achieved an initial inoculum concentration of approximately 3.75 log cfu/mL in both the filled and half-filled containers. We determined SPC by spiral plating (Autoplate 5000; Advanced Instruments, Norwood, MA) immediately after inoculation and after 14 d of storage at 6°C to verify the growth of the inoculum. We selected an incubation temperature of 6°C for both the milk and the cheese, because this represented the semi-abusive conditions that dairy products may realistically encounter in the homes of consumers (Kosa et al., 2007). To determine color change over time, we applied the approach used by Evanowski et al. (2017). Briefly, sample $L^*a^*b^*$ coordinates were measured immediately after inoculation and after 14 d of storage using a HunterLab ColorQuest XE colorimeter (Hunter Associates Laboratory, Reston, VA). In the $L^*a^*b^*$ color space, L^* represents lightness ($L^* = 0$ is black; $L^* = 100$ is a diffuse white), a^* represents the position between green and red (negative values are green; positive values are red), and b^* represents the position between blue and yellow (negative values are blue; positive values are yellow).

We used laboratory-scale mozzarella-style cheese made from 1.9 L (0.5 US gallon) of locally obtained pasteurized, homogenized whole milk for color defect assessment. The cheese was prepared as described previously by Carroll (2002). The milk was heated to 13°C, and 60 mL of 360 mM citric acid solution (Fisher Chemical, Fair Lawn, NJ) was stirred in. The milk was further heated to 32°C, and a solution containing 616 μ L of prepared vegetable rennet (mucorpepsin; New England Cheesemaking Supply Company, South Deerfield, MA) in 30 mL of deionized water was added with gentle stirring. The milk was allowed to coagulate without stirring for 5 min while heating to 40°C before the curd was drained. The curd was then heated in a 1,100 W microwave oven set at full power for 1 min, followed by kneading with gloved hands. Next, the curd was heated for 30 s and kneaded for a second time. Finally,

the curd was heated for 30 s, and 1.18 g of granulated sodium chloride (Fisher Chemical) was added before kneading again. The resulting molten cheese was flattened into a disk approximately 1 cm in height before it was chilled in an ice-water bath and stored at 4°C in a sealed plastic bag to await inoculation. To inoculate, 1-mL bacterial suspensions prepared from overnight cultures of isolates FSL E2-0548, FSL W5-0203, and FSL W5-0299, were pelleted in a microcentrifuge at $18,000 \times g$ for 1 min and resuspended in 1 mL of PBS. Pieces of the prepared cheese approximately 4.5 cm in diameter and 1 cm in height were placed into sterile 90-mm-diameter polystyrene Petri dishes and spot-inoculated with 50 μ L of the bacterial suspensions. The dishes were covered and stored at 6°C for 7 d. At 2 d and 7 d after inoculation, photographs of the cheese samples were taken using an EOS Rebel T3 DSLR camera (Canon) in a darkened room and illuminated by (1) the camera flash (f/5.6, 1/60 s exposure, ISO-800, 55 mm focal length) and (2) a 6 W, 365-nm wavelength UV-A lamp (VWR, Radnor, PA) with full automatic camera settings. We performed color analysis of the photographed cheese samples essentially as described by Yam and Papadakis (2004). Briefly, the pigmented area of the cheese (or the entire surface of the negative control cheese) was selected in Photoshop (CC 2018; Adobe Systems, San Jose, CA), and the histogram window was used to obtain the mean, standard deviation, and median L, a, and b values for the selected area, which were then converted to standard L*, a*, and b* coordinates. For both the inoculated milk and cheese, L*a*b* coordinates across strains and treatments were visualized using ggplot2 (Wickham, 2016).

DNA Extraction and Whole-Genome Sequencing

Extraction of genomic DNA and WGS was performed using select modifications of the procedure described by

Kovac et al. (2016) for all 9 *Pseudomonas* isolates listed in Table 1. Briefly, genomic DNA was extracted and purified from the prepared bacterial suspensions using a QIAamp DNA MiniKit (Qiagen, Valencia, CA) and checked for concentration and purity using a Nanodrop spectrophotometer and a Qubit dsDNA high sensitivity kit (both ThermoFisher Scientific, Waltham, MA). We submitted DNA samples to the Cornell University Institute of Biotechnology Genomics facility, where libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). Samples were pooled and sequenced in 2 Illumina HiSeq 2500 rapid runs with 2×100 -bp paired-end reads or in an Illumina MiSeq with 2×250 -bp paired-end reads. The detailed protocol for genomic DNA extraction and quality analysis for WGS can be accessed at <https://cornell.box.com/v/Reichler2018b>.

Quality Checks, Genome Assembly, Genome Annotation, and Assessment of Orthology

Raw sequence reads were checked for quality using FASTQC v 0.11.5 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and trimmed using Trimmomatic version 0.36 (Bolger et al., 2014). After trimming, sequence reads were rechecked for quality and then used for de novo genome assembly. Genomes were assembled using SPAdes version 3.6.2 or 3.10.1 (Bankevich et al., 2012) using the “-careful” parameter. Final draft assemblies were annotated using Prokka v 1.12 (Seemann, 2014). Orthologs were identified using the program Roary (Page et al., 2015). An identity threshold of 50% was used to account for intrinsic diversity in the isolate set. Whole genome assemblies were deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) under BioProject PRJNA341280. Accession IDs for individual isolates can be found in Table 2.

Table 2. Summary of *Pseudomonas* whole-genome metrics

Strain	Genome size ¹ (bp)	% GC	No. of contigs	N50 ²	Average coverage	GenBank accession ID
FSL E2-8864	6,755,291	59.8	188	95,881	23	RWKC000000000
FSL R10-2514	6,397,553	59.9	57	260,661	108	RWKC000000000
FSL E2-0548	6,901,852	59.6	260	74,043	26	RXHZ000000000
FSL W5-0203	6,284,734	59.7	114	136,285	177	RXHY000000000
FSL R10-3386	6,068,646	62.6	91	175,121	46	RWKE000000000
FSL R10-3389	6,087,214	62.5	87	175,140	43	RWKF000000000
FSL W5-0299	7,139,722	59.2	137	156,053	83	RXHX000000000
FSL R5-0199	6,659,123	59.5	106	194,733	111	RXHV000000000
FSL W7-0098	5,026,399	58.3	35	449,785	145	RXHW000000000

¹This represents the combined size of all contigs.

