Competition of *Listeria monocytogenes* Serotype 1/2a and 4b Strains in Mixed-Culture Biofilms[▽][†]

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The majority of *Listeria monocytogenes* isolates recovered from foods and the environment are strains of serogroup 1/2, especially serotypes 1/2a and 1/2b. However, serotype 4b strains cause the majority of human listeriosis outbreaks. Our investigation of *L. monocytogenes* biofilms used a simulated food-processing system that consisted of repeated cycles of growth, sanitation treatment, and starvation to determine the competitive fitness of strains of serotypes 1/2a and 4b in pure and mixed-culture biofilms. Selective enumeration of strains of a certain serotype in mixed-culture biofilms on stainless steel coupons was accomplished by using serotype-specific quantitative PCR and propidium monoazide treatment to prevent amplification of extracellular DNA or DNA from dead cells. The results showed that the serotype 1/2a strains tested were generally more efficient at forming biofilms and predominated in the mixed-culture biofilms. The growth and survival of strains of one serotype were not inhibited by strains of the other serotype in mixed-culture biofilms. However, we found that a cocktail of serotype 4b strains survived and grew significantly better in mixed-culture biofilms containing a specific strain of serotype 1/2a (strain SK1387), with final cell densities averaging 0.5 $\log_{10}$ CFU/cm^2 higher than without the serotype 1/2a strain. The methodology used in this study contributed to our understanding of how environmental stresses and microbial competition influence the survival and growth of *L. monocytogenes* in pure and mixed-culture biofilms.

A prominent food-borne pathogen, *Listeria monocytogenes* can cause severe infections in humans, primarily in high-risk populations, though the disease (listeriosis) is relatively rare (11, 30, 43). Outbreaks of listeriosis have resulted from the contamination of a variety of foods by *L. monocytogenes*, especially meat and dairy products (27). *L. monocytogenes* is ubiquitous in the environment, able to grow at refrigeration temperature, and tolerant of the low pHs (3 to 4) typical of acidified foods (28, 32, 44). The capacity to produce biofilms confers protection against stresses common in the food-processing environment (13, 33).

Biofilms are characterized by dense clusters of bacterial cells embedded in extracellular polymeric substances which are secreted by cells to aid in adhesion to surfaces and to other cells (4, 5). Strains of *L. monocytogenes* have been known to persist for years in food-processing environments, presumably in biofilms. Of the 13 known serotypes of *L. monocytogenes*, three (1/2a, 1/2b, and 4b) account for >95% of the isolates from human illness (21). Serotype 1/2a accounts for >50% of the *L. monocytogenes* isolates recovered from foods and the environment, while most major outbreaks of human listeriosis have been caused by serotype 4b strains (1, 3, 14, 15, 17, 22, 29, 31, 41, 47, 49,). No correlation between *L. monocytogenes* strain

fitness and serotype has been identified (16, 19). Some studies have reported that strains repeatedly isolated from food and environmental samples (defined as persistent strains) had a higher adherence capacity than strains that were sporadically isolated (2, 36), while this phenomenon was not observed by others (7). Serotype 4b strains exhibited a higher capacity for biofilm formation than did serotype 1/2a strains (36), whereas this was not observed by Di Bonaventura and colleagues (6). It has been suggested that serotype 1/2a strains could be more robust than serotype 4b strains in biofilm formation under a variety of environmental conditions. Furthermore, strains of these serotypes differ in terms of the medium that promotes biofilm formation. Biofilm formation by serotype 4b strains was higher in full-strength tryptic soy broth than in diluted medium, whereas the opposite was observed with serotype 1/2a strains, which produced more biofilm in diluted medium (12).

There is limited information on microbial competition between strains of different serotypes in biofilms or on how the environmental stresses present in food-processing environments may affect the biofilm formation and survival of *L. monocytogenes* of different serotypes. In food-processing plants, the environmental stresses encountered by bacteria are more complex and variable than most laboratory systems used for microbial ecology and biofilm studies. A simulated food-processing (SFP) system has been developed to address this issue (38). The SFP system incorporates several stresses that may affect bacteria in biofilms in the food-processing environment, including exposure to sanitizing agents, dehydration, and starvation. When biofilms were subjected to the SFP regimen over a period of several weeks, the cell numbers of *L. monocytogenes* strains in the biofilms initially were reduced and then

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TABLE 1. *L. monocytogenes* strains used in this study

