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# Synergistic Effects of Sodium Chloride, Glucose, and Temperature on Biofilm Formation by *Listeria monocytogenes* Serotype 1/2a and 4b Strains[▽]†‡

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**Biofilm formation by *Listeria monocytogenes* is generally associated with its persistence in the food-processing environment. Serotype 1/2a strains make up more than 50% of the total isolates recovered from food and the environment, while serotype 4b strains are most often associated with major outbreaks of human listeriosis. Using a microplate assay with crystal violet staining, we examined biofilm formation by 18 strains of each serotype in tryptic soy broth with varying concentrations of glucose (from 0.25% to 10.0%, wt/vol), sodium chloride (from 0.5% to 7.0%, wt/vol) and ethanol (from 1% to 5.0%, vol/vol), and at different temperatures (22.5°C, 30°C, and 37°C). A synergistic effect on biofilm formation was observed for glucose, sodium chloride, and temperature. The serotype 1/2a strains generally formed higher-density biofilms than the 4b strains under most conditions tested. Interestingly, most serotype 4b strains had a higher growth rate than the 1/2a strains, suggesting that the growth rate may not be directly related to the capacity for biofilm formation. Crystal violet was found to stain both bacterial cells and biofilm matrix material. The enhancement in biofilm formation by environmental factors was apparently due to the production of extracellular polymeric substances instead of the accumulation of viable biofilm cells.**

*Listeria monocytogenes*, a Gram-positive bacterium, is capable of causing severe food-borne infections in both humans and animals. The organism is ubiquitous in the environment and can grow in a wide variety of foods, including those stored at refrigeration temperatures. It is particularly difficult to eliminate this bacterium from ready-to-eat foods and food-processing equipment (19). The ability to form biofilms protects the bacterium from stresses in food-processing environments (13, 25). Among the 13 different serotypes described, serotypes 1/2a, 1/2b, and 4b are involved in the majority of human cases of listeriosis. Serotype 4b strains have accounted for most human outbreaks, whereas the majority of *L. monocytogenes* strains isolated from foods or food-processing plants belong to serotype 1/2a (19).

Comparative studies to link the phenotypic attributes of *L. monocytogenes* strains to serotypes have obtained variable results. Buncic et al. (4) have shown that serotype 1/2a isolates were more resistant to antilisterial bacteriocins than serotype 4b strains at 4°C. They also found that 4b isolates exhibited greater resistance to heat treatments at 60°C and were easier to recover than 1/2a strains immediately following cold storage. Bruhn et al. (3) observed that 1/2a strains (lineage II) grew faster than 4b and 1/2b (lineage I) strains in commonly used enrichment broth media (University of Vermont media I and II). However, other studies have indicated that similar differences could not be linked to a serotype (14), and sequencing results have shown a syntenic relationship between strains of the two serotypes (27).

Some *L. monocytogenes* strains have consistently been isolated from food-processing plants over many years (1, 28). Although several studies have been carried out to identify differences in cell adherence and biofilm formation among different serotypes, conflicting results were obtained. Lineage I isolates (including serotypes 4b, 1/2b, 3c, and 3b) were found to produce higher-density biofilms than lineage II isolates (including serotypes 1/2a, 1/2c, and 3a) (8, 28). However, this conclusion was not supported by other studies (1, 7, 18). For serotype 4b strains, the capacity to form biofilms was reduced when the nutrient level in a medium decreased, while serotype 1/2a strains were not similarly affected (11).

It has been suggested that the formation of a biofilm is a stress response by bacterial cells (15, 16). Biofilm research under laboratory conditions may not reflect biofilm formation in the environment. To investigate the behavior of *L. monocytogenes* in biofilms, a simulated food-processing (SFP) system including several stresses was designed (30). The SFP system was used to study 1/2a and 4b strains in mixed-culture biofilms (31). Bacterial cells from a 1/2a cocktail predominated over 4b strains when exposed to the SFP system for 4 weeks, but no competitive inhibition was observed. Environmental factors, including temperature, sugar, salt, pH, and nutrients that are common in foods and food-processing environments, have been demonstrated to have impacts on *L. monocytogenes* ad-

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TABLE 1. *Listeria monocytogenes* strains used in the study

| Strain no.[a] | Origin | Reference | Other ID(s)[b] |
|---|---|---|---|
| Serotype 1/2a | | | |
| SK1387 | Food (frankfurter), 1988 | 2 | G3965, F6854 |
| SK90 | Turkey-processing environment, 2004 | 21 | 90 |
| SK1637 | Turkey-processing environment, 2005 | 21 | 1637 |
| SK600* | Turkey-processing environment, 2004 | 26 | 600 |
| M39503A* | Bulk milk, 2001 | 1 | |
| SK754 | Turkey-processing environment, 2004 | 26 | 754 |
| SK2642 | Turkey-processing environment, 2006 | 26 | 2642 |
| SK2508 | Turkey-processing environment, 2004 | 21 | 2508 |
| RM3023 | Poultry, England; 1998 | | ATCC 19111 |
| RM3316 | Silage, 2002 | | CWD243 |
| RM3349 | Turkey frank, 2002 | | F6854 |
| RM3354 | Pork plant, 2002 | | JL1-6, MFS-1 |
| RM3373 | Human, United States; 2002 | | 1155 |
| RM3834* | Cooked corned beef, 2000 | | 33034 |
| RM3835 | Sausage, 2000 | | 33035 |
| RM4527 | Patient, England; 2004 | | TS33 (L745) |
| RM4543 | Food, United States; 2004 | | TS49 (F7273) |
| RM4561 | Patient, United States; 2004 | | TS67 (F6953) |
| Serotype 4b | | | |
| RM4573 | Patient, Canada; 2004 | | TS79 (L4738) |
| RM 2387 | Mint, 2000 | | |
| RM2992 | Cucumber, 2002 | | 2223 |
| RM2998 | Human, 2002 | | 2207 |
| RM3013 | Human, 1998 | | ATCC 19115 |
| RM4503* | Food, Canada; 2004 | | TS9 (L4707) |
| RM4504 | Food, United States; 2004 | | TS10 (F8353) |
| RM 3302 | Cow brain, 2002 | | CWD874 |
| RM3817 | Oyster, 1999 | | 33007 |
| RM4515 | Food, Switzerland; 2004 | | TS21 (L4486j) |
| SK1450* | Hot dog outbreak, 1998-1999 | 20 | H7550 |
| SK1403 | Food, United States (California outbreak); 1985 | 27 | F2365, G3990 |
| 2140 | 2001 | 1 | |
| SK1495* | Turkey-processing environment, 2003 | 10 | L0315 |
| SK1277 | Turkey-processing environment, 2003 | 10 | 82-2a |
| M35402A | Bulk milk, 2001 | 1 | |
| M33027A | Bulk milk, 2001 | 1 | |
| SK1463 | Turkey-processing environment, 2002 | 20 | J1815 |