²N50 = weighted median statistic such that 50% of the entire assembly is contained in contigs or scaffolds of a length equal to or larger than this value.

Phylogenetic Analysis

We obtained the 16S rDNA sequences of 164 *Pseudomonas* type strains from the Ribosomal Database Project (Cole et al., 2009). The 16S rDNA sequences from the 9 newly sequenced *Pseudomonas* isolates in this study were extracted from their whole genome sequences using RNAmmer (Lagesen et al., 2007). These sequences were aligned using MUSCLE (Edgar, 2004) and used to generate a maximum-likelihood tree in RAxML (Stamatakis, 2014) with the General Time Reversible nucleotide substitution model, an optimization of substitution rates (gamma model of rate heterogeneity), and estimation of proportions of invariable sites. To measure node confidence, 1,000 bootstraps were generated.

Whole genome sequence assemblies of 52 *Pseudomonas* type strains (i.e., denoted as type strains by the American Type Culture Collection, Deutsche Sammlung von Mikroorganismen und Zellkulturen, or other collections) were downloaded from <http://www.pseudomonas.com>. We also obtained 4 whole genome sequences of pigmented and unpigmented *Pseudomonas* strains (Ps_20, Ps_22, Ps_40, and Ps_77; generated by Andreani et al. (2015a) from GenBank. The Ps_22 and Ps_77 strains were isolated in Italy and the United Kingdom, respectively, and are associated with blue color defects similar to those observed for FSL W5-0203. The Ps_20 and Ps_40 strains were 2 isolates that did not produce the color defect and were used for comparison with Ps_22 and Ps_77. We used these sequences along with the whole genome sequences generated in this study to construct an Average Nucleotide Identity by Basic Local Alignment Search Tool (ANiB) distance matrix using pyani (<https://github.com/widdowquinn/pyani>). This distance matrix was visualized as a dendrogram using the gg dendro package (de Vries and Ripley, 2016) in R (R Core Team, 2018). To confirm the phylogeny of a subset of closely related isolates (5 newly sequenced isolates, 4 previously sequenced isolates, type strain *Pseudomonas lactis* DSM 29167, and type strain *Pseudomonas paralactis* DSM 29164) with higher resolution than the ANiB dendrogram, we constructed a maximum parsimony tree from their pan-genome SNPs using kSNP3 (Gardner et al., 2015) with a k-mer size of 19.

Predicted amino acid sequences annotated as the *trpA*, *trpB*, *trpC*, *trpD*, and *trpF* genes were extracted from the 9 newly sequenced genomes and the 4 previously sequenced genomes. These sequences were used to construct maximum-likelihood evolutionary trees for each gene based on the Jones–Taylor–Thornton matrix-based model (Jones et al., 1992) implemented in MEGA

X (Kumar et al., 2018). We used a discrete Gamma distribution with 5 categories to model evolutionary rate differences among sites; all positions containing gaps and missing data were eliminated, and 1,000 bootstrap replicates were performed.

Hidden Markov Models

We downloaded hidden Markov models for proteins hypothesized to be involved in the biosynthesis of pyoverdine, pyocyanin, tryptophan, indigoidine, violacein, and blue copper-binding proteins (see Supplemental Table S1 for models included; <https://doi.org/10.3168/jds.2018-16192>) from the Pfam database (Finn et al., 2016). We used these models to search for their targeted protein families or domains in the annotated predicted protein sequences of the 9 newly sequenced *Pseudomonas* isolates using HMMER v. 3.1b2 (<http://hmmerr.org>) in maximum sensitivity mode. An E-value of $<1 \times 10^{-3}$ for one or more predicted proteins from an isolate was considered significant evidence of the presence of a given protein family or domain for that isolate.

RESULTS

Color Defect Screening on PDA and in Milk

We initially screened the 6 isolates associated with either a gray color defect in milk or a blue color defect in cheese for color formation on PDA (incubated at 6 or 21°C) and in pasteurized milk. On PDA incubated at 21°C, 4 isolates (FSL E2-8864, FSL E2-0548, FSL W5-0203, and FSL R10-2514) showed clearly visible color development after 3 d of incubation, whereas the other 2 isolates showed either no color development (FSL R10-3389) or very weak orange coloration (FSL R10-3386) after 3 and 5 d (Figure 1). The 4 isolates with clear color development showed varied intensities of gray or gray-orange to gray-blue color; isolate FSL E2-8864 showed the weakest color (defined by the <http://html-color-codes.info> web tool as gray-orange after both 3 d and 5 d of incubation) and FSL W5-0203 showed the darkest color (defined as dark gray-blue after both 3 d and 5 d of incubation). Consistent with the data obtained for the same group of isolates at 21°C, incubation of isolates on PDA at 6°C also identified a group of 4 isolates (FSL E2-8864, FSL E2-0548, FSL W5-0203, and FSL R10-2514) with clear color development after 12 d of incubation and a group of 2 isolates (FSL R10-3386 and FSL R10-3389) with very weak growth after 12 d at 6°C and no evidence of color formation (Figure 2). Interestingly, the 4 isolates with

Figure 1. Growth and color phenotype of pigment-associated isolates of *Pseudomonas* on potato dextrose agar after incubation at 21°C for 1, 3, and 5 d.

clear color development showed a wider range of color when incubated at 6°C than when incubated at 21°C: colors displayed at 6°C ranged from light gray-violet (FSL E2-8864) and dark gray-blue (FSL R10-2514) to very dark reddish-brown (FSL E2-0548) and dark blue (FSL W5-0203). Color formation was evident for FSL R10-2514 after 3 d incubation and for FSL E2-8864, FSL E2-0548, and FSL W5-0203 by 6 d incubation. Over time, the color on the plates intensified but did not appear to change from one color to another (e.g., from blue to brown).