Strain ID[a]	Serotype	Persistence[b]	Source, yr	Reference(s)/other ID(s)
SK1450	4b	NA	Hot dog outbreak, 1998-1999	22/H7550
SK1403	4b	NA	Food, United States (California outbreak), 1985	35/F2365, G3990
2140	4b	NP	2001	2
SK1495	4b	NP	Turkey-processing environment, 2003	10/L0315
SK1277	4b	NP	Turkey-processing environment, 2003	10/82-2a
M35402A	4b	NP	Bulk milk, 2001	2
M33027A	4b	NP	Bulk milk, 2001	2
SK1463	4b	P	Turkey-processing environment, 2002	22/J1815
SK1387	1/2a	P	Food (frankfurter), 1988	21, 35/G3965, F6854
SK90	1/2a	P	Turkey-processing environment, 2004	23/90
SK1637	1/2a	NP	Turkey-processing environment, 2005	23/1637
SK600	1/2a	P	Turkey-processing environment, 2004	34/600
M39503A	1/2a	P	Bulk milk, 2001	2
SK754	1/2a	NP	Turkey-processing environment, 2004	34/754
SK2642	1/2a	P	Turkey-processing environment, 2006	34/2642
SK2508	1/2a	NP	Turkey-processing environment, 2004	23/2508

[a] The strains with identifications (IDs) that begin with SK are from the culture collection of S. Kathariou; others are from the culture collection of D. Call's laboratory at the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman.

[b] The strains were designated as persistent (P) or nonpersistent (NP) based on the frequency of the same PFGE patterns of the isolates from the same source, as discussed by Kathariou (21). Others are not available (NA).

increased as the culture adapted (38). The development of resistance to sanitizing agents was specific to the biofilm-associated cells and was not apparent in the detached cells (38). This suggested that extracellular polymeric substances present in the biofilm matrix were responsible for the resistance to sanitizing agents. It was subsequently found that real-time PCR, in combination with propidium monoazide (PMA) treatment of samples prior to DNA isolation, was an effective method for enumerating viable cells in biofilms (37).

The objective of this study was to determine if strains of serotype 1/2a or 4b have a selective advantage under stress conditions. We investigated and compared the initial attachment and biofilm formation capabilities of *L. monocytogenes* strains of these two serotypes and analyzed the survival and growth of bacteria of each serotype in mixed-serotype biofilms in the SFP system by using PMA with quantitative PCR.

MATERIALS AND METHODS

Strains and growth conditions. Eight *L. monocytogenes* serotype 4b strains and eight serotype 1/2a strains from different sources were used in this study (Table 1). The serotype of each strain was confirmed by PCR with serotype-specific primers (8). The strains chosen for this study consisted of both persistent and nonpersistent (21) strains from a variety of sources, characterized as described in the references in Table 1. The bacterial cultures were prepared as described by Pan et al. (38), with tryptic soy agar supplemented with 0.7% yeast extract or tryptic soy broth supplemented with 0.7% yeast extract (TSBYE; Difco Laboratories, Detroit, MI). Overnight cultures were diluted (1:100) in TSBYE and reincubated either at 37°C for 12 h or at 30°C for 18 h to prepare cells for biofilm formation as described below. Each culture was harvested and washed with sterile saline (0.85% NaCl) by centrifugation (3,500 × *g* for 10 min at 10°C) and resuspended in saline or in TSBYE diluted 10-fold with sterile water (TSBYE/10). The cell density of each strain was adjusted to an optical density at 600 nm (OD$_{600}$) of 0.5 (ca. 10^8 CFU/ml). An equal volume of each cell suspension for each serotype was pooled to make cocktails of serotype 4b or 1/2a. In the course of this work, strain SK1387 was found to exhibit unique biofilm-forming properties, and therefore this strain was used separately. The mixture of strains of serotype 1/2a therefore consisted of seven strains, as described below.