[a] Strains with designations beginning with SK are from the culture collection of S. Kathariou at the Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, NC 27695-7624. Strains with designations beginning with RM are from the culture collection of L. Gorski. The others are from the culture collection of D. Call's lab at the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164. Strains marked with asterisks were used as components in a six-strain cocktail for time course monitoring and crystal violet staining.

[b] ID, identification.

hesion and biofilm formation (25). The objectives of this study were to investigate and compare biofilm formation between *L. monocytogenes* serotype 1/2a strains and serotype 4b strains under a variety of environmental conditions, including different temperatures and varying concentrations of salt, sugar, and ethanol, and to examine the synergistic effects of these factors on biofilm formation by both serotypes.

## MATERIALS AND METHODS

**Strains and growth conditions.** Eighteen *L. monocytogenes* serotype 1/2a strains and 18 serotype 4b isolates from diverse sources were used in the study (Table 1). The methods for the storage and preparation of strains have been described previously (30). Briefly, each strain was transferred from a frozen stock (−80°C) to a petri plate of tryptic soy agar containing 0.6% yeast extract (TSAYE; BD Biosciences) and was incubated at 37°C for 20 to 24 h. One or two typical colonies from the recovery plate were inoculated into 8 ml of tryptic soy broth containing 0.6% yeast extract (TSBYE) and were incubated statically for approximately 8 h at 30°C to generate fresh late-exponential-stage cultures.

**Microplate assays.** The capacity of each individual strain to form biofilms was measured by a microplate assay (8, 31). Four microliters of each late-exponential-stage culture (optical density at 600 nm [$OD_{600}$], 0.5) was diluted 1:50 with TSBYE (196 μl) supplemented with either glucose, sodium chloride, or ethanol in 96-well polystyrene microplates (catalog no. 163320; Nunclon Delta, Denmark). Replicate plates were incubated statically at different temperatures. The procedure described previously for staining, washing, drying, destaining, and plate reading was followed (8, 31). Briefly, the biofilms were stained with 0.8% crystal violet (CV; Acros Organics, NJ). The stained biofilms in the microplate wells were then flushed with tap water and air dried. The wells were filled with 95% ethanol to destain the biofilms, and the $OD_{580}$ of the ethanol was determined in a microplate reader (Tecan Safire, Austria) with Magellan software (version 6.5; Tecan, Austria). The absorbances of well contents were read at 580 nm, the wavelength at which the CV (Acros Organics, NJ) used had the maximum absorbance in a 96-well microplate reader (Tecan Safire, Austria) with Magellan software (version 6.5; Tecan, Austria). The mean absorbance from control wells containing medium only was subtracted from the mean absorbance of the other wells. Data from the absorbance of individual strains of each serotype were analyzed using box plots (below). Data for each group (18 strains each) are presented as means ± standard deviations (STDEV) in the text.

**Factors affecting biofilm formation.** Biofilm formation was analyzed individually for each strain by using the microplate assay with various concentrations of sodium chloride (0.5 to 7.0%, wt/vol), glucose (0.25 to 10.0%, wt/vol), or ethanol (1.0 to 5.0%, vol/vol), which may be present in foods, beverages, and food-