All 6 isolates additionally showed evidence of gray pigment formation in pasteurized milk after 3 d at 21°C (Figure 3). As well, FSL E2-8864, FSL R10-2514, FSL E2-0548, and FSL W5-0203 exhibited fluorescence under UV light (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-16192>). Extensive proteolysis, evidenced by coagulation and separation of the milk samples, appeared after 3 d of incubation and became more pronounced after 4 d of incubation. An uninoculated milk sample showed neither color change nor coagulation after 4 d incubation.

Figure 2. Growth and color phenotype of pigment-associated isolates of *Pseudomonas* on potato dextrose agar after incubation at 6°C for 3, 6, and 12 d.

Color Defect Demonstration and Measurement in Milk and Cheese

We selected 2 blue- or gray-pigmented isolates and 1 yellow-pigmented isolate for more detailed analysis of color defects produced in milk and on cheese. All 3 isolates were inoculated into filled and half-filled containers of pasteurized, homogenized, 2% milk followed by incubation at 6°C for 14 d. Milk from full containers that were inoculated with FSL E2-0548 or FSL W5-0203 were not substantially different in L*a*b* coordinates from uninoculated milk in half-full or full containers,

or from milk inoculated with the control strain FSL W5-0299 in half-full or full containers. This indicated no detectable color change (Figure 4a). However, half-full containers inoculated with FSL E2-0548 and FSL W5-0203 exhibited decreased values in L*a*b* relative to the uninoculated half-full container control. These decreased values indicated that these samples were darker, greener, and less yellow than the uninoculated negative control sample. Only small differences existed between the L*a*b* coordinates of FSL E2-0548 and FSL W5-0203, but all coordinate values were numerically lower for FSL E2-0548 than they were for FSL

W5-0203. Half-full and full containers of milk inoculated with these 3 *Pseudomonas* isolates showed SPC between 7.3 and 8.5 log cfu/mL after 14 d of incubation (data not shown). All isolates reached a higher plate count in half-filled containers than they did in full containers, and the population difference between full and half-full containers ranged from 0.3 to 1.1 log cfu/mL. The SPC of the full and half-full uninoculated control containers did not increase over the 14 d of incubation.

When the same 3 isolates were inoculated onto the surface of mozzarella-style cheese followed by incubation at 6°C for 2 d, blue pigment was present on the cheeses inoculated with FSL E2-0548 and FSL W5-0203 when viewed under white light, and the pigmented areas fluoresced under UV light (Figure 5). The cheese inoculated with the control strain FSL W5-0299 and the uninoculated (negative control) cheese showed no visible pigmentation under white light and did not fluoresce under UV light after 2 d. After 7 d of incubation, dark gray-blue pigmentation was visible on the cheeses inoculated with FSL E2-0548 and FSL W5-0203 under white light, but only the pigmented areas on the cheese inoculated with FSL W5-0203 fluoresced under UV light. The cheese inoculated with FSL W5-0299 had a yellow pigment after 7 d of incubation, and the pigmented areas fluoresced under UV light.

The pigmented regions of photographs of the cheese inoculated with FSL E2-0548 and FSL W5-0203 both exhibited decreased values of $L^*a^*b^*$ when compared with the negative control, indicating that they were darker, greener, and less yellow than the control cheese (Figure 4b). Cheese inoculated with FSL E2-0548 was lower in L^* and b^* and higher in a^* than cheese inoculated with FSL W5-0203, indicating that FSL E2-0548 produced a pigment that was darker, less green, and bluer than that produced by FSL W5-0203. The pigmented regions of cheese inoculated with FSL W5-0299 exhibited a decreased value of L^* and increased values of a^* and b^* compared with the negative control, indicating that they were darker, redder, and yellower than the control cheese.

WGS Metrics

The WGS of the 9 isolates yielded genomes that ranged from 5,026,339 bp (FSL W7-0098) to 7,139,722 bp (FSL W5-0299; Table 2). The GC content ranged from 58.3% to 62.6%. The average coverage achieved was lowest for FSL E2-8864 with 23× and highest for FSL W5-0203 with 177×. The number of contigs in the final high-quality draft genomes ranged from 35 (FSL W7-0098) to 260 (FSL E2-0548), and the N50 (defined

Figure 3. Growth of pigment-associated isolates of *Pseudomonas* in pasteurized, homogenized 2% fat milk after incubation in half-filled containers at 21°C for 2, 3, and 4 d.

as a weighted median statistic such that 50% of the entire assembly is contained in contigs or scaffolds of a length equal to or larger than this value) ranged from 74,043 bp (FSL E2-0548) to 194,733 bp (FSL R5-0199).

Phylogenetic Analysis of 16S rDNA Sequences

Initial phylogenetic characterization of the 6 *Pseudomonas* isolates previously linked to blue or gray color

defects in dairy products and that were characterized by the initial tests on PDA and in fluid milk showed that these isolates represented 2 distinct clusters in a 16S rDNA phylogenetic tree (Supplemental Figure S2; <https://doi.org/10.3168/jds.2018-16192>). Of these isolates, 4 (FSL W5-0203, FSL E2-8864, FSL R10-2514, and FSL E2-0548) represented a monophyletic clade that clustered with the 16S rDNA sequence for the *P. azotoformans* type strain. The other 2 isolates previ-

Figure 4. Color (expressed as L*a*b* coordinates; L* = lightness, a* = red-green color, b* = yellow-blue color) of (A) pasteurized fluid milk after inoculation with pigment-associated strains of *Pseudomonas* and incubation at 6°C for 14 d, and (B) mozzarella-style cheese after inoculation with pigment-associated strains of *Pseudomonas* and incubation at 6°C for 7 d.

ously linked to blue or gray color defects in dairy products (FSL R10-3386 and FSL R10-3389) also clustered together but were very distinct from the clade described above and formed a monophyletic clade with the *P. putida* type strain. Low bootstrap support (<15%) was generally achieved throughout the tree, which suggests that the 16S sequences may not provide enough resolution to clearly delineate *Pseudomonas* species, including among the isolates characterized here.

