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Following Pathogen Development and Gene Expression in a Food Ecosystem: the Case of a *Staphylococcus aureus* Isolate in Cheese

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Human intoxication or infection due to bacterial food contamination constitutes an economic challenge and a public health problem. Information on the *in situ* distribution and expression of pathogens responsible for this risk is to date lacking, largely because of technical bottlenecks in detecting signals from minority bacterial populations within a complex microbial and physicochemical ecosystem. We simulated the contamination of a real high-risk cheese with a natural food isolate of *Staphylococcus aureus*, an enterotoxin-producing pathogen responsible for food poisoning. To overcome the problem of a detection limit in a solid matrix, we chose to work with a fluorescent reporter (superfolder green fluorescent protein) that would allow spatiotemporal monitoring of *S. aureus* populations and targeted gene expression. The combination of complementary techniques revealed that *S. aureus* localizes preferentially on the cheese surface during ripening. Immunochemistry and confocal laser scanning microscopy enabled us to visualize, in a single image, dairy bacteria and pathogen populations, virulence gene expression, and the toxin produced. This procedure is readily applicable to other genes of interest, other bacteria, and different types of food matrices.

Humans are susceptible to numerous food-borne diseases that are transmitted via water and food consumption. There are an estimated 48 million cases of food-borne illnesses in the United States every year, resulting in 128,000 hospitalizations, 3,000 deaths (1), and large economic and productivity losses (2). In Europe, the European Food Safety Authority reported a total of 5,363 outbreaks of food-borne illness in 2012, affecting almost 55,000 people and causing 41 deaths (3). Food-borne infection or intoxication is attributed to the pathogen itself or to toxins released in the food product.

Staphylococcus aureus is a worldwide cause of food-borne infections (4, 5). This bacterium is a leading cause of gastroenteritis resulting from the consumption of foods in which enterotoxigenic staphylococci have grown and produced toxins (6, 7). Thus, even if the bacteria are killed, e.g., by heat treatment, the heat-resistant enterotoxins can persist, leading to staphylococcal food poisoning (SFP) (6, 8). A notable example is staphylococcal enterotoxin D (SED), which is clearly involved in both cheese official controls performed in France (9) according to European Union regulation 1441/2007 (10) and in SFP outbreaks (11–17). The *sed* gene is carried by a plasmid (6, 18, 19) and controlled by two regulators, the accessory gene regulator (*agr*) quorum-sensing system, a main regulatory system controlling virulence gene expression in *S. aureus* (6, 12, 20) and the staphylococcal accessory regulator *sarA* (21).

In Europe, 777 outbreaks in 2012 were caused by bacterial toxins produced by *Bacillus* spp., *Clostridium* spp., and coagulase-positive staphylococci; the latter are the second most common causative agents of food-borne outbreaks (3). Among them, 346 were due to staphylococcal enterotoxins, of which 20% correlated with cheese as the food vehicle. Among all cheese families, soft and uncooked semihard cheeses are most often involved in SFP outbreaks (22, 23).

Cheese is a complex environment, constituted principally by water, proteins, fat, minerals, and a dynamic microbial ecosystem characterized by the presence of a large variety of bacteria, yeasts, and molds. The monitoring of minority bacterial populations of food pathogens and specific genes that they express within such a complex microbial physicochemical ecosystem is a major challenge in food microbiology. Indeed, the detection of microbial pathogens in food is complicated by low bacterial counts, which may not be recovered by using traditional culturing and sampling techniques. Traditional culture-based approaches may be affected by large populations of lactic acid bacteria in the cheese matrix (24). Implementation of DNA microarray technology has proven effective for the profiling of microbial communities (25, 26) and for *S. aureus* gene expression analysis in pure and mixed cultures with *Lactococcus lactis* in a simplified model cheese matrix (27). More recently, next-generation sequencing technologies (24, 28) and real-time reverse transcription-quantitative PCR (RT-qPCR) (29) have been used to measure enterotoxin and virulence gene expression and regulation in simulations of environmental conditions (27, 30, 31). While these methods can be sensitive and give both qualitative and quantitative information about the microorganisms tested, there is also a need to evaluate the distribution of microbial populations *in situ*. Fluorescence *in situ* hybridization with 16S rRNA provides microbial identification and physical detection of uncultivable microorganisms in fragile matrices like cheese (32), and its use is being expanded to pathogens in different environments (33–35). The spatial distribution of bacterial flora in cheese has also been explored by using scanning electron mi-

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croscopy, fluorescence and light microscopy, and laser scanning microscopy (36–40). An *in situ* approach used to investigate the spatial distribution of bacterial colonies of fluorescent *Lactococcus lactis* in a solid-food matrix with a model system has been recently developed (41), demonstrating that live cells can be visualized in cheese. However, *in situ* approaches to visualize minority gene expression in food matrices are still lacking.

Green fluorescent protein (GFP) variants have been used to quantify the expression of numerous *S. aureus* genes under various conditions, including different stages of biofilm maturation and dispersal (42–48). To date, all of these studies have been conducted either with a single bacterial population at high cell concentrations or under laboratory conditions.

The aim of the present study was to monitor the distribution of *S. aureus* and the spatial and temporal expression of virulence genes during the manufacture and ripening of semihard cheeses. To best approach a real-life situation, a fluorescent reporter was applied for use in *S. aureus* cheese isolates. The procedures developed for cheese sample preparation, immunochemistry, and confocal laser scanning microscopy (CLSM) enabled the visualization of dairy bacteria and minor pathogen populations, pathogenic gene expression, and the toxin produced, all in a single image.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strain CIM433 (*sed*, *sej*, and *ser* enterotoxin genes), from the ARILAIT collection (La Roche-sur-Foron, France), was used throughout this study. CIM433 is a cheese isolate that produces SED, which can be quantified by the confirmatory method of the European Union Reference Laboratory for coagulase-positive staphylococci (EU RL for CPS) (49).