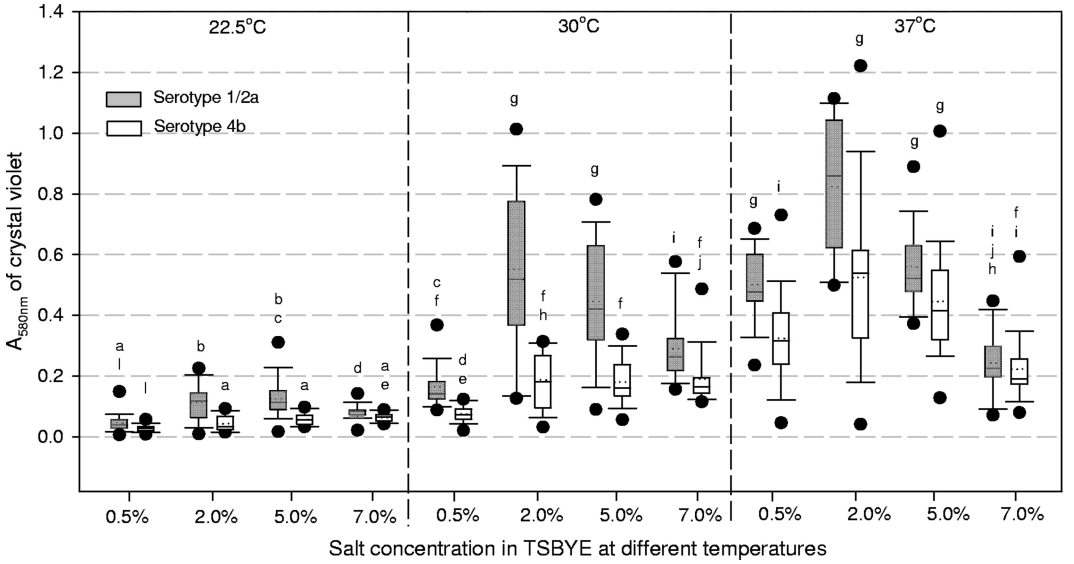


FIG. 1. Box plot of absorbance ($A_{580}$) of crystal violet from destained *L. monocytogenes* biofilms formed by serotype 1/2a ($n = 18$) and 4b ($n = 18$) strains in TSBYE containing the indicated concentrations of sodium chloride at each temperature. (Data for individuals are provided in Fig. S1.1, S1.2, and S1.3 in the supplemental material.) Boxes labeled with same letters at the top are not significantly different from each other ($P \geq 0.05$).

processing facilities. For the analysis of synergistic effects, combinations of these solutes were prepared as described below. For glucose-NaCl mixtures, the type and concentration of each medium preparation are reported with subscripts; for example, $TSBYE_{gluc1\%+NaCl2\%}$ stands for TSBYE containing 1% glucose and 2% NaCl. For all experiments, replicate microplates were prepared and incubated at 22.5°C, 30°C, or 37°C for 40 h prior to CV staining.

**Growth rate analysis.** The growth rate of each strain was determined individually using a microplate assay. The cultures were prepared as described above. Bacterial cells at late-exponential phase were inoculated into microplate wells at approximately $1 \times 10^7$ CFU/ml (final volume, 200 μl/well). The optical density in each well was monitored at 600 nm by periodic measurements using a 96-well microplate reader (Tecan Safire, Austria) with Magellan software (version 6.5; Tecan, Austria). To determine the maximum growth rate of each single strain, the slope of the linear part of the growth curve ($R^2$, >0.98) was determined for at least five data points of the semilogarithmic plot of optical density ($\ln[OD_{600}]$) versus incubation time (in hours). The maximum growth rates for individual strains for the 1/2a or 4b serotypes were presented and analyzed using box plots as described below; the rates for the two groups (1/2a and 4b serotypes; 18 strains per group) are expressed in the text as means ± STDEV per hour.

**Biofilm formation.** In order to determine how biofilm density changed over time, a six-strain cocktail (as indicated in Table 1) was used to form biofilms in microplate wells for 40 h in two types of media (TSBYE and $TSBYE_{gluc1\%+NaCl2\%}$) at 22.5°C, 30°C, and 37°C. Replicate samples were taken at the specified intervals for the measurement of total biofilm mass (absorbance of crystal violet), viable biofilm cell numbers, planktonic cell numbers, and the pH of the cell suspension in the microplate wells. To determine viable cell numbers in the biofilms, 200 μl of a mixed enzyme solution containing lipase (100 U/ml; catalog no. 62285; Sigma-Aldrich, Switzerland), cellulase (100 U/ml; catalog no. C0615; Sigma-Aldrich, Japan), and protease K (100 μg/ml; catalog no. 19131; Qiagen, CA) in Tris-HCl buffer (20 mM; pH 7.8) with 150 mM NaCl, 1 mM $CaCl_2$, and 2 mM $MgCl_2$ was added to each well, followed by incubation at 37°C for 1 h. This enzymatic treatment was previously found to be comparable to a swabbing method for removing and enumerating viable cells (30, 31; Y. Pan and F. Breidt, Jr., unpublished data).

**CV staining of fresh bacterial cells.** A six-strain cocktail prepared as described above was washed twice by centrifugation at 3,500 × *g* for 10 min at 10°C and was then resuspended in saline (8.5 g of NaCl/liter). The cell suspension was then serially diluted, and cell counts were determined by plating on TSAYE. Four 1.0-ml aliquots of each of the diluted cell suspensions were filtered using 13-mm syringe filters (pore size, 0.2 μm; catalog no. 09-720-5; Fisher, Ireland). The filters with bacterial cells were stained with CV solution for 15 min at room temperature. The stained filters were then flushed with deionized water until the filtrate was clear, followed by drying in air overnight in a biosafety cabinet. Five milliliters of 95% ethanol was used to solubilize CV bound to the filter and bacterial cells. The absorbance at 580 nm ($A_{580}$) for 100 μl of ethanol containing CV from each destained filter was assayed using the 96-well microplate reader as described above. The $A_{580}$ value from the filter without bacterial cells was used as a blank and was subtracted from the $A_{580}$ values for the other filters. The relationship between logarithms of bacterial cell numbers (CFU) and the corresponding adjusted $A_{580}$ values for CV were analyzed using linear regression.

**Statistics and reproducibility of results.** At least three replicates for each of three independent repeats were performed for each experiment. The data presented are the means of data generated from three independent trials. Box plots were used to summarize the data for the individual strains of different serotypes; each box represents the range of values for the 18 individual strains of an indicated serotype. 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 ($n = 18$); and the boundary of the box farthest from zero indicates the 75th percentile. The horizontal bars above and below the box indicate the 95th and 5th percentiles, respectively. Solid dots represent the data beyond the 5th and 95th percentiles (in Fig. 1, 2, and 3). Comparisons of multiple means were done using Student's *t* test.