The 3 control isolates, which had not been linked to blue or gray color defects and were included in our WGS to allow for comparative genomics approaches, were also included in the 16S rDNA phylogeny. Among these 3 isolates, FSL R5-0199 grouped into the same monophyletic clade that included the *P. azotoformans* type strain and the 4 *Pseudomonas* isolates previously linked to blue or gray color defects in dairy products. The other 2 control isolates clustered separately from

Figure 5. Photographs under white and UV light of pigment-associated *Pseudomonas* isolates inoculated onto mozzarella-style cheese and incubated at 6°C for 2 and 7 d. Red dashed lines represent the areas in each image from d 7 that were selected for color analysis (results in Figure 4).

each other and from the other 7 isolates characterized here. The closest relatives of FSL W7-0098 were *P. fragi* and *P. psychrophila*, whereas FSL W5-0299 did not cluster with any of the *Pseudomonas* type strains included in the Ribosomal Database Project.

ANiB Data Analysis and Clustering

To facilitate better species classification than is possible with 16S rDNA data alone, we constructed an ANiB dendrogram using WGS data (Figure 6). This dendrogram confirmed that the 6 *Pseudomonas* isolates previously linked to blue or gray color defects in dairy products represented 2 distinct groups. The 4 newly sequenced pigment-forming isolates that grouped with *P. azotoformans* based on 16S rDNA sequence data grouped with (1) the *P. lactis* type strain (the most closely related type strain based on ANiB data); (2) our control strain FSL R5-0199; (3) 2 other European strains (Ps_22 and Ps_77) previously reported to cause blue color defects in food; and (4) 2 European strains (Ps_20 and Ps_40) that had not been shown to cause blue color defects when inoculated into cheese. However, the ANiB dendrogram also indicated that this group of 10 isolates represented 2 clusters: cluster I with 4 isolates (including the *P. lactis* type strain DSM 29167) that were all within a pairwise ANiB of >95.2%, and cluster II with 6 isolates (all within a pairwise ANiB of >95.0%) that further separated into 2 distinct subclusters (IIa and IIb) of 3 isolates each. In subcluster IIa, pigmented isolate FSL E2-0548 clustered closely with Italian isolates Ps_20 and Ps_22 (unpigmented and pigmented, respectively; ANiB >98.2%). Notably, FSL E2-0548 and Ps_22 shared >99.7% ANiB. In subcluster IIb, FSL W5-0203 (pigmented) and FSL R5-0199 (unpigmented) clustered closely with each other and with the pigmented isolate Ps_77 from the United Kingdom (ANiB >98.7%). In cluster I, the pigmented isolates FSL E2-8864 and FSL R10-2514 clustered with the unpigmented Italian isolate Ps_40 and with the unpigmented type strain *P. lactis* DSM 29167 (ANiB >95.2%). The 9 non-type strain isolates from clusters I and II had ANiB ranging from 94.2 to 95.9% with the type strain *Pseudomonas lactis* DSM 29167, and they therefore fell close to the lower bound of the suggested average nucleotide identity for species delineation of 95 to 96% (Richter and Rosselló-Móra, 2009). The ANiB of these 9 isolates with the type strain *P. azotoformans* (the closest match in the 16S rDNA phylogenetic tree) ranged from 86.7 to 87.1%, and the ANiB with the type strain *P. fluorescens* DSM 50090 ranged from 86.4 to 86.8%. To determine whether or not the isolates from clusters I and II would have been identified as *P. lactis* had its 16S rDNA sequence been included in the Ribosomal

Database Project, we constructed a second 16S rDNA maximum-likelihood tree using the 5 newly sequenced isolates from clusters I and II, the 7 *Pseudomonas* type strains from the Ribosomal Database Project that clustered with the newly sequenced isolates, and *P. lactis* DSM 29167 (Supplemental Figure S3; <https://doi.org/10.3168/jds.2018-16192>). *P. lactis* DSM 29167 clustered more closely with the newly sequenced isolates than the *P. azotoformans* type strain, indicating that the 5 newly sequenced isolates were more closely related to *P. lactis* than to *P. azotoformans* by 16S rDNA as well. A maximum parsimony tree constructed using the pan-genome SNPs from the isolates in clusters I and II supported the groupings of the ANiB dendrogram with more resolution (Supplemental Figure S4; <https://doi.org/10.3168/jds.2018-16192>).

The ANiB dendrogram also confirmed that 2 additional *Pseudomonas* isolates previously linked to blue or gray color defects clustered closely to *P. putida* (consistent with the 16S rDNA-based clustering). Isolates FSL R10-3386 and FSL R10-3389 clustered very closely with each other (ANiB >99.9%) and showed an ANiB of 94.7% relative to the type strain *P. putida* NBRC 14164, which places these isolates close to the lower bound for species delineation.

Genes Associated with the Gray Color Defect in Milk and the Blue Color Defect in Cheese

We used subtractive genomics to identify genes that were specifically present in the 4 newly sequenced *P. cf. lactis* isolates (FSL E2-0548, FSL W5-0203, FSL E2-8864, and FSL R10-2514) and in the 2 newly sequenced *P. putida* isolates (FSL R10-3386 and FSL R10-3389) but missing from the other 3 newly sequenced isolates (FSL W7-0098, FSL R5-0199, and FSL W5-0299). We identified 18 genes using this approach (Supplemental Table S2; <https://doi.org/10.3168/jds.2018-16192>). Among these, 14 genes (including *trpA*, *trpB*, *trpC*, *trpD*, and *trpF*) were in the same locus and were annotated with functions mainly associated with the tryptophan biosynthesis pathway. An additional 2 genes were found in this locus only among the 4 pigmented *P. cf. lactis* isolates and were not found in the 2 pigmented *P. putida* isolates. The genes in the *trpABCDF* operon are involved in all reactions carried out in the tryptophan biosynthesis pathway, beginning from anthranilate (Figure 7). Interestingly, further analyses found that these 5 genes showed homology to 5 divergent genes, present in all genomes analyzed, where they are separated into 3 distinct loci containing 2 (*trpAB*), 1 (*trpF*), and 2 (*trpCD*) genes. Also present in the 6 pigmented isolates sequenced here and absent in the unpigmented isolates were genes encoding toxin and antitoxin pro-

Figure 6. Dendrogram of the whole-genome-sequenced *Pseudomonas* isolates generated using the whole-genome average nucleotide identity by basic local alignment search tool (ANIb) distance matrix. Newly sequenced isolates are in bold text, and isolates known to produce a gray or blue pigment are in blue text. The red vertical line represents 95% ANIb, and the numbers along the horizontal axis represent pairwise nucleotide distance. Clusters and subclusters identified in the *Pseudomonas fluorescens* group are denoted using curly braces.

