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Formation of Biofilm at Different Nutrient Levels by Various Genotypes of *Listeria monocytogenes*

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ABSTRACT

Strains of *Listeria monocytogenes* differ in their ability to form biofilms. The objectives of this study were to determine whether genetically related strains have similar biofilm-forming capacities and what effect nutrient concentration has on the ability of different strains to produce biofilms. Biofilms of 30 strains of *L. monocytogenes*, obtained from a variety of sources were grown on stainless steel in tryptic soy broth (TSB) or in a 1:10 dilution of TSB (DTSB) for 24 h at 32°C. The amount of biofilm formed was determined with image analysis after cells were stained with bisBenzimide H 33258 (Hoechst 33258). The strains were genetically subtyped by repetitive element sequence–based PCR (rep-PCR) with the primer set rep-PRO_{Dt} and rep-PRO_{G5}. Data were analyzed with an analysis of variance and Duncan's multiple range test. Eleven strains produced the same amount of biofilm in both media. Fourteen strains produced more biofilm in TSB than in DTSB. Five strains produced more biofilm in DTSB than in TSB. Serotype 4b strains produced more biofilm in TSB than did serotype 1/2a strains, whereas serotype 1/2a strains produced more biofilm in DTSB than did serotype 4b strains. Growth in DTSB resulted in decreased biofilm accumulation for serotype 4b strains. There was no correlation between genetic subtype and the amount of biofilm accumulation. These results indicate that strains of serotype 1/2a and serotype 4b differ in the regulation of their biofilm phenotype. The poor biofilm accumulation of serotype 4b isolates when grown in DTSB could be a factor in the predominance of serogroup 1/2 strains in food processing plants, where nutrients may be limited.

Strains of *Listeria monocytogenes* are variable in their ability to produce biofilms; this ability largely depends on the growth conditions, the media, and the strain. Previous research has produced conflicting results regarding the ability of *L. monocytogenes* to produce biofilms, with reports ranging from no production *(28)* to prolific production *(8)*. *L. monocytogenes* strains also vary in the amount of biofilm formed when grown on tryptic soy broth or in modified Welshimer's broth. Some strains produce more biofilm in one medium, whereas others produce more biofilm in another medium *(19)*. In one study, lineage I produced more biofilm than lineage II when grown in modified Welshimer's broth *(9)*, whereas in another study lineage II strains produced more biofilm than did lineage I strains when grown in the same medium using a similar procedure *(3)*.

Although the amount of biofilm produced by strains of *L. monocytogenes* is affected by growth conditions and strain variation, biofilm formation behavior is even more complex. Strains that achieve the same cell density in older biofilms exhibit different biofilm growth kinetics in the early stages of biofilm formation *(7)*. Although some strains attach to surfaces more effectively than others, they will not necessarily produce more biofilm after attachment, and even strains from a similar source do not exhibit similar biofilm behavior *(14)*. The biofilms of some strains are more difficult to remove by swabbing than are those of other strains *(7)*, which in environmental studies may result in reduced estimates of biofilm prevalence. Lower nutrient conditions may stimulate biofilm production by *Listeria (12, 27)*, whereas other data suggest that starvation does not affect biofilm production *(28)*. Blackman and Frank *(2)* found that under low-nutrient conditions the amount of biofilm produced on several surfaces was reduced, but growth on Teflon was unaffected at 21°C but reduced at 10°C even though biofilm production on other surfaces was greater.

This study reported here was undertaken to determine the effect of nutrient level on biofilm production of different strains of *L. monocytogenes* and to assess whether genetically similar isolates of diverse origin have similar abilities to form biofilms.

MATERIALS AND METHODS

Strains. The 30 strains of *L. monocytogenes* used in this project are described in Table 1. These isolates were obtained from food outbreaks, from processing plant environments, and from Yerkes National Primate Center environmental and animal clinical samples. The cultures were maintained on Microbank cryogenic storage beads (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) at −70°C and were recovered by transferring one bead into 5 ml of the appropriate medium for each experiment. Cultures were transferred three times before use because Briandet et al. *(4)* found that the surface properties of *L. monocytogenes* became more uniform with successive serial transfers. Each strain was recovered before each experiment because additional transfers could lead to phenotypic adaptations associated with laboratory maintenance *(29)* that could affect biofilm formation. Cultures

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TABLE 1. *Strains of* Listeria monocytogenes *used in this study*

Strain	Source[a]	Serotype
12375	Monkey (clinical)[b]	4b
YM-32	Environmental (YNPC)	4b
106	Environmental (FPC)	4b
Ym-15	Environmental (YNPC)	4b
70	Environmental (FPC)	4b
32	Environmental (FPC)	4b
17	Environmental (FPC)	4b
Ym-84	Environmental (YNPC)	4b
YM-87	Environmental (YNPC)	4b
SAF	ScottA	4b
SA	ScottA	4b
G3990	Vaucherin cheese outbreak	4b
12374	Monkey (clinical)	4b
YM-7	Environmental (YNPC)	4b
LCDC	Cabbage outbreak	4b
G3982	Jalisco cheese outbreak	4b
18	Environmental (FPC)	UD[c]
961	Monkey (clinical)	1/2a
960	Monkey (clinical)	1/2a
YM-112	Environmental (YNPC)	1/2a
302	Monkey (clinical)	1/2a
303	Monkey (clinical)	1/2a
12378	Monkey (clinical)	1/2a
12443	Monkey (clinical)[d]	1/2a
YM-2	Environmental (YNPC)	1/2a
YM-6	Environmental (YNPC)	1/2a
Ym-19	Environmental (YNPC)	1/2a
YM-96	Environmental (YNPC)	1/2a
YM-54	Environmental (YNPC)	1/2a
YM-3	Environmental (YNPC)	1/2a

[a] YNPC, outdoor animal facilities at Yerkes National Primate Center; FPC, various food processing environments (isolates obtained from the Department of Food Science and Technology, University of Georgia, Athens).