## RESULTS

**Influence of salt concentration on biofilm production.** Serotype 1/2a strains and serotype 4b strains formed biofilms with similar densities when they were grown in TSBYE with 0.5% salt at 22.5°C for 40 h ($P > 0.05$) (Fig. 1). Almost all strains showed enhanced biofilm formation when the salt concentration was increased from 0.5% to 7.0% at 22.5°C and 30°C, although this was not the case at 37°C (Fig. 1). The optimal salt concentrations for biofilm formation were 5% at 22.5°C and 2% at 30°C and 37°C. Most (12/18) of the 1/2a strains formed significantly higher density biofilms than the 4b strains in TSBYE supplemented with 2% to 5% sodium chloride at 22.5°C and 30°C ($P \leq 0.04$) (see Fig. S1.2 in the supplemental material). Similarly, 72% (13/18) of the 1/2a strains formed significantly higher density biofilms than 83% (15/18) of 4b strains in $TSBYE_{NaCl2\%}$ at 37°C ($P < 0.005$); the exceptions were 4b strains RM2387, RM4504, and RM3013 (see Fig. S1.3

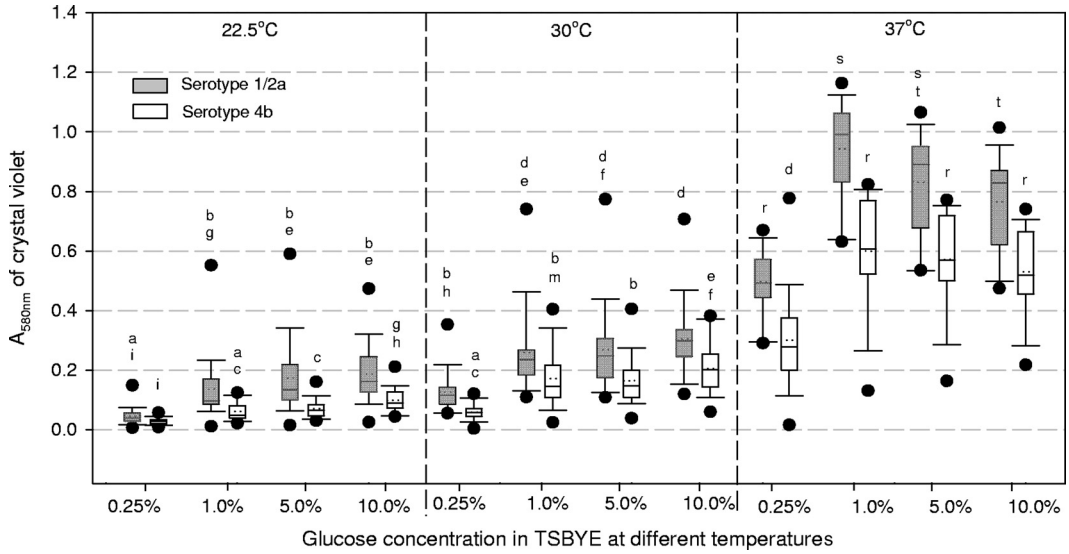


FIG. 2. Box plot of absorbance ($A_{580}$) of crystal violet from destained *L. monocytogenes* biofilms formed by serotype 1/2a ($n = 18$) and 4b ($n = 18$) strains in TSBYE containing the indicated concentrations of glucose at each temperature. (Data for individuals are provided in Fig. S2.1, S2.2, and S2.3 in the supplemental material.) Boxes labeled with same letters at the top are not significantly different from each other ($P \geq 0.05$).

in the supplemental material). With the noted exceptions (in the base medium at 22.5°C and in TSBYE with ≥5% sodium chloride at 37°C), the *L. monocytogenes* serotype 1/2a strains formed higher-density biofilms than the serotype 4b strains when the NaCl concentration was 2% to 7% at 30°C and below, or below 2% NaCl at 37°C.

**Influence of glucose concentration on biofilm formation.** With the addition of glucose in a range from 1.0% to 10.0% in TSBYE, 97% (35/36) of the strains formed higher-density biofilms at 22.5°C, 30°C, and 37°C than in TSBYE alone (Fig. 2). The 4b strain RM3013, however, formed the highest-density biofilms in TSBYE without the addition of glucose at 37°C (see Fig. S2.3 in the supplemental material). Serotype 1/2a strains generally formed higher-density biofilms than serotype 4b strains ($P < 0.05$) with glucose concentrations of 1.0% to 10.00% (Fig. 2). The average absorbance values (0.14 ± 0.12 and 0.06 ± 0.03 for 1/2a and 4b, respectively) for the biofilms of the 18 individual strains in each serotype grown in $TSBYE_{gluc1\%}$ at 22.5°C increased more than 2-fold at 30°C and more than 7-fold at 37°C (Fig. 2). As with the salt effect, the serotype 1/2a strains formed higher-density biofilms than the serotype 4b strains as the glucose concentration and temperature increased.

**Effect of ethanol on biofilm formation.** The biofilm formation of 61% (11/18) of the 1/2a strains and 22% (4/18) of the 4b strains was enhanced by ethanol at 22.5°C (Fig. 3; see also Fig. S3.1 in the supplemental material). For the range tested, the concentrations of ethanol that resulted in the densest biofilm formation by the 1/2a and 4b strains were 3.0% and 5.0%, respectively, at 22.5°C. At 37°C, ethanol had a pronounced inhibitory effect on biofilm formation. In the presence of ethanol in TSBYE, the 1/2a strains consistently formed higher-density biofilms than the 4b strains at 22.5°C, 30°C, and 37°C ($P \leq 0.01$), unless the ethanol concentration was 5.0% (vol/vol) (Fig. 3; see also Fig. S3.1 to S3.3 in the supplemental material).