Industrial starter and ripening cultures (Ets COQUARD, Villefranche sur Saône, France) lyophilized Beta 1 (homofermentative *Lactococcus lactis*/*Lactococcus cremoris* strains), Lambda 5/2 (*Lactobacillus bulgaricus*/*Streptococcus thermophilus* 25/75), Sigma 63 (*Brevibacterium linens*), and liquid Sigma 52 (*Geotrichum candidum*) were used for cheese making. Cultures were stored at −20°C prior to use.

Plasmid construction. Plasmid construction was performed with *Escherichia coli* strain TG1. Plasmid pCM11 (47) contains a *sarA* promoter that drives the expression of a synthetic version of the gene encoding superfolder GFP (sGFP), which gives a strong fluorescent signal in *S. aureus* (44, 46, 47). pCM11 is derived from pE194, which has an estimated copy number of 55 per cell (50). A promoterless version of *sgfp*, called pIF1, was constructed as a negative control by digesting pCM11 with HindIII and KpnI, followed by filling in and ligation steps (New England BioLabs, Ipswich, MA). To monitor *sed* expression, the *sed* promoter was fused to *sgfp* by the Gibson assembly method, giving rise to plasmid pIF2 (51). The oligonucleotides used for *sed* amplification were 5′ GTAAAAC GACGGCCAGTGCCAAGCTTGGTACCCCGGCGTAGAGGATCAAA TATATTG 3′ and 5′ CATCCTCCTAAGGTACCCGGGGATCCGCCTT TTTTTCAATAAATTTGAGCACC 3′, such that the transcription start site and −35 and −10 promoter elements were amplified. The pCM11 vector, including the *sgfp* ribosome binding site, was amplified with oligonucleotides 5′ GGTGCTCAAATTTATTGAAAAAAAGGCGGATCCC CGGGTACCTTAGGAGGATG 3′ and 5′ CAATATATTTGATCCTCTA CGCCGGGGTACCAAGCTTGGCACTGGCCGTCGTTTTAC 3′. All constructs were verified by DNA sequencing (GATC Biotech, Constance, Germany). The pIF1 and pIF2 plasmids were introduced into *S. aureus* RN4420 (52), extracted, and used to electrotransform *S. aureus* CIM433 as described previously (53).

Growth conditions in reconstituted milk. *S. aureus* CIM433, CIM433/pIF1 (*sgfp* without promoter), CIM433/pCM11 (*sarA* promoter-driven *sgfp*), and CIM433/pIF2 (*sed* promoter-driven *sgfp*) were cultured in brain heart infusion (BHI; Oxoid, Dardilly, France) broth; plasmid-carrying strains were grown in medium with erythromycin (Ery) at 10 μg/ml. Precultures were grown at 37°C with shaking (200 rpm) for 8 h. A preculture containing 10^6 CFU/ml was used to inoculate 50 ml of sterile reconstituted milk (100 g of semiskim milk powder [Régilait, Saint-Martin-Belle-Roche, France] per liter of distilled water sterilized at 108°C for 10 min). Bacterial growth in shaking cultures was followed for 24 h. Serial dilutions of milk cultures were prepared in sterile 1% (wt/vol) peptone water and plated on BHI solid medium, supplemented or not for antibiotic selection, for differential *S. aureus* count determination. Plates prepared in duplicate were incubated for 48 h at 37°C before bacterial enumeration.

Inoculum preparation for cheese manufacture. Strains were cultured in 10 ml of BHI broth with antibiotic (except for CIM433) at 37°C with shaking for 8 h. A 100-μl volume of this preculture was then added to 100 ml of BHI broth without antibiotic, and the culture was incubated for 4 h at 37°C without shaking so that *S. aureus* was in the exponential growth phase at the time of milk inoculation for cheese manufacture. The lactic acid bacterial starter culture was prepared according to the manufacturer's instructions and suspended, just before use, in pasteurized milk from the cheese vat to ensure homogenization (see below).

Cheese manufacture. Raw bulk milk (pH 6.5) cooled at 4°C and collected from a local farm (Viltain, Jouy-en-Josas, France) was pasteurized (30 s at 72°C). Cheeses were prepared in four automated 20-liter tanks in a P2-level experimental cheese plant at INRA (Jouy-en-Josas, France). Following the addition of $CaCl_2$ (12.5 g/100 liters of milk), 15 liters of pasteurized milk preheated at the maturation temperature was inoculated with *S. aureus* (to a final concentration of 10^4 CFU/ml) and with starter culture (to a final level of 10^6 CFU/ml). The equivalent of 33 ml of filtered rennet extract (520 mg of chymosin/liter; Berthelot) for 100 liters of milk was added after 1 h of milk maturation at 34°C. Coagulation then proceeded for about 40 min before the curd was cut into small cubes to corn grain size, and after 20 min of slow stirring, 10 min with no stirring, and then 10 min of rapid stirring at 34°C, 33% (5 liters) of the whey was drained. The curd was then poured into molds and pressed for 3 h with 1.5-kg weights. After being taken out of the molds (molding lasted 4 h), cheeses were salted for 1 h in sterile brine (24% NaCl, pH 5.10, 13°C), turned over daily, and dried for 4 days at 16°C. Cheeses were washed and smeared at 12°C with brine solution including *B. linens* and *G. candidum* at days 6 and 12 during ripening. Cheeses were approximately 13 to 15 cm in diameter and 2.5 cm thick. The mean levels of moisture on a fat-free basis and fat in dry matter of 1-day-old cheeses were, respectively, 64.7 and 46.2%; the curd pH was around 5.2. The mean moisture on a fat-free basis of 15-day-old cheeses was 52.3%, and the mean NaCl content was 2.2%. The core pH reached values of around 4.8, and the surface pH was around 6.9.

Bacterial enumeration in cheeses. The absence of *S. aureus* was checked in all pasteurized milk samples before inoculation. *S. aureus* and starter bacteria in cheeses were estimated over the 24 h of the cheese-making procedure and after 15 days of ripening (after core-surface separation) by plating as described previously (31).