A

Locus	Gene name	Annotation
FSL_E2-0548_02093	<i>wbpA</i>	UDP-N-acetyl-D-glucosamine 6-dehydrogenase
FSL_E2-0548_02094	<i>trpD</i>	Anthranilate phosphoribosyltransferase
FSL_E2-0548_02095	<i>trpF</i>	N-(5'-phosphoribosyl)anthranilate isomerase
FSL_E2-0548_02096	<i>trpA</i>	Tryptophan synthase alpha chain
FSL_E2-0548_02097	<i>trpB</i>	Tryptophan synthase beta chain
FSL_E2-0548_02098	group_6931	hypothetical protein
FSL_E2-0548_02099	<i>gfo</i>	Glucose--fructose oxidoreductase
FSL_E2-0548_02100	<i>yjmC</i>	putative oxidoreductase YjmC
FSL_E2-0548_02101	<i>lysK</i>	[LysW]-lysine hydrolase
FSL_E2-0548_02102	<i>ioIE</i>	Inosose dehydratase
FSL_E2-0548_02103	<i>wbpE</i>	UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase
FSL_E2-0548_02104	<i>wbpE</i>	UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase
FSL_E2-0548_02106	<i>trpC</i>	Indole-3-glycerol phosphate synthase
FSL_E2-0548_02108	<i>rhtB</i>	Homoserine/homoserine lactone efflux protein

Figure 7. The putative roles of accessory tryptophan biosynthesis genes found in *Pseudomonas* isolate FSL E2-0548 in the tryptophan and indigo biosynthetic pathways. All pigmented *Pseudomonas* isolates possessed these accessory genes.

teins (*ChpB-ChpS*) and an L-homoserine lactone exporter (*rhtB*).

Evolution of Genes Involved Both in Tryptophan Biosynthesis and Indigo Biosynthesis

As was indicated by our own data from the preceding section and by a previous study on 2 *Pseudomonas* isolates linked to a blue color defect in food products (Andreani et al., 2015a), there appears to be a linkage between blue or gray color phenotypes and the presence of genes encoding part of the tryptophan biosynthesis pathway. The 8 pigmented isolates, FSL E2-8864, FSL R10-2514, FSL E2-0548, FSL W5-0203, FSL R10-3386, FSL R10-3389, Ps_22, and Ps_77, harbored 2 copies of *trpA*, *trpB*, *trpC*, *trpD*, and *trpF*; the other 5 isolates, which showed no color defect, harbored 1 copy of each gene. Maximum-likelihood trees created from multiple alignments of the predicted amino acid sequences of the genes showed that 1 of the 2 copies of the *trpA*, *trpB*, *trpC*, *trpD*, and *trpF* genes from all 8 of the pigmented isolates clustered together (Figures 8a–e). To differentiate the 2 distinct copies of these genes, we designated the *trp* genes found in all isolates as *trpA*-1 through *trpF*-1, and we designated the *trp* genes found only in the pigmented isolates as *trpA*-2 through *trpF*-2. We did not include Ps_22 in the *trpB* tree because its *trpB*-2 sequence was incompletely sequenced in the previously published Ps_22 genome (Andreani et al., 2015a); *trpA*-2, *trpC*-2, *trpD*-2, and *trpF*-2 for Ps_22 clustered with the remaining 7 pigmented isolates as detailed below. With this exception, in all 5 gene trees, 1 copy of each gene consistently grouped the 8 pigmented isolates together in a cluster consisting of 1 branch (*trpD*-2) or 2 branches (*trpA*-2, *trpB*-2, *trpC*-2, and *trpF*-2). In cases of 2 branches, 1 branch consisted of *P. cf. lactis* isolates FSL E2-8864, FSL R10-2514, FSL E2-0548, FSL W5-0203, Ps_22, and Ps_77, and 1 branch consisted of *P. putida* isolates FSL R10-3386 and FSL R10-3389. For *trpA*-1, *trpB*-1, *trpC*-1, *trpD*-1, and *trpF*-1, all of which were found in all 13 isolates, the genes for pigmented and unpigmented isolates did not represent separate clusters.

Hidden Markov Models

We ran hidden Markov models for 13 protein families or domains on the translated protein-coding sequences from the whole genome sequences of each of the 9 newly sequenced *Pseudomonas* isolates. We found significant evidence using 6 of the models for the presence of protein families or domains associated with pyocyanin, tryptophan, and plastocyanin copper-binding proteins in all 9 genomes tested, whether from pigmented or

unpigmented isolates. We found a lack of evidence for the presence of 5 other protein families or domains that were associated with pyoverdine, indigoidine, or violacein biosynthesis in all 9 tested genomes. The remaining 2 models, a protein family encoding the N-terminal of a protein putatively involved in pyoverdine biosynthesis (*HpaB*) and a protein domain encoding membrane-bound blue copper proteins (*CopC*), were present in 1 or more predicted proteins for 7 of the 9 isolates (*P. putida* isolates FSL R10-3386 and FSL R10-3389 were missing these predicted proteins). Overall, the hidden Markov models failed to detect any protein families or domains associated with pigment production that were present only in the pigmented isolates and absent in the unpigmented isolates. Corroborating the results found in the comparative genomics analysis, 2 copies of predicted tryptophan biosynthesis domain *Trp_syntA* were found in pigmented isolates, whereas only 1 copy was found in unpigmented isolates.

DISCUSSION

A Diversity of *Pseudomonas* Can Cause Gray Defects in Fluid Milk

The *Pseudomonas* spp. that can cause either a gray color defect in milk or a blue color defect in cheese can represent at least 2 very distinct taxonomic groups, including (1) a group that we can currently classify as *P. cf. lactis* [we used the designation *cf.* (confer) because these isolates are closely related to the *P. lactis* type strain but border the currently accepted cutoff for speciation by whole genome average nucleotide identity] and (2) a group that we can likely classify as *P. putida*. These findings illustrate the improved ability of taxonomic and species classification when using WGS data compared with using 16S rDNA data, which have not allowed for reliable classification of *Pseudomonas* isolates in several studies (Gomila et al., 2015; Garrido-Sanz et al., 2016; Peix et al., 2018).