Biofilm formation by individual strains. The biofilm formation capacity of each strain was determined with a microtiter plate assay as described previously (7), with a few modifications. Briefly, 200 μl of each overnight culture diluted with TSBYE (1:50) was transferred into 96-well microplates (catalog no. 163320; Nunclon Delta, Denmark). Replicate plates were incubated statically for 40 h at

22.5°C, 30°C, and 37°C. The biofilms were stained with 0.8% crystal violet (CV; Acros Organics, NJ), which was filtered with 0.22-μm-pore-size filters prior to use. The stained biofilms in the microplate wells were then flushed with tap water. The air-dried wells were filled with 95% ethanol to destain the biofilms. To determine the CV density, 100 μl from each well was transferred into a new 96-well flat-bottom microplate (catalog no. 353075; BD Falcon) and the OD$_{580}$ of the well content was assayed in a 96-well microplate reader (TECAN Safire, Austria) with Magellan 6.5 (TECAN, Austria) software. The mean OD$_{580}$ of the wells containing sterile medium only was used as a blank measurement to adjust the OD$_{580}$s of all of other wells.

Cell attachment, biofilm formation, and treatment in the SFP regimen. The procedure for cell attachment and biofilm formation was as described previously (38), with minor modifications. All cultures were grown at 30°C. The cell suspensions were added (230 ml/box) into sterile Rainin pipette tip boxes (without the tip-holding grid) in which stainless steel coupons were positioned as described previously (38). Biofilms were formed on the stainless steel coupons (T-304L, no. 4 finish, 780 by 25 by 1.2 mm) by the serotype 1/2a and 4b cocktails separately (230 ml/box of each cocktail) or mixed together (115 ml/box of each cocktail). Similarly, biofilms were prepared from serotype 1/2a strain SK1387 used alone and in combination with the serotype 4b cocktail. Following 3 h of incubation at 30°C, the cell suspension was removed from the boxes and the coupons were rinsed three times with sterile saline to remove loosely attached cells. Sterile boxes were then filled with TSBYE/10 and placed at 22.5°C for 7 days. The TSBYE/10 was replaced every 24 h. The coupons were then subjected to the SFP regimen, which consisted of consecutive 24-h cycles of sanitation, starvation and dehydration, and growth in diluted medium (38). Sample coupons (four replicates per box) were removed to determine viable cell densities (mean ± standard deviation log CFU/cm²) at the start (day 0), in the middle (day 14), and at the end (day 28) of the SFP regimen.

Measurement of viable cell density of strains of the same serotype in single or mixed biofilms in the SFP regimen. To determine total viable cell counts, cells were detached from the surfaces of the stainless steel coupons with sterile cotton-tipped swabs. A designated area (55 by 25 mm) on the surface of each coupon was swabbed with six separate swabs to remove cells from the surface. The swab tips from each sample were placed in a plastic screw-cap tube (50 ml; Corning) containing 20 ml of sterile saline and six to eight solid glass beads (4 mm in diameter). The bead-cell mixture was vortexed for 60 s. The total viable cell density of each sample was enumerated by plating as described previously (38). Because serotype-specific enumerations in mixed-serotype biofilms could not be achieved by plate counting, bacteria of each serotype (1/2a or 4b) were enumerated by quantitative real-time PCR with serotype-specific primers (Table 2). To prevent DNA amplification from dead cells or cell-free DNA, the cell suspension was treated with PMA prior to DNA isolation as described previously (37), with minor modification. A 650-W halogen light source was used to optimize PMA-DNA binding efficiency. The use of the 650-W light source could achieve 90% more free DNA bound with PMA than the 600-W light source used

TABLE 2. Oligonucleotides used in this study

Serotype and gene or use	Oligonucleotide[a]	Target fragment size (bp)	Reference
4b			
ORF0799F	5′-GCTGGGTTTCTTACGA-3′	83	9
ORF0799R	5′-CAACCGTTCATTTAGCTCAT-3′		
Probe	FAM-TCTGCTGTTCAGTTATTGGAGTGGGA-BHQ-1		
1/2a			
lmo0737F	5′-GCGGATGTGATTGATTTAC-3′	78	8
lmo0737R	5′-AAACTGCACTAACTCTTGAAT-3′		
Probe	FAM-TGCTCCAGGATCAAGACACGGTA-BHQ-1		

[a] FAM, 6-carboxyfluorescein; BHQ-1, Black Hole Quencher 1.

previously (37). This afforded increased discrimination between viable cells and dead cells and allowed the enumeration of viable bacterial cells in a live-dead mixture in which the ratio of dead cells to live cells could be up to 10^5:1 (F. Breidt and Y. Pan, unpublished data). The subsequent DNA extraction and real-time PCR assay were carried out as previously described (37). The relationship between threshold cycle times (using the mean of duplicates for each sample) and log_{10} CFU/ml was determined by using a standard curve with 10^3 to 10^8 CFU/ml live cells spiked with dead cells (up to 10^8 cells/ml; data not shown) as described previously (37).