FCM. Cell pellets were recovered from 1-ml milk cultures by centrifugation (6,000 × *g* for 2 min), immediately frozen in liquid nitrogen, and stored at −80°C. For flow cytometry (FCM), cell pellets from cheese were prepared as for RNA extraction (see below). All cell pellets were suspended in 1 ml of sterile 1% (wt/vol) peptone water, and the cell density was adjusted to 10^6 CFU/ml after filtering with a 50-μm CellTrics filter (Partec, Ste. Geneviève des Bois, France). Fluorescence levels of 20,000 cells were determined with a CyFlow Space cytometer (Partec) equipped with a blue laser (488-nm emission). Fluorescence signals (from the sGFP reporter) were collected with a 527-nm bandpass filter (512 to 542 nm) (FL1 channel). FCM analyses were performed by using logarithmic gains and specific detector settings as adjusted on CIM433, the nonfluorescent parental strain, to correct for autofluorescence. Data were collected and analyzed with FlowMax software (Partec).

TABLE 1 Cell counts of *S. aureus* CIM433 (parental strain without *sgfp* plasmid), CIM433/pIF1 (*sgfp* without promoter), CIM433/pCM11 (*sarA* promoter-driven *sgfp*), and CIM433/pIF2 (*sed* promoter-driven *sgfp*) in shaking reconstituted milk without antibiotic for 24 h[a]

	Cell count on BHI (CFU/ml)			
Time (h)	CIM433	CIM433/pIF1	CIM433/pCM11	CIM433/pIF2
0	1.2×10^6	1.2×10^6	1.1×10^6	1.3×10^6
2	3.0×10^7	3.5×10^7	2.0×10^7	3.2×10^7
4	3.5×10^8	2.3×10^8	1.4×10^8	1.9×10^8
6	2.3×10^8	3.1×10^8	1.7×10^8	2.5×10^8
24	3.2×10^8	4.1×10^8	2.2×10^8	3.4×10^8

[a] Bacterial cultures were plated on BHI solid medium. Data are expressed as the means of two biological replicates.

TABLE 2 Stability of plasmids pIF1 (*sgfp* without promoter), pCM11 (*sarA* promoter-driven *sgfp*), and pIF2 (*sed* promoter-driven *sgfp*) carried by *S. aureus* CIM433 in milk for 24 h[a]

	Cell count on BHI + Ery as % of cell count on BHI alone		
Time (h)	pIF1	pCM11	pIF2
0	103	105	94
2	101	117	96
4	105	105	103
6	101	116	118
24	86	55	93

[a] Bacterial cultures were plated in duplicate on BHI and on BHI supplemented with 10 μg/ml erythromycin. Cell counts on BHI with antibiotic selection are expressed as percentages of cell counts on BHI alone.

Gene expression analysis. *sed* gene expression was analyzed by RT-qPCR analysis of total RNA extracted from cheese (same homogenate as for bacterial cell counts) as described previously (31). Three reference genes shown to be stably expressed during cheese manufacturing, *pta*, *gyrB*, and *rpoB*, were used in this study (31). Primers 5′ GATCTCCTGT ACTTTTATTTTCTCC 3′ and 5′ AAACGTTAAAGCCAATGAAAAC 3′, designed for *sed* real-time PCR, were purchased from Eurogentec SA (Seraing, Belgium).

Total RNA extracted from cheese (375 ng) was annealed with Random Nonamers (Eurogentec) for 10 min at 20°C after a denaturation step (5 min at 65°C) to remove RNA secondary structures. cDNA was synthesized by 1 h of RT at 42°C with PrimeScript reverse transcriptase and Ultrapure deoxynucleoside triphosphate (Clontech-TaKaRa Bio Europe, Saint Germain en Laye, France) in a 30-μl final volume, followed by enzyme inactivation (15 min at 70°C). cDNA levels were analyzed by qPCR with the ABI Prism 7900 HT Sequence detection system (SDS; Applied Biosystems, Foster City, CA). Each sample was tested in duplicate in a 96-well plate (Applied Biosystems). The reaction mixture (20 μl, final volume) consisted of 10 μl of SYBR green PCR master mix (Applied Biosystems), 1.2 μl of each primer (300 nM, final concentration), 2.6 μl ultrapure H_2O, and 5 μl of a 2.5-fold dilution of the relevant cDNA as the template. Absence of genomic DNA in RNA samples was checked by real-time PCR before cDNA synthesis (minus RT control). A blank (no-template control) was incorporated into each assay. The thermocycling program was as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Melting-curve data were then collected to check PCR specificity, contamination, and the absence of primer dimers. To minimize interrun variations, a calibrator sample (RNA extracted from a pure culture of strain CIM433) was used to determine and fix the fluorescence threshold. The C_T values calculated by the SDS software were exported to Excel for relative quantification analysis as described previously (31).

Enterotoxin determination. Cheese samples (25 g) were tested for the presence of SED after molding for 4 h and after 1 and 15 (core and surface separately analyzed) days of ripening, according to the European Screening Method of the EU RL for CPS, consisting of extraction followed by a dialysis concentration step coupled to detection with the Vidas SET2 kit, a qualitative detection test (bioMérieux, Marcy l'Etoile, France). Staphylococcal enterotoxin-positive samples were further analyzed by quantitative double-sandwich enzyme-linked immunosorbent assay, the confirmatory method of the EU RL for CPS (49), to quantify the amount of enterotoxin produced.