Members of the group of 6 pigmented *P. cf. lactis* isolates sequenced previously and in this work had been reported as *P. azotoformans* (Evanowski et al., 2017), *P. fluorescens* biovar IV (Martin et al., 2011), and *P. fluorescens* (Andreani et al., 2015a). Based on ANIb analysis of WGS data, our data clearly support the concept that these isolates form a single group of related strains that likely represent a single species or perhaps 2 closely related species. Specifically, this group includes 9 total isolates along with the *P. lactis* type strain, with overall pairwise ANIb of $\geq 94\%$ (slightly below the species cutoff of 95%). Separation of this overall group into 2 clusters yielded cluster I with 3 isolates (along with the *P. lactis* type strain) and cluster II

Figure 8. Phylogeny of proteins encoded by (a) *trpA*, (b) *trpB*, (c) *trpC*, (d) *trpD*, and (e) *trpF* involved in tryptophan biosynthesis from all newly sequenced *Pseudomonas* isolates and in previously sequenced isolates Ps_20, Ps_22, Ps_40, and Ps_77. The maximum-likelihood trees are drawn to scale, with branch lengths and the scale bar measured in the number of AA substitutions per site. Isolate names in blue text have been shown to produce gray or blue pigment. Curly braces have been used to identify phylogenetic clusters.

with 6 isolates. Each cluster had pairwise ANIb values of >95.0%. Although one could argue that cluster II represents a new species (when applying the previously proposed 95% cutoff as a strict rule), we elected to classify all 9 isolates as a single species (*P. cf. lactis*), at least for the time being, allowing for collection and

characterization of additional isolates and generation of further data before making a taxonomic proposal that contributes to the already rapidly expanding number of new *Pseudomonas* spp. Previous studies that classified isolates in this group to the species level did not use WGS data for classification. For example, our group

reported that, based on 16S rDNA data, a *Pseudomonas* isolate that caused a gray defect in milk (FSL E2-0548) could be classified as *P. azotoformans* (Evanowski et al., 2017). This finding is consistent with the 16S data analysis we have reported here, but the ANIb data generated for this study showed that this isolate had only 86.8% similarity to the *P. azotoformans* type strain and 94.2% similarity to the *P. lactis* type strain and classified it into *P. lactis* cluster II. Similarly, our group used 16S rDNA sequence data to classify a single isolate obtained from blue-pigmented cheese (FSL W5-0203) as *P. fluorescens* biovar IV (Martin et al., 2011), whereas it showed only 86.9% ANIb similarity to the *P. fluorescens* type strain. An Italian group also previously reported whole genome sequences for 2 isolates (Ps_22 and Ps_77) that caused blue color defects in cheese as well as 2 closely related isolates (Ps_20 and Ps_40) that did not cause this type of defect (Andreani et al., 2015a). These 4 isolates were classified into the *P. fluorescens* group without reporting a more specific species classification. The WGS data analyses we have reported here support the idea that *P. lactis*, which was first reported in 2017 (von Neubeck et al., 2017), represents a species that includes several distinct strains that can cause blue or gray color defects in dairy products. Several *Pseudomonas* species have been linked to the spoilage of dairy products, but identification of *P. lactis* isolates in products or processing environments may indicate an enhanced risk of color defects. It is, however, important to note that many currently used species classification approaches for *Pseudomonas* may not correctly identify a given isolate as *P. lactis* (e.g., as of December 5, 2018, a *P. lactis* 16S sequence was not included in the Ribosomal Database Project) and may misidentify *P. lactis* isolates as closely related species including, but not limited to, *P. azotoformans*.

Interestingly, we also found 2 isolates that caused a color defect when inoculated into milk, did not cause a color defect on PDA, are not closely related to *P. lactis*, and likely represent the species *P. putida*. These 2 pigmented isolates were very closely related to each other and showed an ANIb similarity of 94.7% to the *P. putida* type strain. Importantly, this clarifies that *Pseudomonas* strains closely related to *P. putida* are also able to cause blue or gray color defects in dairy products. Interestingly, these 2 historical isolates were described by Hammer (1914), who investigated an “outbreak” of blue milk in Iowa and originally identified these isolates as *Bacillus cyanogenes*. *Bacillus cyanogenes* was later reclassified as *Pseudomonas syncyanea* or *P. cyanogenes* (Hammer, 1957), but neither of these species is present in the most recent edition of *Bergey’s Manual* (Palleroni, 2015). In addition, the 2 isolates characterized here were submitted to the USDA Agri-

cultural Research Service Culture Collection by B. W. Hammer under the names *P. mildenbergii* and *P. cyanogena*, but these species do not appear in the current *Bergey’s Manual* either (Haynes, 1961; Palleroni, 2015). The collection currently lists these isolates as *P. putida* (USDA Agricultural Research Service, 2018), and we verified that this classification is accurate by WGS. Overall, our findings indicate that a larger diversity of *Pseudomonas* species than is generally assumed can cause color defects in dairy products, and they illustrate the value of accessible long-term strain collection maintenance.

Some *Pseudomonas* Strains Can Cause Both Gray Color Defects in Milk and Blue Color Defects on Cheese

Through inoculation and WGS experiments involving isolate FSL E2-0548 from fluid milk and isolate FSL W5-0203 from cheese, we demonstrated that these 2 isolates are both closely related to each other and are able to produce color defects in dairy products other than those from which they originated (i.e., FSL E2-0548 produced blue pigment on cheese and FSL W5-0203 produced gray pigment in milk). This inoculation experiment, along with the >99.7% ANIb shared between pigment-producing isolates FSL E2-0548 (milk) and Ps_22 (cheese), strongly suggests that many blue- or gray-pigment-producing *Pseudomonas* associated with dairy foods should be able to produce pigment defects in multiple dairy products. The reason why fluid milk appears gray when contaminated with these *Pseudomonas* whereas cheese turns blue is relatively unexplored, but is supported by historical reports. A gray color in milk similar to what we observed was reported by Duckwall (1905) and by Hammer (1914) when samples of sterilized milk were inoculated with their respective isolates of blue-pigmented *Pseudomonas*. Both authors observed blue pigment in their *Pseudomonas*-inoculated sterilized milk when the milk was acidified — chemically in the case of Duckwall, and when cocultured with lactic acid bacteria in the case of Hammer. The efficacy of both approaches to acidification suggests that pH, rather than other fermentation-related changes in the milk, determines the ultimate appearance of the pigment. It is likely that modern high-temperature short-time pasteurized milk appears gray when contaminated due to its near-neutral pH and its low prevalence of lactic acid bacteria that reach spoilage levels (i.e., reduce the pH) over shelf life (Reichler et al., 2018). By extension, it could be surmised that blue, rather than gray, pigment was produced on mozzarella cheese by these *Pseudomonas* because of the acidification of the cheese during production, having much the same ef-