**Synergistic effects.** The addition of either glucose or sodium chloride to TSBYE stimulated *L. monocytogenes* strains to form higher-density biofilms (Fig. 1 and 2). The combination of salt and glucose resulted in even higher density biofilms at all three temperatures than individual treatments (Fig. 4). The CV absorbance ($A_{580}$) for the biofilms formed by the 1/2a strains was three times more in $TSBYE_{gluc1\%+NaCl2\%}$ (0.38 ± 0.29) than either in $TSBYE_{gluc1\%}$ (0.14 ± 0.13) or in $TSBYE_{NaCl2\%}$ (0.12 ± 0.06) at 22.5°C (Fig. 4). Furthermore, the biofilm densities of all strains increased with an increase in the incubation temperature (Fig. 4). The same trend was observed at 30°C and 37°C. The data suggest that temperature, glucose, and salt have synergistic effects on biofilm formation and that the 1/2a strains formed higher-density biofilms than the 4b strains in $TSBYE_{gluc1\%+NaCl2\%}$ at all three temperatures ($P < 0.01$).

**Growth rates in TSBYE.** The mean growth rate of the 18 individual serotype 1/2a strains was 0.42 ± 0.04 $h^{-1}$ in TSBYE at 22.5°C, which was similar to that of the 4b strains (0.45 ± 0.04 $h^{-1}$) ($P = 0.05$). The addition of glucose to TSBYE stimulated the growth of several 4b strains, making the growth rate of the 4b strains higher than that of the 1/2a strains (0.48 ± 0.06 $h^{-1}$ versus 0.43 ± 0.04 $h^{-1}$; $P < 0.01$) at 22.5°C (Fig. 5). Increasing the salt concentration (from 0.5% to 2%) in TSBYE did not significantly affect the growth of either serotype at all three temperatures, and the growth rate of each serotype was similar to that in $TSBYE_{gluc1\%+NaCl2\%}$ ($P > 0.1$). Unexpectedly, more than 75% of the 4b strains had higher growth rates than most (75%) of the 1/2a strains under all conditions tested ($P < 0.01$), excluding TSBYE at 22.5°C. The growth rates of all strains were increased by approximately 0.2 $h^{-1}$ on average when the temperature increased from 22.5°C to 30°C and were approximately 0.12 $h^{-1}$ higher at 37°C than at 30°C. A few of the 4b strains, including SK1403 (1.31 ± 0.05 $h^{-1}$), RM2992 (1.33 ± 0.02 $h^{-1}$), and RM4515 (1.19 ± 0.02

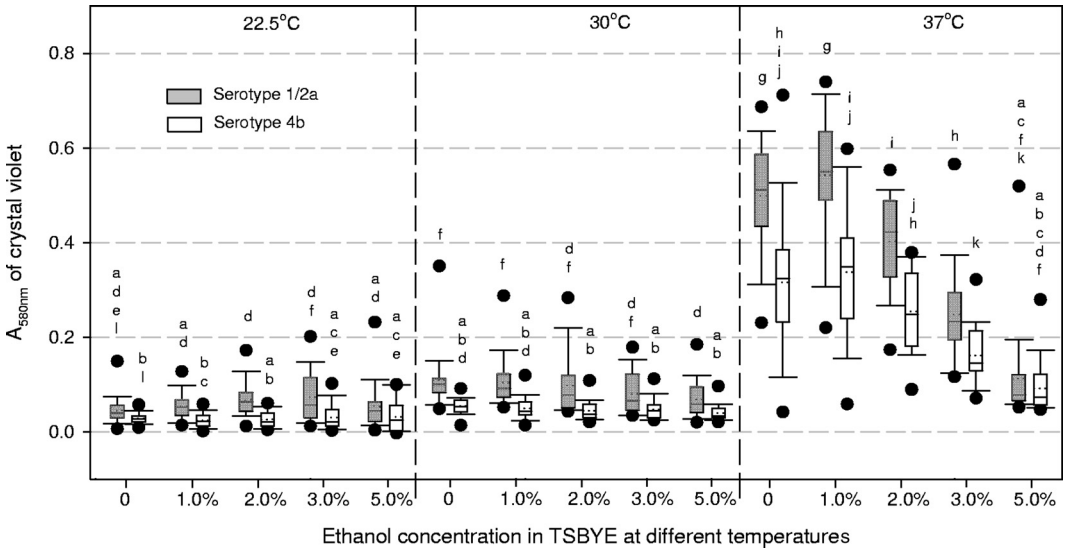


FIG. 3. Box plot of absorbance ($A_{580}$) of crystal violet from destained *L. monocytogenes* biofilms formed by serotype 1/2a ($n = 18$) and 4b ($n = 18$) strains in TSBYE containing the indicated concentrations of ethanol at each temperature. (Data for individuals are provided in Fig. S3.1, S3.2, and S3.3 in the supplemental material.) Boxes labeled with the same letters at the top are not significantly different from each other ($P \geq 0.05$).

$h^{-1}$), grew significantly faster than the average (0.81 ± 0.1 $h^{-1}$) for the 4b strains in $TSBYE_{gluc1\%}$ at 37°C ($P < 0.001$) (see Fig. S5 in the supplemental material). Interestingly, the slowest-growing strain in the 4b group was RM3013 (0.58 ± 0.04 $h^{-1}$), although it formed the highest-density biofilms in TSBYE at 37°C (see Fig. S2.3 in the supplemental material). These data suggest that the 4b strains generally grow faster than the 1/2a strains, and they indicate that growth rate and biofilm formation are not directly related.