Microstructural analysis. Cheese pieces of approximately 1 by 1 by 0.5 cm were cut from fresh samples with a scalpel and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature. Samples were rinsed and immersed in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 20 h at 4°C as described previously (54). After direct freezing with liquid nitrogen vapor, 15-μm-thick embedded cheese sections were cut at −20°C with a Cryostat (Leica CM 1950) and stored at −80°C for immunohistochemistry. Each section was placed on a SuperFrost ULTRA PLUS slide (Thermo Scientific Menzel, Illkirch, France), blocked in 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 2% bovine serum albumin (Sigma-Aldrich) for 1 h at 4°C, rinsed, and incubated overnight at 4°C with *S. aureus*-specific rabbit antibody (1:200) (Biodesign International, Saco, ME) and sheep antibody specific to SED (1:120; Toxin Tech, Sarasota, FL). After rinsing, slides were incubated with the following secondary polyclonal antibodies: diluted (1:200) goat anti-rabbit IgG antibody coupled with cy5 (Rockland Immunochemicals Inc., Gilbertsville, PA) and donkey anti-sheep IgG antibody (1:200) coupled with tetramethyl rhodamine isocyanate (TRITC; Jackson ImmunoResearch, West Grove, PA). Nucleic acids were stained with a 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) solution at 2 μg/ml for 30 min at 4°C. Microstructural analysis was performed with Zen 2011 software and a Zeiss confocal laser scanning microscope 700 (MIMA2 Platform; INRA, Jouy-en-Josas) with an immersion 63× objective (numerical aperture 1.40, oil M27) at zoom 2.0. GFP fluorescence was excited with a 488-nm laser diode (at 5% intensity), cy5 was excited with a 639-nm laser diode (at 2% intensity), TRITC was excited with a 555-nm laser diode (at 5% intensity), and DAPI was excited with a 405-nm laser diode (at 5% intensity). Fluorescence was detected with a 490- to 555-nm band-pass filter for GFP, a 560- to 630-nm band-pass filter for TRITC, a 640-nm-and-higher filter for Cy5, and a 410-nm-and-higher filter for DAPI. Sequential tracking was performed, i.e., each fluorescent marker was detected sequentially, to minimalize potential spectral overlap.

RESULTS AND DISCUSSION

Fitness and stability of genetically modified food isolates in milk. The main objective of this work was to monitor targeted gene expression with the use of the fluorescent reporter sGFP. We chose the *S. aureus sarA-sgfp* fluorescent reporter fusion in plasmid pCM11 to initiate this project, as the *sarA* promoter region is a well-studied transcriptional element and is frequently used as a constitutive promoter in biofilm and host labeling experiments (42, 44, 45, 47, 55). Using this plasmid, we constructed pIF1, which lacks the *sarA* promoter, to evaluate basal fluorescence and the background and pIF2, in which the *sed* promoter is fused to *sgfp*, to monitor enterotoxin expression under simulated food contamination conditions. The three plasmids were successfully introduced into *S. aureus* food isolate CIM433, demonstrating that this strain is transformable.

We tested the transformed isolates in milk without antibiotic to mimic the conditions encountered in cheese, in which dairy bacteria are antibiotic sensitive and antibiotic addition is forbidden. The growth kinetics of the parental CIM433 strain and transformed strains in aerated reconstituted milk without antibiotic

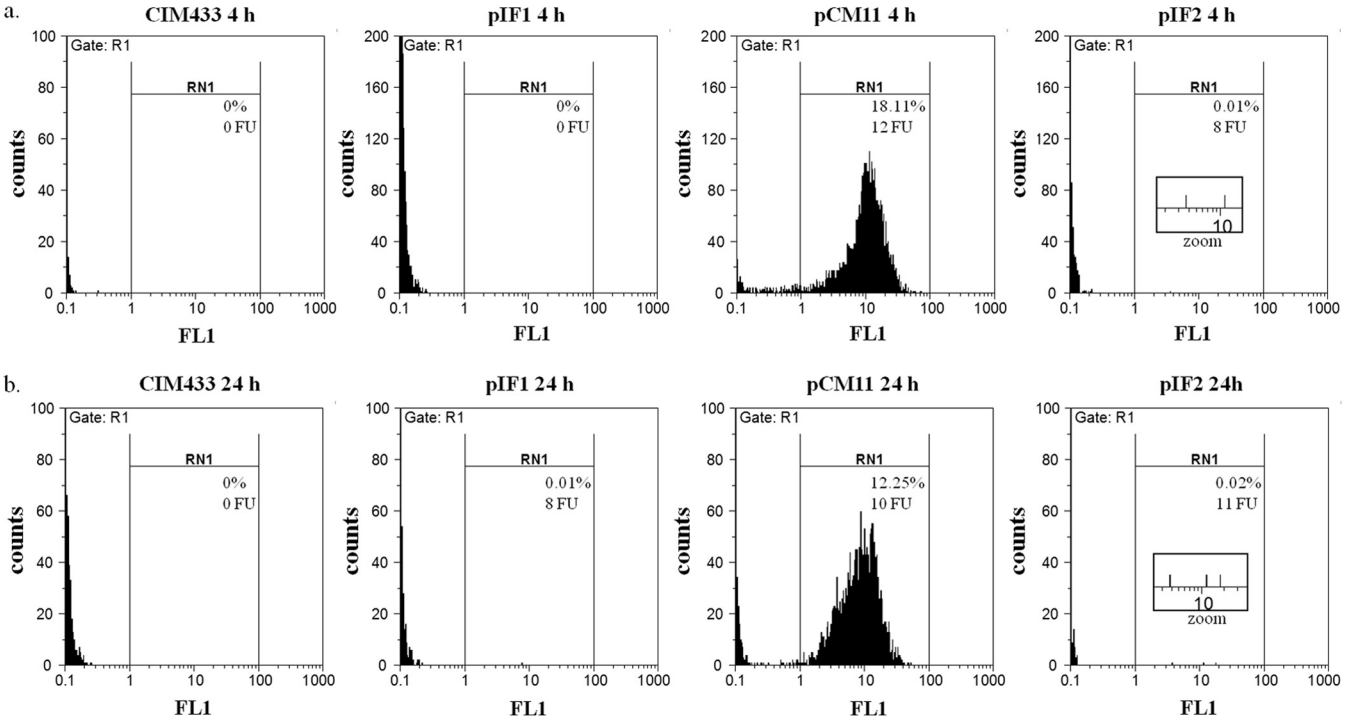

FIG 1 Cell flow sorting of *S. aureus* labeled with a fluorescent reporter under growth conditions in milk. CIM433 served as a negative gating control. CIM433 carrying plasmid pIF1, pCM11, or pIF2 was used for the FCM test. Each strain was grown for 24 h in reconstituted milk, the cell density was adjusted to 10^6 CFU/ml after filtering, and populations of 20,000 cells were separated by FCM after 4 (a) and 24 (b) h of growth (insets show zoom on cytographs). The percentage of fluorescent cells collected with a 527-nm bandpass filter (FL1 channel) and the fluorescence intensity of each signal are indicated.