To determine the cell density of serotype 1/2a and 4b strains in the medium following 8 h of incubation in TSBYE/10 in each 24-h cycle of the SFP treatment, aliquots of the cell suspension in the box for mixed-serotype biofilms were sampled. The total cell density was determined by plating and PMA-quantitative PCR analysis was employed as described above to determine the concentration of bacterial cells of each serotype as described above.

Attachment and motility. Bacteria were grown in TSBYE at 30°C for 18 h or at 37°C for 12 h. Cells were harvested by centrifugation (3,500 × g for 10 min at 10°C), resuspended in fresh TSBYE/10, and adjusted to an OD_{600} of 0.5 (ca. 10^8 cells/ml). Multistrain mixtures of each serotype were prepared as described above. Cell suspensions of serotype 1/2a and 4b cultures grown at 37°C and at 30°C were added (230 ml) into the Rainin pipette tip boxes containing stainless steel coupons. Following 3 h of incubation at 30°C, the cell suspension was removed from the boxes and the coupons were rinsed with saline three times. The cell density of each suspension was determined by plating. Attached cells

were enumerated by using six replicate coupons from each mixture as described above. To determine the motility of individual *L. monocytogenes* strains, stationary-phase cell suspensions were inoculated into 5 ml of TSBYE with 0.3% agar in a 10-ml glass tube by stabbing down through the center of the agar cylinder with an inoculation needle. The tubes were incubated at 30°C and 37°C for 48 h, respectively, and motility was determined by visual inspection of the growth of cells surrounding the stab.

Statistical analysis. The cell densities (log_{10} CFU/cm^2) of seven biofilm treatments (serotypes 1/2a and 4b in single- and mixed-serotype biofilms, strain SK1387 in single-culture biofilms, and a serotype 4b strain cocktail along with strain SK1387 in mixed-culture biofilms) were measured at three time points (days 0, 14, and 28) in the SFP regimen. The experiment was repeated three times. All data from the three repeats were statistically analyzed by using the General Linear Models Procedure of SAS version 9.1 (SAS Inc., Cary, NC). The significant differences among the treatment means were computed by Tukey's method.

RESULTS

Cell motility and initial attachment. To optimize the conditions for biofilm formation, each of the 16 strains was examined for motility and attachment to stainless steel coupons
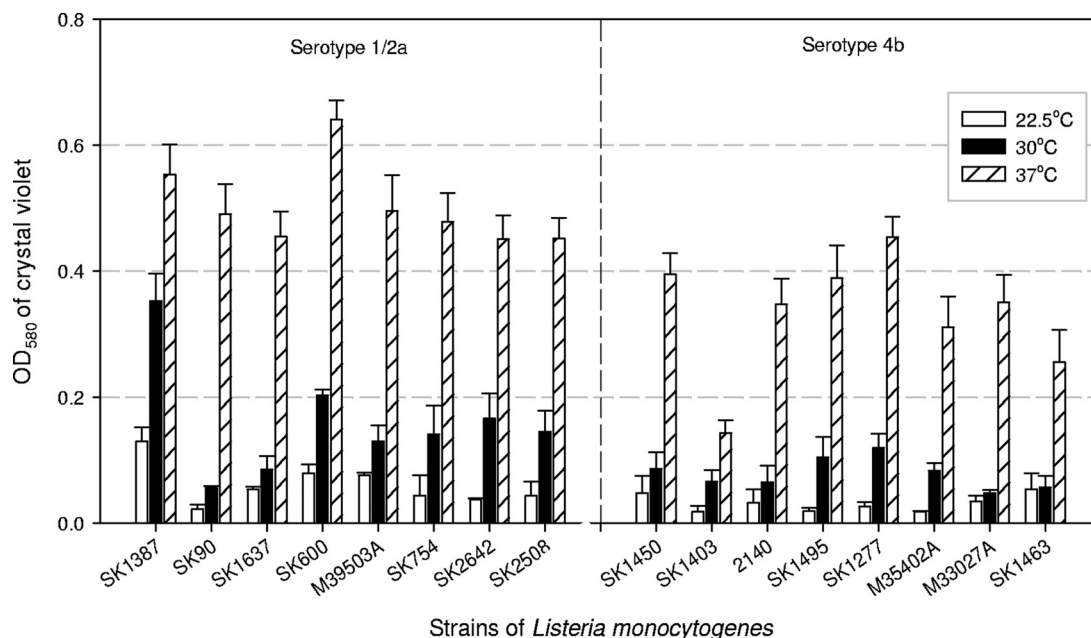