fect as has been reported in fluid milk. Other potential explanations for the differences in the color produced in milk and on cheese are that multiple pigments are produced, that the pigment is altered after its synthesis, and that the perceived color of the pigment or pigments depends on environmental conditions other than pH (e.g., nutrients, oxygen).

Importantly for the dairy industry, isolates associated with blue or gray pigment defects have been isolated from dairy and other food products both in the United States and throughout the European Union, including Great Britain, Spain, Germany, and Italy (Andreani et al., 2015b; del Olmo et al., 2018). The occurrence of these strains in global regions that are noteworthy, even famous, for their dairy products leaves open the risk of future high-profile mass spoilage events similar to what occurred in Italy in 2010 (Greenhalgh, 2010). In the United States, we have heard anecdotally that periods of contamination at dairy processing facilities have led to increased levels of consumer complaints due to gray pigment defects in fluid milk. Because even a single spectacularly spoiled unit of product has the potential to cause damage to a brand if disseminated in photographs or videos via social media, it is imperative that producers of fresh, uncultured milk and cheese products take the necessary precautions to prevent postpasteurization contamination with pigment-producing *Pseudomonas*. Such precautions include the implementation or improvement of sanitation standard operating procedures, preventive maintenance protocols, and employee training.

It is important to note that the gray pigmentation in milk does not result from an unrealistically large population of *Pseudomonas*. In our study, the gray color defect was observed in milk that reached approximately 8 log cfu/mL. This level of *Pseudomonas* can realistically be achieved in commercial fluid milk over 21 d of storage at refrigeration temperatures, particularly under conditions of slight temperature abuse. For example, Reichler et al. (2018) observed that milk contaminated with *Pseudomonas* at the processing facility showed an average SPC of 8.23 log cfu/mL after 21 d of storage at 6°C.

Tryptophan and Indigo Biosynthesis Genes Are Likely Associated with the Formation of Color Defects in Foods by *Pseudomonas* spp. Strains

Our comparative genomics analysis identified only 18 genes that were present in all 6 genomes associated with the gray color defect in fluid milk and absent in all 3 genomes not associated with this color defect. Among those genes, 14 were clustered in a single locus that had been previously found in a European *Pseudomonas*

isolate that produced a blue color defect in mozzarella cheese (Andreani et al., 2015a). Interestingly, the 16 genes of this locus reported by Andreani et al. were present in all of our newly sequenced *P. cf. lactis* isolates, but only 14 genes were detected in the newly sequenced *P. putida* isolates. This may be because of extensive evolutionary divergence between the *P. cf. lactis* isolates and the *P. putida* isolates, or it may represent either the gain of these 2 genes by the ancestor of the *P. cf. lactis* isolates or the loss of them by the ancestor of the *P. putida* isolates. This locus includes 5 genes that encode for proteins involved in the tryptophan biosynthesis pathway. All *Pseudomonas* shown to produce the gray color defect in fluid milk or the blue color defect in cheese present 2 copies of these 5 genes, whereas *Pseudomonas* that do not produce either of these color defects harbor a single copy of the genes. Interestingly, our data are consistent with the previous suggestion (Andreani et al., 2015a) that this second tryptophan operon copy may have been transferred horizontally across a diverse population of *Pseudomonas*, resulting in its presence in 2 distinct *Pseudomonas* groups. The horizontal gene transfer hypothesis is supported by the observation that the genes in the second copy of this operon, which is present only among pigmented isolates, are phylogenetically closely related and show much lower nucleotide diversity than the copies present in all *Pseudomonas*. Interestingly, indole is produced during tryptophan biosynthesis, which can then be converted into indigo or an indigo-like molecule (which typically exhibit a color that is close to blue) in a reaction that is oxygen-dependent (Ensley et al., 1983). The oxygen dependence of the indigo production, combined with the observation that the gray color defect was only observed in half-empty containers of fluid milk (which leads to increased oxygen exposure) and previous reports that the defect often showed up as a ring on the milk surface, makes it highly plausible that this indigo reaction is responsible for the production of the gray milk defect. Functionally, an additional tryptophan operon may be regulated in a way that facilitates tryptophan synthesis in refrigerated fluid milk, whereas the broadly distributed operon is not expressed in milk. Further experimental work is needed to test this hypothesis. Another alternative hypothesis that could be tested experimentally is that the second horizontally transferred *trp* operon encodes proteins with enhanced activity in a dairy environment or at low temperatures. In addition to the 5 genes encoding enzymes involved in tryptophan biosynthesis, the locus specifically present in the pigmented isolates also included the gene *rhtB*, which encodes for an L-homoserine and L-homoserine lactone exporter. Because L-homoserine lactone has been shown to function as a quorum-sensing molecule

(i.e., its presence in the environment signals that a high population density has been reached; Zakataeva et al., 1999; Dunstall et al., 2005), it is possible that it serves as a signal for biosynthesis of the pigment as the cell density reaches a minimum threshold. No other gene within the locus appears to be involved in gene regulation.