over a 24 h-period show that the fitness of the *S. aureus* food isolate is not affected by plasmid transformation (Table 1). However, after 24 h in milk, about 45, 14, and 7% of the cultivable CIM433 bacteria carrying pCM11, pIF1, and pIF2, respectively, lost their plasmids (Table 2). This loss of stability will be considered in further analyses. While plasmid loss would clearly lower signal detection, the fact that strain fitness was essentially unaffected in milk led us to use these strains and evaluate their behavior in the cheese matrix.

Detection of sGFP fluorescence in milk. The fluorescence levels of each strain in milk were measured by FCM after 4 or 24 h. CIM433 and CIM433/pIF1 served as a negative gating control to gauge the green fluorescence intensity shifts (Fig. 1). In comparison with control strains (Fig. 1a and b), the presence of pCM11 in CIM433 conferred robust fluorescence in the late logarithmic growth (4 h) and stationary phases (24 h), as already observed for the *sarA* promoter-*sgfp* fusion under laboratory conditions (44). These observations demonstrate that pCM11 can be used to monitor *S. aureus* in milk even if the fluorescence levels are underestimated because of plasmid loss. A very weak fluorescent signal was detected for CIM433 carrying the *sed* reporter plasmid (pIF2) (8 fluorescence units [FU] at 4 h and 11 FU at 24 h) relative to the GFP background level of pIF1 (0 FU at 4 h and 8 FU at 24 h) (Fig. 1a and b, insets), indicating that *sed* promoter activity is very low under the conditions tested.

Fitness and stability of genetically modified food isolates in cheese. *S. aureus* fitness and plasmid stability were evaluated all along the cheese-making process. Bacterial cell counts in cheese showed <5.5-fold differences between parental and plasmid-car-

TABLE 3 Cell counts of *S. aureus* CIM433, CIM433/pIF1, CIM433/pCM11, and CIM433/pIF2 in cheese for 15 days[a]

	Cell count (CFU/g of cheese) on BHI			
Stage and location	CIM 433	CIM433/pIF1	CIM433/pCM11	CIM433/pIF2
Molding for 4 h	3.0×10^7	2.3×10^7	1.9×10^7	2.4×10^7
Day 1	1.1×10^8	4.5×10^7	4.3×10^7	5.2×10^7
Day 15				
Core	2.8×10^7	2.5×10^7	1.9×10^7	8.3×10^6
Surface	5.9×10^8	1.1×10^8	1.2×10^8	1.6×10^8

[a] After 15 days of ripening, the core had separated from the surface. Bacterial cultures were plated on BHI solid medium. Data are expressed as the means of two biological replicates.

TABLE 4 Stability of plasmids pIF1, pCM11, and pIF2 carried by *S. aureus* CIM433 during cheese manufacturing[a]

	Cell count on BHI + Ery as % of cell count on BHI alone		
Stage and location	CIM433/pIF1	CIM433/pCM11	CIM433/pIF2
Molding for 4 h	114	94	88
Day 1	95	41	55
Day 15			
Core	61	63	46
Surface	67	61	60

[a] Bacterial cultures were plated in duplicate on BHI and on BHI supplemented with 10 μg/ml erythromycin. Cell counts on BHI with antibiotic selection are expressed as percentages of cell counts on BHI alone.