FIG. 1. Biofilm production of strains of *L. monocytogenes* as determined by microplate assay. The bars represent the means of triplicate replications, and error bars represent the standard deviations.
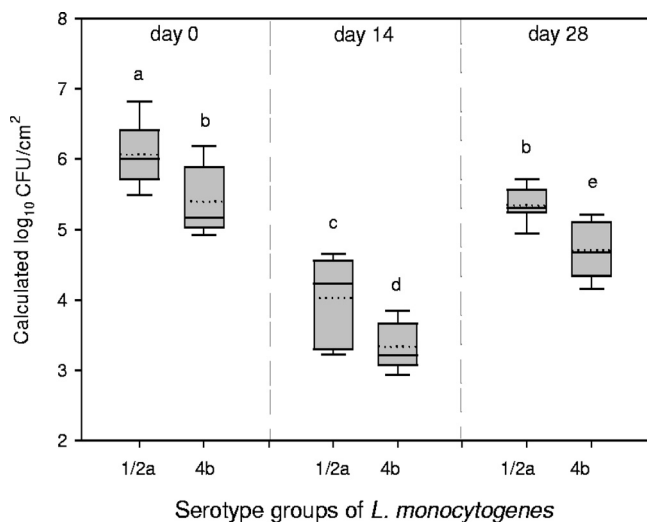
FIG. 2. Calculated cell density of *L. monocytogenes* serotype 1/2a and 4b strains in mixed-culture biofilms on stainless steel in the SFP system. Each box represents the range of cell densities of the indicated serotype from 12 replicate samples (4 replicates for each of three repeats). The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively ($n = 12$); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentiles. Boxes with the same letter have no significant difference ($P > 0.05$).
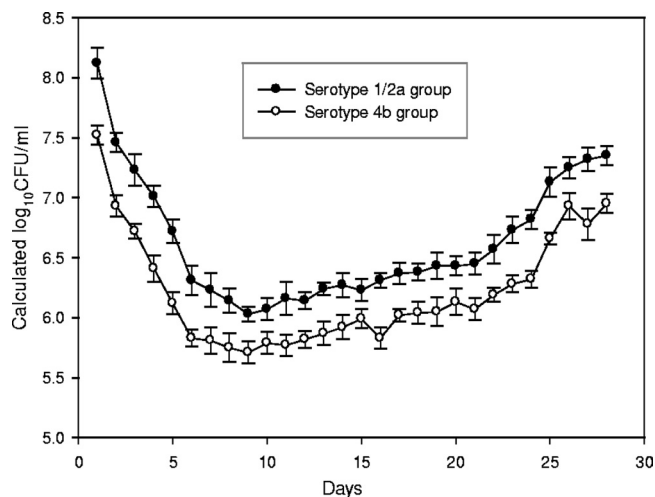

FIG. 3. Planktonic cell density of *L. monocytogenes* serotype 1/2a and 4b strains in diluted medium (TSBYE/10) in the group for serotype mix biofilms at the end of each 8-h incubation period in each 24-h cycle of the SFP system. The filled symbols represent serotype 1/2a strains; the open symbols represent serotype 4b strains. The error bars represent the mean and standard deviation ($n = 3$).

when grown at 30°C and 37°C (Fig. 1). As expected, the motility of all of the strains was suppressed at 37°C regardless of serotype, while at 30°C, motility was evident (data not shown). Cell cocktails ($\sim 5 \times 10^8$ CFU/ml total) were prepared from cells grown at 30°C and 37°C and examined for attachment to stainless steel coupons at 30°C. The attached cell densities of the serotype 1/2a group grown at 30°C and 37°C were 5.1 ($\pm$ 0.35) and 5.5 ($\pm$ 0.42) log CFU/cm², respectively. Similarly, the attached cell densities of the serotype 4b group were 5.2 ($\pm$ 0.12) and 5.5 ($\pm$ 0.22) log CFU/cm² for the cultures grown at 30°C and 37°C, respectively. These data show that there was no significant difference in cell density between the serotype 1/2a and 4b strains on stainless steel surfaces ($P > 0.05$) or for a given serotype with cells prepared at 30°C or 37°C ($P > 0.05$).