**Biofilm formation.** Biofilm formation by a six-strain cocktail containing three strains of each serotype (as indicated in Table 1) was monitored to determine the relationship between viable cells and CV absorbance data. The CV absorbance values ($A_{580}$) increased during 40 h of incubation for biofilm formation for all treatments. The viable cell density in biofilms increased from 6.4 ± 0.12 $\log_{10}$ CFU/well at 8 h to 6.9 ± 0.13 $\log_{10}$ CFU/well at 40 h in TSBYE at 30°C (Fig. 6A). In contrast to the biofilms formed in TSBYE, the viable cell density in-

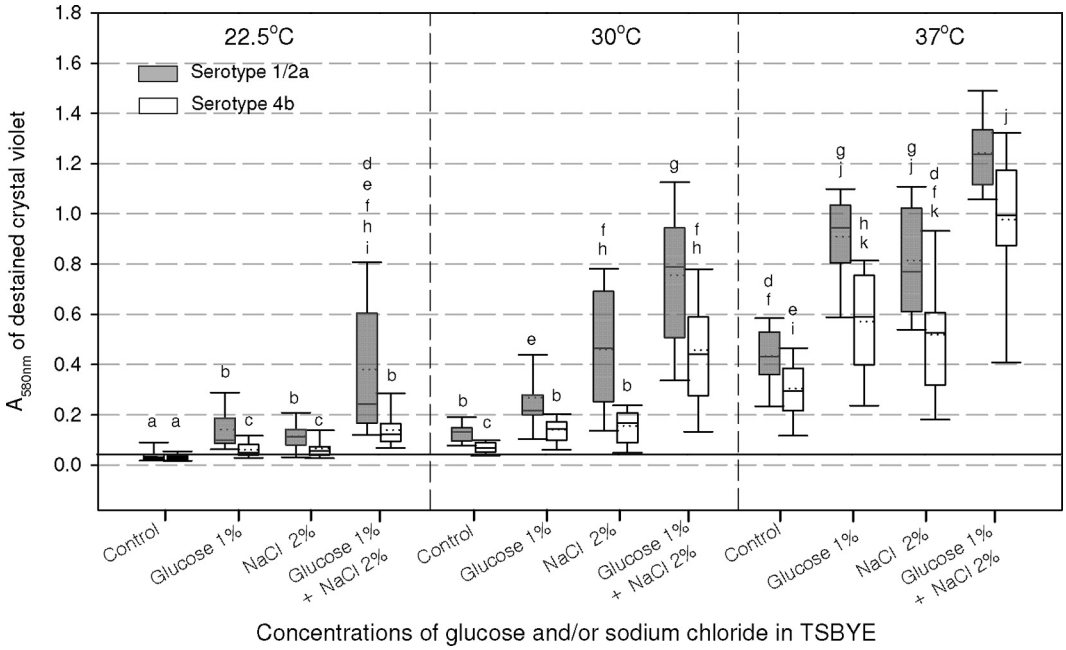


FIG. 4. Box plot of absorbance ($A_{580}$) of crystal violet from destained *L. monocytogenes* biofilms formed by serotype 1/2a ($n = 18$) and 4b ($n = 18$) strains in TSBYE containing the indicated concentrations of glucose and sodium chloride at each temperature. Boxes labeled with the same letters at the top are not significantly different from each other ($P \geq 0.05$).

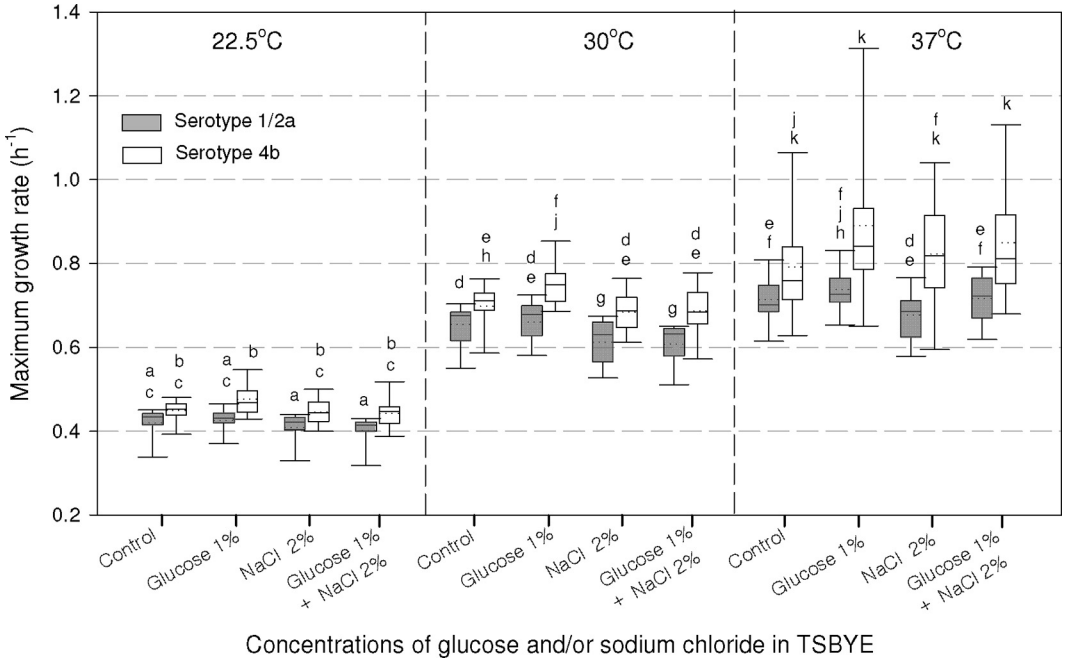


FIG. 5. Box plot of maximum growth rate of *L. monocytogenes* serotype 1/2a ($n = 18$) and 4b ($n = 18$) strains in TSBYE containing the indicated concentrations of glucose and sodium chloride at each temperature. Boxes labeled with the same letters at the top are not significantly different from each other ($P \geq 0.05$).