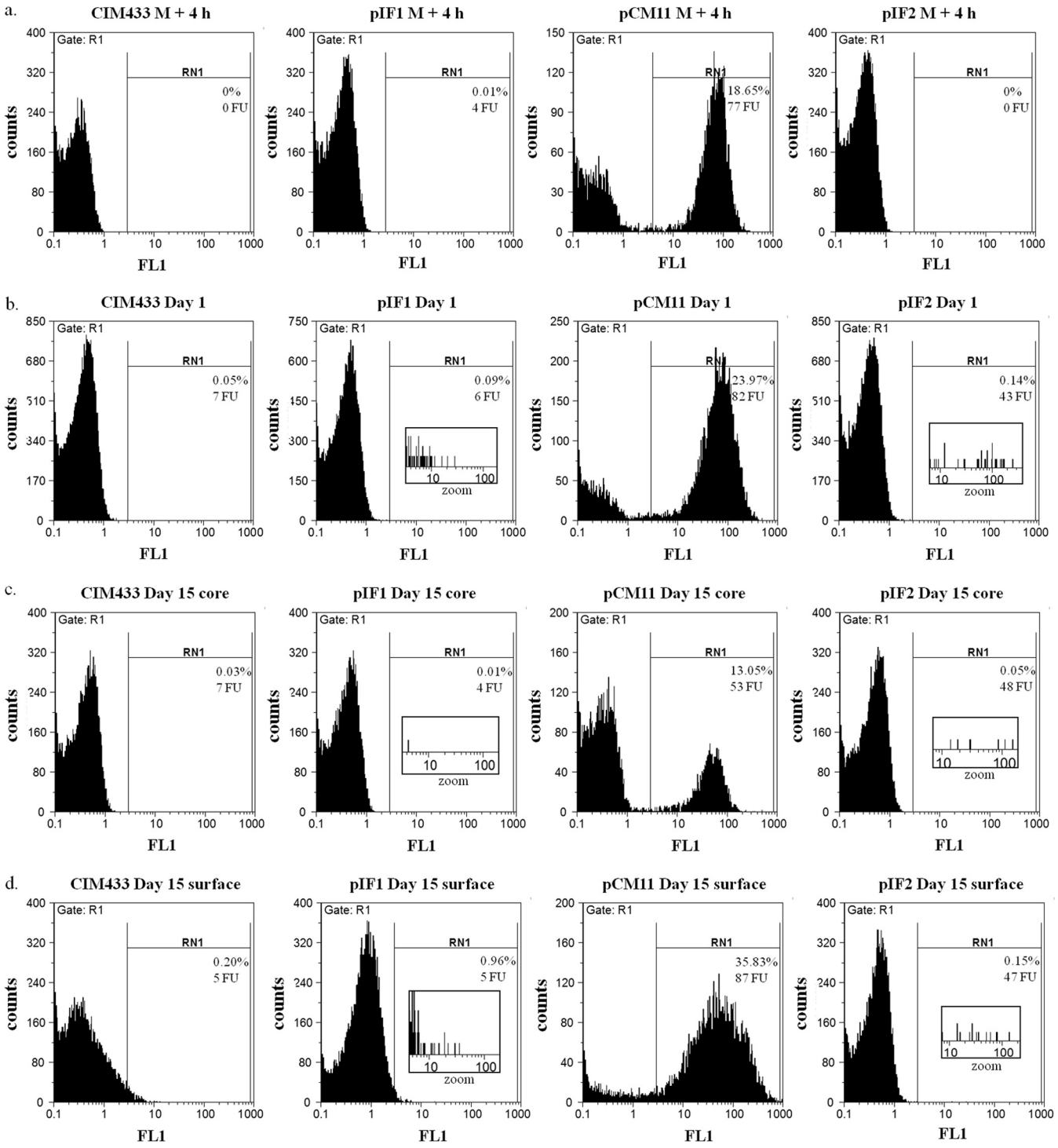

FIG 2 Cell flow sorting of *S. aureus* labeled with a fluorescent reporter in cheese. CIM433 served as a negative gating control. CIM433 carrying plasmid pIF1, pCM11, or pIF2 was used for the FCM test. The cell density was adjusted to 10^6 CFU/ml after filtering, and populations of 20,000 cells were separated by FCM after molding for 4 h (a) and after 1 (b) and 15 (after core [c]-surface [d] separation) days (insets show zoom on cytographs). The percentage of fluorescent cells collected with a 527-nm bandpass filter (FL1 channel) and the fluorescence intensity of each signal are indicated.

rying strains after 15 days, indicating that plasmid maintenance has a minor impact on bacterial fitness (Table 3).

Independently of the presence of a plasmid, we observed that *S. aureus* counts were about 10-fold higher on the cheese surface than in the core, which can be attributed to more favorable aerobic conditions and higher pH values on the cheese surface than in the core (56, 57). This difference was also observed in soft cheeses (58) and in other semihard cheese studies in our laboratory (data not shown).

In 1-day-old cheese, plasmids pCM11 and pIF2 were lost by

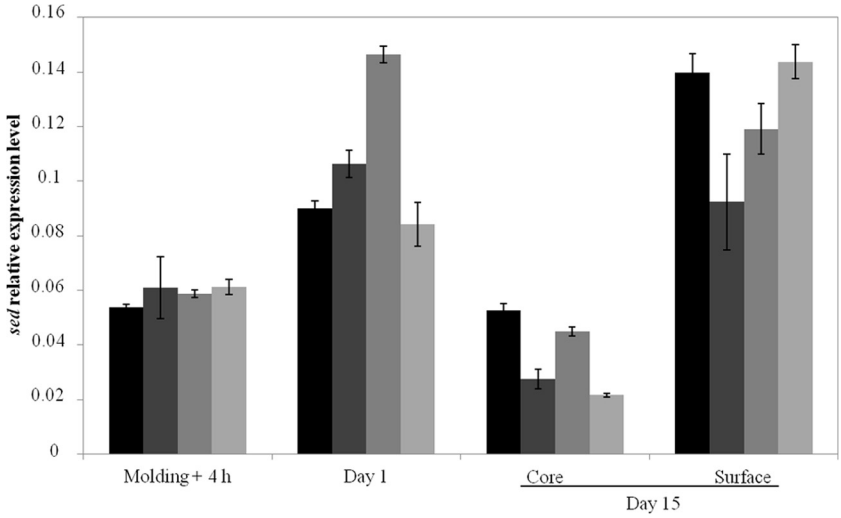

FIG 3 *sed* expression levels quantified by RT-qPCR during growth of *S. aureus* CIM433 (black), CIM433/pIF1 (dark gray), CIM433/pCM11(medium gray), and CIM433/pIF2 (light gray) in cheese. Vertical bars indicate standard deviations.

about half of the *S. aureus* CIM433 populations (Table 4). However, the levels of all three plasmids were stable for up to 15 days (46 to 67% according to plasmid and core versus surface sampling; Table 4). This stability likely reflects the absence of significant bacterial growth (i.e., cell divisions) in the core and little growth on the cheese surface, during which plasmid loss would occur. We concluded that the presence of reporter plasmids in at least half of the bacterial population is sufficient to address the question of *S. aureus* localization and *sed* expression in the cheese matrix.