Biofilm formation by individual strains. Biofilm density was determined for each strain by using the microtiter plate assay at 22.5°C, 30°C, and 37°C. Strain SK600 formed the highest-density biofilm at 37°C, as measured by the OD of the CV dye, whereas strain SK1387 formed the highest-density biofilm at ≤30°C. Strain SK1387 was therefore selected individually for use in mixed-culture biofilm experiments (see below), which were carried out at 22.5°C.

Mixed-culture biofilms with serotype 1/2a and 4b strain cocktails. Following cell growth and attachment to stainless steel coupons at 30°C and subsequent biofilm formation at 22.5°C, the cocktail of the seven serotype 1/2a strains formed higher-density biofilms on stainless steel than did the serotype 4b strain cocktail ($P < 0.05$). The serotype 1/2a strain cocktail had a mean cell density of 6.07 ($\pm$ 0.45) log CFU/cm² versus 5.40 ($\pm$ 0.47) log CFU/cm² for the 4b strain cocktail (Fig. 2, day 0 of the SFP regimen). After 14 days of the SFP regimen,

all cell densities in the biofilms were reduced, although the cell density of the serotype 1/2a group remained higher than that of the serotype 4b group (4.03 $\pm$ 0.6 versus 3.34 $\pm$ 0.33 log CFU/cm²). The observed differences in biofilm formation between the serotype 1/2a and 4b strain cocktails were small (ca. 0.6 log) but were consistently observed and statistically significant ($P < 0.05$). By the end of the SFP regimen at 28 days, cell numbers for strain cocktails increased, with cell densities of 5.34 ($\pm$ 0.25) log CFU/cm² for serotype 1/2a strains and 4.71 ($\pm$ 0.38) log CFU/cm² for serotype 4b strains. Interestingly, the difference between the log of the cell numbers for the two serotype cocktails remained constant (0.6 to 0.7) during the 28-day experiment. These results indicated that the serotype 4b strains experienced the same growth pattern as the serotype 1/2a strains in mixed biofilms during the 28-day SFP regimen (i.e., an initial decrease in cell numbers and then an increase). Similar results were observed for the release of biofilm cells into the TSBYE/10 during the SFP treatment. After every 8-h incubation period in TSBYE/10 (in every 24-h cycle), the cell density of each serotype in the liquid medium was determined (Fig. 3). The cell density of serotype 1/2a strains was consistently higher (0.3 to 0.6 log CFU/ml) than that of serotype 4b strains during the 28-day experiment.