creased from 6.5 ± 0.1 $\log_{10}$ CFU/well at 8 h to 7.2 ± 0.17 $\log_{10}$ CFU/well at 16 h in $TSBYE_{gluc1\%+NaCl2\%}$ and then declined to 4.5 ± 0.2 $\log_{10}$ CFU/well at 40 h at 30°C (Fig. 6C). With planktonic cells, the pH declined faster in $TSBYE_{gluc1\%+NaCl2\%}$ than in TSBYE. In TSBYE, the pH decreased to 5.2 within the first 8 h and then remained constant at 30°C (Fig. 6B). The cell density increased from 6.8 ± 0.08 $\log_{10}$ CFU/ml to 9.25 ± 0.05 $\log_{10}$ CFU/ml in the first 16 h and then decreased to 8.6 ± 0.05 $\log_{10}$ CFU/ml after 24 h (Fig. 6B). In $TSBYE_{gluc1\%+NaCl2\%}$, the pH declined from 6.9 to 4.3 in 24 h of incubation and remained constant at 30°C (Fig. 6D). The planktonic cell counts decreased following 8 h of incubation, and cell numbers were 5.35 ± 0.09 $\log_{10}$ CFU/ml at 40 h (Fig. 5D). Similar trends were found at 22.5°C and 37°C (data not shown). As pH decreased, the viability of both planktonic cells and biofilm cells declined accordingly. The CV values increased while the viable cell counts in the biofilms decreased (Fig. 6C).

**Cell counts and crystal violet staining.** To determine the relationship between the absorbance of CV extracted from bacterial cells and cell number, planktonic bacterial cells were stained using a filtration method. The amount of crystal violet adsorbed onto the filters during cell staining, as measured by absorbance, was constant until the number of bacterial cells retained on a filter membrane was more than 2.8E+07 CFU. A curve representing the relationship between OD values and corresponding cell counts was plotted and analyzed using linear regression. A linear relationship ($R^2$, 0.93) was found between the logarithms of cell counts and the corresponding absorbance values when the cell numbers were in the range from 2.8E+07 CFU to 4.5E+08 CFU (Fig. 7).

## DISCUSSION

The objectives of this research were to determine if there is a difference in biofilm formation between serotypes 1/2a and 4b of *L. monocytogenes* under a variety of conditions and to examine the effects of environmental factors on biofilm formation. Eighteen serotype 1/2a strains and 18 serotype 4b strains from a wide range of sources were examined individually for their abilities to form biofilms (as shown in the supplemental material). Despite the differences in the abilities of individual strains to form biofilms, consistent differences were observed in the biofilms of the 1/2a and 4b strains. Serotype 1/2a strains generally formed higher-density biofilms than serotype 4b strains under a variety of conditions (Fig. 1, 2, 3, and 4). However, the 4b strains exhibited higher maximum growth rates than the 1/2a strains (Fig. 5), further supporting the findings in previous studies that the growth rate is not directly correlated with biofilm formation (5, 8). The transition from the planktonic and free-swimming state to the sessile state of biofilms has been considered a regulated developmental process (29), resulting in a complex surface-attached bacterial community in which the physiological status of cells is distinct from that in the planktonic state.

In addition to the intrinsic properties of individual strains, numerous extrinsic factors, including the physiochemical characteristics of surface materials, temperature, nutrients, pH, salt, sugar, and the presence of other bacteria, have been shown to influence initial cell attachment and subsequent biofilm formation by *L. monocytogenes* (25). *L. monocytogenes* formed higher-density biofilms when the growth medium was supplemented with sugar and/or salt (17). Similar results have

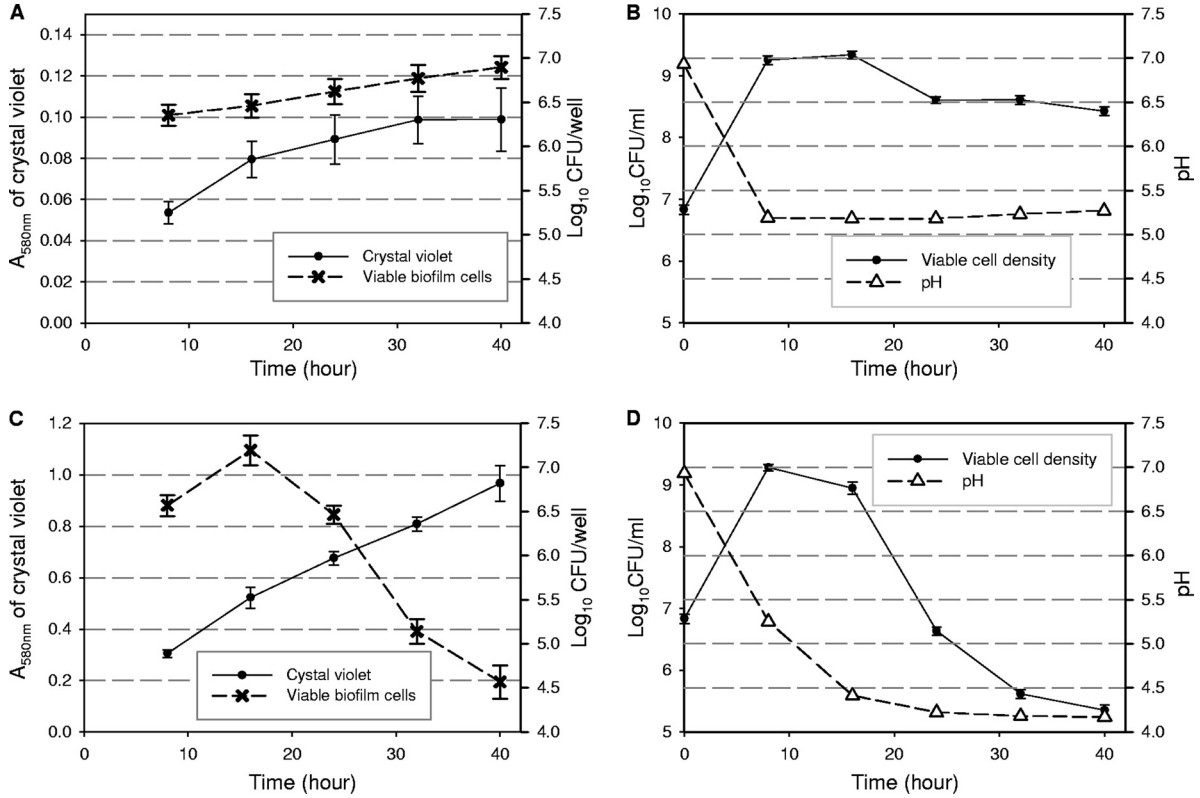


FIG. 6. Time course monitoring of cell vitality in biofilms and absorbance values of CV from destained biofilms (A and C) or cell vitality and pH in cell suspension (B and D) during biofilm formation in TSBYE (A and B) or $TSBYE_{gluc1\%+NaCl2\%}$ (C and D) for 40 h at 30°C. Each data point is presented as the mean of six replicates. Error bars represent the standard deviations of the means.