Detection of sGFP fluorescence in cheese. The fluorescence levels of the CIM433 strain and plasmid-carrying derivatives in cheeses were monitored by FCM after molding for 4 h and after 1 and 15 days. CIM433 and CIM433/pIF1 served as negative gating controls (Fig. 2). Compared to CIM433 or CIM433/pIF1, CIM433/pCM11 displayed robust fluorescence after molding for 4 h (77 FU) (Fig. 2a), at day 1 (82 FU) (Fig. 2b), and during ripening (Fig. 2c and d), with a lower fluorescence level in the core (53 FU) (Fig. 2c) than on the cheese surface (87 FU) (Fig. 2d). We observed a very slight but significant fluorescence level (43 FU) of the CIM433 strain carrying the *sed-sgfp* reporter fusion (pIF2) relative to the GFP background of pIF1 (6 FU) in 1-day-old cheese (Fig. 2b, insets). The same low but significant level of *sed* expression as measured by GFP was maintained in 15-day-old cheese in both core and surface samples (48 FU versus 4 FU in the core and 47 FU versus 5 FU on the surface) (Fig. 2c and d, insets). We note that the fluorescence intensities were likely underestimated by at least 2-fold because of plasmid loss (Table 4). Weak fluorescence from pIF2 may reflect low *sed* promoter activity under our conditions. The expected lower plasmid copy number in the absence of selection may further account for the weak signal. The above factors may also explain the differences between the FCM (Fig. 2) and culture counts (Table 3) of CIM433/pIF2. Our results reveal that *S. aureus* cell counts were about 10-fold higher on the cheese surface than in the core (Table 3). As FCM is corrected for a given cell number, we project that the cheese surface would contain about 10-fold more SED, accordingly.

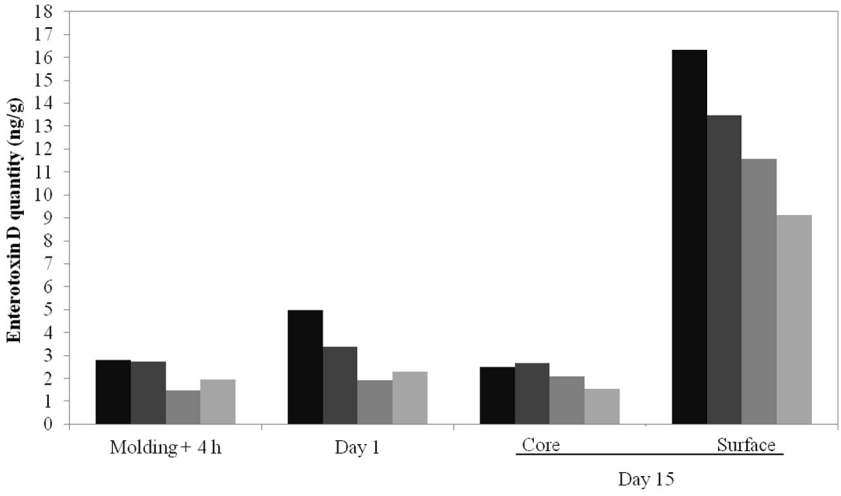

FIG 4 SED produced by *S. aureus* CIM433 (black), CIM433/pIF1 (dark gray), CIM433/pCM11(medium gray), and CIM433/pIF2 (light gray) in cheese was quantified by the confirmatory method.

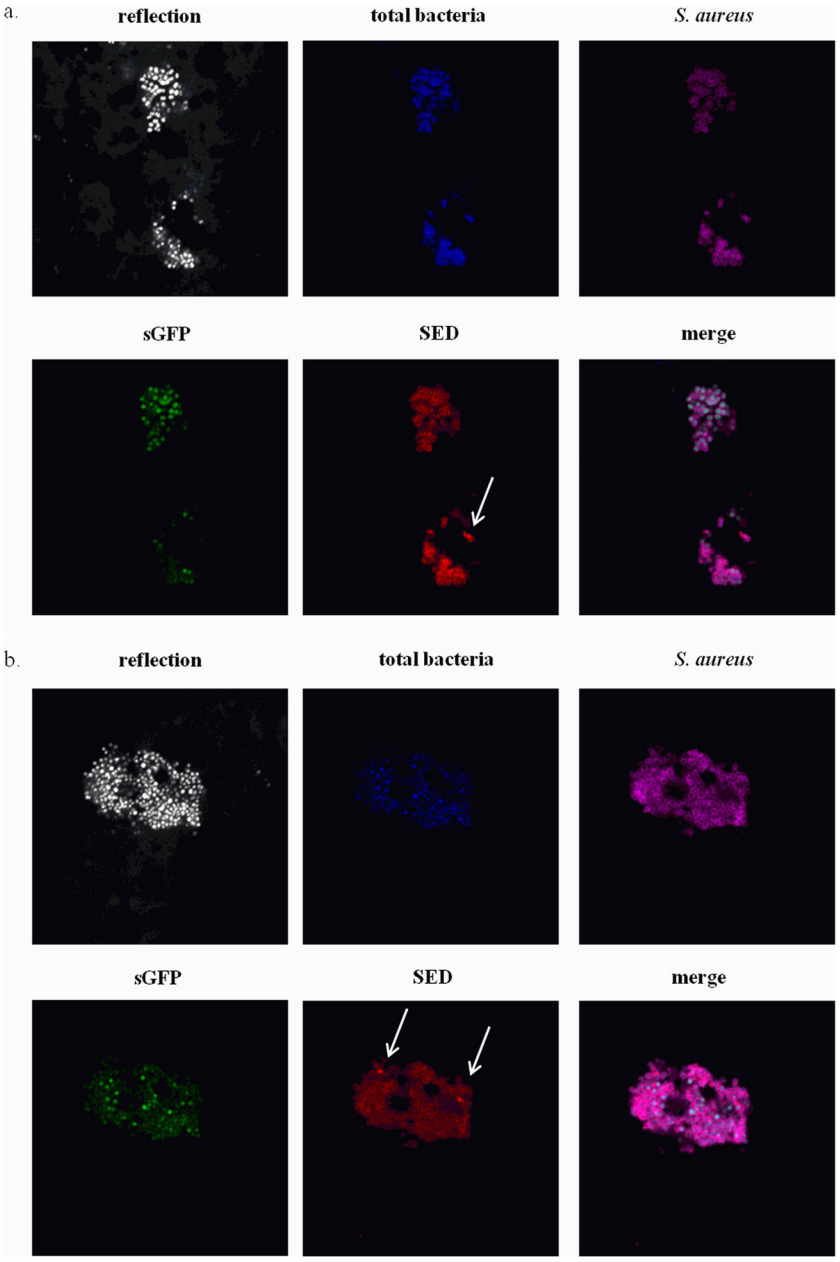

FIG 5 Confocal laser scanning micrographs of *S. aureus* CIM433/pCM11 in the cheese core (a) and on the cheese surface (b) at day 15. The cheese sample structure is visualized by the reflection of the 405-nm laser diode in a grayscale image. Dairy bacteria and pathogens are blue, *S. aureus* strains are magenta, *sgfp* expression is green, and SED is red. Representative images of merged channels are also shown. SED-positive bacteria are indicated by arrows.