Mixed-culture biofilms with serotype 4b strains and serotype 1/2a strain SK1387. Biofilms of the serotype 1/2a strain cocktail (excluding SK1387), strain SK1387 alone, strain SK1387 with the serotype 4b strain cocktail, and the serotype 4b strain cocktail were prepared and subjected to the SFP regimen. The results are shown in Fig. 4. The initial biofilm densities, in CFU per cm², for the serotype 1/2a cocktail (excluding strain SK1387), SK1387 with the serotype 4b cocktail, and strain SK1387 alone were 6.2 ($\pm$ 0.19), 6.9 ($\pm$ 0.45), and 7.2 ($\pm$ 0.3) log CFU/cm², respectively. However, after 14 or 28 days, there was no significant difference in biofilm cell density ($P > 0.05$) among the serotype 1/2a cocktail (1/2a), strain SK1387 (SK1387), and strain SK1387 in mixed biofilms with
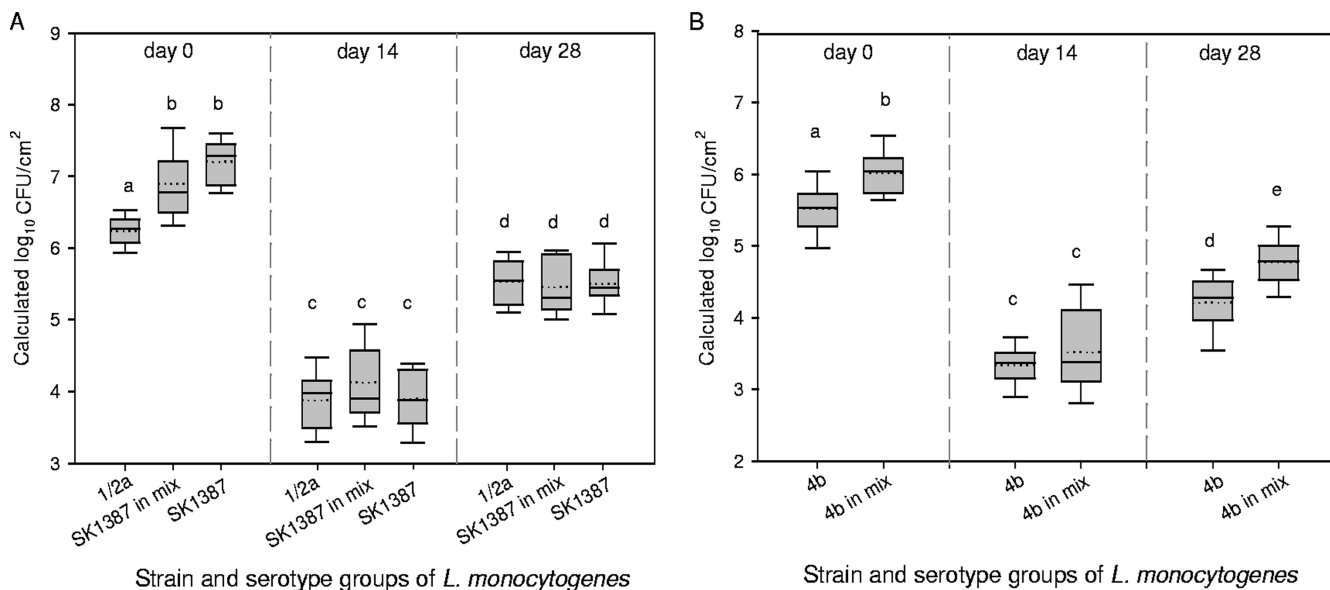
FIG. 4. Calculated cell density of *L. monocytogenes* in biofilms on stainless steel in the SFP system. A serotype 1/2a cocktail of seven isolates (excluding strain SK1387), strain SK1387 (single isolate), and strain SK1387 with the serotype 4b cocktail (eight isolates) are grouped in panel A. A serotype 4b (4b cocktail of eight isolates) along with the serotype 4b cocktail mixed with strain SK1387 are shown in panel B. Each box represents the range of cell densities of a certain serotype from 12 replicate samples at a specific time. The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively ($n = 12$); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentiles. Boxes with the same letter have no significant difference ($P > 0.05$).

the serotype 4b cocktail (SK1387 in mix) (Fig. 4A). The presence of serotype 4b strains (4b cocktail) in mixed biofilms with strain SK1387 or the serotype 1/2a cocktail (excluding SK1387) did not affect the biofilm density compared to that of strain SK1387 alone or the serotype 1/2a cocktail alone. There were no significant differences in biofilm density between the serotype 1/2a cocktail and strain SK1387, irrespective of the presence or absence of the serotype 4b cocktail, after 14 days in the SFP system. Interestingly, the biofilm density of the serotype 4b strains was initially greater when biofilms were prepared by using the mixture with strain SK1387 than when the 4b cocktail alone was used ($P < 0.05$; Fig. 4B, day 0). Similarly, the biofilm density of the serotype 4b strain cocktail with strain SK1387 was significantly higher than without strain SK1387 after 28 days of the SFP regimen ($P < 0.05$; Fig. 4B, day 28).

DISCUSSION

Research on *L. monocytogenes* biofilms has focused on attachment and biofilm formation with strains of different serotypes, but consistent trends have not emerged. The inconsistent results obtained could be due to differences in strains, media, and environmental conditions during biofilm formation (45). It is interesting to investigate the ability of serotype 1/2a and 4b strains to form biofilms because serotype 1/2a strains are frequently isolated from food-processing environments but serotype 4b strains are commonly implicated in food-borne outbreaks of listeriosis (1, 3, 14, 15, 17, 22, 29, 31, 41, 47, 49).