also been observed with *Staphylococcus* species (9, 12, 22, 24). We compared the effects of the medium components at different temperatures. The combination of sugar (1%), salt (2%), and increasing temperature resulted in a stimulation of biofilm formation. Particularly, serotype 1/2a strain SK1387 formed the highest-density biofilms in the presence of different concentrations of glucose, salt, and ethanol at 30°C and below (see Fig. S1 to S4 in the supplemental material). It is interesting that this strain (SK1387) was involved in several sporadic outbreaks and was consistently isolated from a turkey deli meat-processing facility for more than 10 years (19). The superior ability of this strain (compared to that of the other strains tested) to form biofilms at low temperatures may also contribute to its persistence in food-processing plants. Serotype 4b strain RM3013 was able to form high-density biofilms at 37°C without added glucose in TSBYE. However, the formation of biofilms by this strain could be significantly stimulated in medium supplemented with 2 to 3% sodium chloride (see Fig. S4.3 in the supplemental material). These results suggest that the mechanisms involved in the stimulation of biofilm formation by glucose and sodium chloride for *L. monocytogenes* may be different for different strains or serotypes. Further research may be needed to understand how biofilm production is enhanced by environmental factors.

Biofilm formation by both serotypes was generally enhanced with increasing temperature at certain levels of salt (0.5% to 2.0%, wt/vol) and sugar (0.25% to 10.0%, wt/vol), in agreement with the findings from previous studies (2, 6, 7, 23). It has been suggested that the increased hydrophobicity at high temperatures (e.g., 37°C) may enhance the initial cell adherence, contributing to a higher biofilm density (7). However, our data suggest that biofilm cells may generate and secrete more extracellular polymeric substances in response to temperature and other factors, which would also be seen as an increase in CV absorbance in the microplate assay.

The microplate assay with CV has been widely used in biofilm research due to the convenience, rapidity, simplicity, and reproducibility of the assay. Although CV staining can be used to enumerate planktonic bacterial cells (Fig. 7), the generation of extracellular polysaccharide (EPS) in biofilms may confound data interpretation. We observed that biofilm mass, as measured by CV, can increase while the viable cell counts decrease (Fig. 6C). Absorbance values for biofilms formed in $TSBYE_{gluc1\%+NaCl2\%}$ were higher than the values for cells grown in TSBYE, but the biofilm cell densities were similar, suggesting that the addition of glucose and sodium chloride stimulated bacterial cells to produce more extracellular matrix material. The data also indicate that stresses from starvation, toxic metabolite accumulation, and low pH may provide biofilm cells extra stimuli to generate EPS.

The data from this study show that serotype 1/2a strains form higher-density biofilms than serotype 4b strains and may help to explain the higher percentage of 1/2a isolates from foods and the environment. The higher production of EPS by 1/2a strains than by 4b strains may aid survival by conferring greater resistance to sublethal stress encountered by the bac-

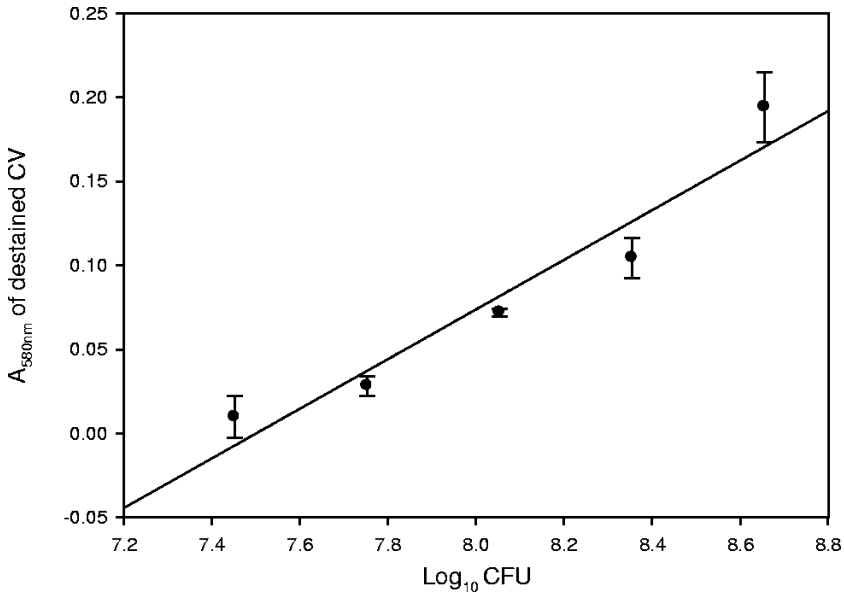


FIG. 7. Relationship between the $\log_{10}$ values for bacterial cell counts and absorbance values of crystal violet from corresponding replicate samples. Each data point is presented as the mean of four replicates. Error bars represent the standard deviations of the means.

teria in food-processing environments. Further research may be needed to identify and characterize the genes that regulate biofilm formation for 1/2a and 4b strains and to investigate how biofilm formation is regulated in response to environmental stimuli.

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