Gene expression correlated with enterotoxin production in cheese. To validate the measurements above of *sed* expression with pIF2, we also quantified endogenous *S. aureus sed* transcripts and protein production in cheese. First, gene expression in the food matrix was quantified by RT-qPCR as described previously (27, 30, 31). Transcript levels were comparable in the four CIM433 strains, indicating that the introduced plasmids do not significantly impact *sed* expression (Fig. 3). A slight variation in *sed* pCM11 expression at day 1 (~1.6-fold) compared to that of the other strains was not considered significant. A more marked difference in *sed* expression between the core and surface observed was observed for all four strains in 15-day-old cheeses, with ~5-fold greater expression on the surface (Fig. 3). Importantly, the results obtained by direct measurement of *sed* expression (Fig. 3) are consistent with those obtained with the *gfp* reporter fusions (Fig. 2), i.e., weak expression that increased from day 1 (Fig. 2b) and a fluorescent subpopulation that was slightly more numerous on the surface (0.15%) than in the core (0.05%) (Fig. 2c and d). These results allowed us to correlate fluorescence intensities with gene expression.

SED protein was measured after molding for 4 h and after 1 and 15 days (Fig. 4) (49, 59). At day 1, low toxin production did not correlate with *sed* gene expression. Importantly, the four strains tested behaved similarly, suggesting that SED is produced late in

the cheese production process. Amounts of SED expectedly represent its accumulation since the start of the cheese-making process. The presence of ~5-fold larger amounts of SED on the surface than in the cheese core may indicate that SED production occurs during ripening, when bacteria are compartmentalized. Greater *sed* expression and SED production correlate with higher counts of CIM433 CFU on the surface (Table 3). In keeping with these results, staphylococcal enterotoxin production is often positively correlated with the growth of *S. aureus* (17, 60), and we also observed a correlation between *sed* mRNA levels and SED production during semihard cheese manufacturing (31).

Combined visualization of bacterial localization, gene expression, and toxin production. In this part of the work, our goal was to visualize *in situ S. aureus* localization, gene expression, and toxin production. Nondestructive techniques using viability staining in conjunction with CLSM have been used for direct observation of bacteria in foods (61, 62) and detection of bacterial microcolonies in cheese (40, 41). Despite this progress, none of these reports aimed at discriminating a minority pathogen among a highly dense population, i.e., of dairy bacteria. Our additional goal was to detect SED production in this system, which required thin-slice sample preparation and multiple compatible immunohistochemistry markers.

We first verified that there was no inherent autofluorescence in the matrix in the absence of bacteria by using cheese models obtained by chemical acidification. The model cheese was prepared with glucono δ-lactone (food additive E575), an organic acid authorized for use as an acidity regulator in the dairy industry (CODEX STAN 283-1978). No fluorescent background was observed in cheeses at any stage when they were contaminated with parental strain CIM433 or CIM433/pIF1 (data not shown). The cheese sample structure was visualized by the reflection of a 405-nm laser diode in a grayscale image (Fig. 5). DAPI staining was used to visualize the total bacteria (i.e., dairy and pathogen bacteria) (stained blue in Fig. 5). At day 15, *S. aureus* clusters were numerous on the surfaces of all of the cheeses contaminated with CIM433, CIM433/pIF1, CIM433/pIF2 (data not shown), and CIM433/pCM11 (Fig. 5b). In contrast, *S. aureus* clusters in the core (Fig. 5a) or in cheeses at day 1 and after molding for 4 h were rarer and more scattered (data not shown). These observations support the enumeration results (Table 3) and suggest that *S. aureus* imprisoned in the curd mass continued to multiply on the aerated surface, which is not the case in the core.

The strong *sarA* promoter in pCM11 allowed GFP visualization in real cheese during manufacturing (data not shown) and ripening (Fig. 5). However, the low fluorescence of pIF2 (carrying the *sed-sgfp* fusion) in cheeses at days 1 and 15 was insufficient for detection by CLSM (data not shown).

We also employed an immunohistochemistry-based method to detect SED protein. Fluorescence appeared in scattered spots (Fig. 5), probably reflecting the weak subpopulation expressing *sed* (Fig. 2) and producing toxin. This approach allowed us to obtain a qualitative image of bacterial distribution with a distinction among dairy bacteria, *S. aureus*, and the toxin produced (Fig. 5).

In conclusion, the present study demonstrated that a combined set of tools can be used to monitor the *in situ* behavior, including population distribution and gene expression, of a pathogen within a complex ecosystem and food matrix. The simulated contamination involved a real high-risk cheese produced with a complete starter culture and an enterotoxin-producing *S. aureus* food isolate. While the hazard of this pathogen is attributed to enterotoxin production, our results suggest that ~10-fold higher bacterial density on the cheese surface is correlated with higher SED production, indicating that cell density is nevertheless an important factor in predicting intoxication.

To our knowledge, this is the first time that a single image reveals dairy bacteria and pathogen populations, pathogenic gene expression, and the toxin produced. This procedure could be easily transferred to other genes of interest and other bacteria within different types of matrices, such as contaminated meat or infected tissues.

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