We also tested the hypothesis that all strains that produce gray milk or blue cheese could express specific blue or gray pigments other than indigo. This hypothesis was considered appropriate, because several other blue or violet pigmented molecules have been described in *Pseudomonas* (e.g., pyocyanin, indigoidine relatives, hydroxamic acids, blue copper proteins; Cha and Cooksey, 1991; Budzikiewicz, 2010; Palleroni, 2015). Although hidden Markov model searches identified several genes that were predicted to encode pathways for potential blue or gray pigments, none of these was specifically associated with the strains that caused the gray milk pigment defects. For example, although genes involved in pyoverdine biosynthesis were found among the genomes of the pigmented isolates, these genes were also found among the genomes of the unpigmented isolates. Of note, however, no models existed for hydroxamic acid or hydroxamate biosynthesis, and hydroxamates are known for their pH-dependent formation of reddish-brown to purple complexes with iron (Neilands, 1967). Hydroxamates have been reported both in *P. fluorescens* group isolates (Shirahata et al., 1970; Philson and Llinás, 1982) and in *P. mildenbergii* (Hulcher, 1982), a currently invalid species that once included our 2 historical *P. putida* isolates.

Closely Related *Pseudomonas* cf. *lactis* Isolates that Cause Near-Identical Color Defects on Cheese and in Milk Differ in Their Color Phenotypes on PDA

Our analyses identified the duplication of a tryptophan biosynthesis operon as a likely mechanism linked to the gray milk and blue cheese defects, but we also found that the *Pseudomonas* isolates we tested caused several different color phenotypes when grown on PDA at 6°C, even though they showed the same phenotype when inoculated in milk incubated at 21°C. For example, isolate FSL E2-0548, which originated from gray milk but also produced a blue pigment on cheese, produced a reddish-brown pigment on PDA after 5 d of incubation at 6°C. On the other hand, isolate FSL W5-0203 produced a blue pigment on both cheese and PDA. The difference in pigmentation on PDA compared with cheese and milk, as well as the diversity of color phenotypes on PDA, may be attributed to several, not necessarily mutually exclusive, causes, including (1) production of additional or different pigments on

PDA; (2) different color appearance of a given pigment on cheese and PDA due to compositional differences (e.g., pH); (3) production of a supplemental factor that either stabilizes or degrades the pigment on PDA; or (4) strains differing in their ability to alter their growth environment in different media (e.g., acid production from glucose), resulting in different visible colors. Some of these hypotheses have been raised by previous papers (Duckwall, 1905; Hammer, 1914; Andreani et al., 2015b) that reported expression of gray and brown pigments by blue-pigment-producing *Pseudomonas* strains grown on agar plates. These authors suggested that these observations could be due to additional pigments being produced or to the blue pigment being altered upon exposure to oxygen. In our experiments, the hypothesis that oxygen exposure is responsible for the alteration of the pigment from blue to brown did not seem relevant, because both cheese (where a blue color was observed) and PDA (where a gray color was observed) were held without immersion in brine or other liquid in unsealed Petri dishes with ample oxygen exposure. Additionally, other isolates have been reported to change color from blue to gray or brown over the course of incubation (Andreani et al., 2015b), but this was not the case for FSL E2-0548, which never exhibited blue pigment on PDA media, or for FSL W5-0203, which has been observed to produce only a stable blue pigment (Martin et al., 2011).

The hypothesis that the color-producing *Pseudomonas* strains studied here produce additional pigments on PDA seemed initially attractive, but hidden Markov model searches did not identify any pigment production pathways that could explain our findings. However, the available hidden Markov models are unlikely to identify all pigment pathways that may be responsible for the different color phenotypes observed. The hypothesis that a given pigment may show different color on cheese and PDA is supported by historical publications that indicate that lower pH (as found in cheese) has been reported to enhance blue pigmentation in dairy spoilage *Pseudomonas* (Hammer, 1914; Mildenberg, 1922). With respect to the hypothesis that a supplemental factor could stabilize or degrade color formed in dairy or PDA, it is possible that the citric acid used to acidify the milk for cheesemaking exerts a protective role on the blue pigment, because citric acid is known to inhibit enzymatic browning in fruits and vegetables and to chelate metal ions that could directly catalyze oxidative reactions of pigments or act as enzymatic cofactors (Lindsay, 1996; Whitaker, 1996; Brady, 2013). Interestingly, historical reports also suggested that increasing amounts of glucose (which is a major ingredient of PDA) create more intense pigments without acid production in blue-pigment-producing

Pseudomonas (Duckwall, 1905; Hammer, 1914); however, mechanisms of this color intensification have not been reported or explored and could be multifold. UV fluorescence disappeared from the cheese inoculated with FSL E2-0548 between d 2 and 7 of incubation, whereas fluorescence remained after 7 d for the cheese inoculated with FSL W5-0203. This difference suggests that the blue pigment and the fluorescent pigment are mutually exclusive, and that some factor differs between these 2 isolates that may explain the colors of the isolates on PDA. Overall, our findings, as well as the previous literature referenced in this section, clearly indicate that production of pigments and color by *Pseudomonas* present in dairy products is complex and may involve several pathways that directly and indirectly relate to color formation. Although control of color defects will not necessarily require future research into relevant pigment formation pathways, this type of future research will likely provide insights that could be used to help enhance rapid detection of color-producing *Pseudomonas* (e.g., through molecular methods targeting appropriate genes or through media that facilitate and enhance color production) or prevent color formation in dairy products (e.g., through inhibitors of pigment production pathways).

CONCLUSIONS

Our findings, combined with those of other studies conducted mainly in Italy, show that a range of *Pseudomonas* strains are likely able to cause visual color defects in dairy products that could result in their broadcast via social media. One may argue that control of these types of visually apparent spoilage issues is simple and only requires prevention of any post-kill step recontamination, but this may not be practically achievable considering that, in theory, contamination of a large volume of milk (e.g., 1 US gallon, slightly less than 4 L) with a single viable cell of *Pseudomonas* is sufficient to allow for the gray color defect to occur over a 21-d shelf life under slightly abusive conditions. Therefore, further development of detection methods to rapidly identify pigmented *Pseudomonas* that could cause color defects is important. In this context, our finding that certain *P. putida* strains cause color defects in milk without showing coloration on plating media (e.g., PDA) is highly relevant, because classical microbiological methods may not easily detect these strains. Fortunately, our data provide molecular targets, such as the accessory tryptophan biosynthesis genes (e.g., *trpD*), that could be used to develop initial molecular tests for the identification of color-producing *Pseudomonas* that belong to both *P. putida* and *P. cf. lactis*.

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