The formation of a biofilm has been considered a stress response of bacterial cells to withstand unfavorable conditions such as starvation, changes in osmosis and pH, oxygen radicals, disinfectants, and antibiotics (18). General experimental conditions utilized for biofilm research in a laboratory are quite different from the conditions that exist in food-processing plants, where bacteria survive and grow in a less-than-ideal environment. We used the SFP system, which consists of several stresses, to determine if *L. monocytogenes* strains of serotype 1/2a or 4b would have a selective advantage in the presence of environmental stresses in single- and mixed-culture biofilms. The use of quantitative PCR with serotype-specific primers and PMA treatment made it possible to selectively enumerate viable cells of each serotype in mixed-culture biofilms. Our results confirm the observations of Folsom et al. (12) that serotype 1/2a strains form higher-density biofilms than serotype 4b strains in diluted medium. The differences were small but statistically significant, and listeriae may behave differently in mixed-culture environments in food-processing facilities. When individual strains were tested, a direct correlation between increasing temperature and increasing biofilm density was observed (Fig. 1), which is in accordance with the results of Di Bonaventura et al. (6). The CV staining results, however, may reflect changes both in the cell density of *L. monocytogenes* at 30°C and in extracellular polymeric substance production (Pan and Breidt, unpublished).

The cell counts of bacteria of each serotype released to the medium from biofilms during the growth phase of the SPF system paralleled the cell counts in the biofilms, suggesting that the more cells there are in a biofilm, the more cells could be released to the environment. These data suggest that serotype 1/2a strains may have a competitive advantage over serotype 4b strains due to the ability of serotype 1/2a strains to form higher-density biofilms once attached to stainless steel surfaces. Once biofilms were formed, however, there was no evidence for the

inhibition of growth due to competition between strains of the two serotypes in mixed-culture biofilms. Strain SK1387 and other serotype 1/2a strains formed and maintained similar-density biofilms regardless of whether they were in the presence or the absence of serotype 4b strains at each time point throughout the SFP system. No difference between the growth rates of serotype 1/2a and 4b strains was observed in planktonic cells by Gorski et al. (16). Surprisingly, we found that the mixed-culture serotype 4b strains formed higher-density biofilms in the presence of serotype 1/2a strain SK1387 in mixed-culture biofilms. This may indicate that the extracellular matrix produced by strain SK1387 conferred greater protection from stress, allowing better growth of the serotype 4b cells in these mixed-culture biofilms.

Motility has been demonstrated to be essential for initial cell attachment and biofilm formation of several bacteria, e.g., *Escherichia coli* (40), *Campylobacter jejuni* (20), *Vibrio cholerae* (50), and *Yersinia enterocolitica* (24). On the other hand, some studies show that flagella were not necessary for initial attachment or biofilm formation of *Pseudomonas aeruginosa* (25, 42). It has been observed that *L. monocytogenes* does not generate flagella and is nonmotile at 37°C but is motile at 30°C and lower temperatures, although there is variation from strain to strain (39, 51). The relationship between the presence of flagella and attachment and biofilm formation has been studied (6, 7, 16, 26, 46, 48). Mutagenesis studies revealed that flagellum-mediated motility was essential for initial attachment (16, 26, 48), although other studies showed that motility was not required for attachment (6, 7). We found no significant difference in attachment to stainless steel between serotype 1/2a and 4b strains regardless of the growth temperature (30 versus 37°C). Other mechanisms may compensate for the lack of flagella in initial attachment for biofilm formation at 37°C.

The use of PMA prior to DNA isolation enabled us to enumerate viable cells in biofilms by quantitative PCR. Using this method, we were able to enumerate cells of a specific serotype detached from stainless steel coupons. The resulting data from these experiments and data from a related study (38) support the hypothesis that biofilms control the adaptation and survival of *L. monocytogenes* on stainless steel surfaces in the presence of environmental stresses. Strain SK1387 was first isolated from a sporadic case of illness associated with the consumption of contaminated turkey franks in 1988, and a strain with an indistinguishable genotype was implicated in a multistate outbreak associated with the same processing plant 12 years later. These data suggested that strain SK1387 was a persistent strain in turkey deli meat-processing facilities (21). Our data suggest that the higher capacity for biofilm formation of strain SK1387 at low temperature may contribute to the persistence of this strain in the processing environment. The enhanced growth seen with the serotype 4b cocktail in the presence of strain SK1387 may be attributed to the benefit provided by strain SK1387, which supports the hypothesis that selfless behavior may occur in a community mode of growth such as in biofilms (18). The biochemical composition of the extracellular matrix of strain SK1387 and other serotype 1/2a and 4b strains of *L. monocytogenes* is a subject for future investigations. The methods used in this study may also be applied for the study of other mixed-culture biofilms, if appropriate primers are available.

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