[b] Monkey clinical strains were isolated from animals at YNPC and were obtained from the Center for Food Safety, Griffin, Ga. *(30).*

[c] UD, serotype undetermined.

[d] Research on this strain was previously reported *(13, 30).*

were incubated statically at 32°C and used or transferred after 20 h.

Biofilm formation. New stainless steel coupons (2 by 5 cm, type 304, finish 4b) were degreased in acetone and sonicated for 60 min at 55°C in alkali detergent (Micro, International Products Corp., Burlington, N.J.). After an additional 16 to 20 h, the coupons were removed from the detergent solution and rinsed with deionized water, sonicated for 20 min in a commercial phosphoric acid–based cleaner (30 ml/liter; Formula 3586, Zep, Atlanta, Ga.) or in phosphoric acid (15 ml/liter), and rinsed again in deionized water. After cleaning, coupons were autoclaved in deionized water.

Sterile coupons were placed into test tubes (1,500 by 25 mm) and submerged in a 20-h culture of *L. monocytogenes* grown on Bacto tryptic soy broth (TSB; Becton Dickinson, Sparks, Md.) or diluted (1:10) TSB (DTSB). Coupons were incubated 4 h at 32°C, vigorously agitated in 50 ml of sterile phosphate buffer (0.015 M KH_2PO_4, pH 7) using sterile tongs, and then transferred to fresh medium. After 24 h of incubation, the coupons were again rinsed in phosphate buffer and then analyzed. Negative controls underwent the same treatment but without inoculation.

Growth curves. Liquid culture aliquots (50 μl) of each individual strain were used to inoculate 5 ml of culture medium. The absorbance at 600 nm was measured hourly with a DU 350 spectrophotometer (Beckman, Fullerton, Calif.) until stationary phase was reached and then again the next day. Growth curves were obtained from TSB and DTSB cultures incubated at 32°C.

rep-PCR. *Listeria* cultures were prepared for repetitive element sequence–based PCR (rep-PCR) after growth in TSB at 37°C for 18 h and streaking for isolation on tryptic soy agar (TSA; Becton Dickinson). After incubation at 37°C for 18 h, isolated colonies were selected and duplicate lawns on TSA were prepared using the same incubation conditions. The resulting cells were suspended in 2 ml of phosphate-buffered saline (PBS; Gibco BRL, Rockville, Md.) and washed twice in PBS. Genomic DNA was extracted using an UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, Calif.) according to the supplier's instructions. DNA concentration was determined using a GeneQuant spectrophotometer (Amersham Biosciences, Piscataway, N.J.). PCR was performed using RepPRO DNA fingerprinting kits with primers rep-PRO_{G5} and rep-PRO_{Dt} with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Calif.) and a PTC 225 thermocycler (MJ Research, Watertown, Mass.) according to the suppliers' instructions (Bacterial Barcodes, Inc., Houston, Tex.). PCR amplicons were separated by electrophoresis in 1.5% (wt/vol) Seakem LE agarose (Cambrex Corporation, East Rutherford, N.J.) gels with a 0.5× TBS buffer system. Electrophoresis was carried out in a subcell model 192 (Bio-Rad Laboratories, Hercules, Calif.) with circulated buffer. Gel images were captured with a FluorChem 8000 (Alpha Innotech, San Leandro, Calif.). Duplicate reactions were done, and amplicons from both reactions were run in separate lanes on the same gel.

Pearson product-moment correlation coefficients (Pearson) were calculated and cluster analyses were carried out using the unweighted pair-group method by the arithmetic averages (UPGMA) within the Bionumerics version 2.5 (Applied Maths, Inc., Austin, Tex.) software package. To obtain a dendrogram from fingerprints from both primer sets, the similarity matrices were averaged and a dendrogram was produced by UPGMA. Bionumerics settings required for processing included setting spot removal to 4 and estimating disk size for background subtraction and the least square filtering using the background scale and Wiener cutoff value, respectively, of the spectral analysis feature. For the calculation of dendrograms, optimization and band tolerance were estimated using the best cluster separation method and then adjusted to accommodate variances in the normalizing of the gels.

Measurement of biofilm formation. Biofilms were stained by submersion in 0.05 mg/ml bisBenzimide H 33258 (Hoechst 33258, Sigma Chemical Co., St. Louis, Mo.) for 20 min. Excess dye was rinsed by dipping coupons in a beaker of water; excess water was wicked away with Kim Wipes (Kimberly Clarke, Neenah, Wis.). After air drying, the stained biofilms were visualized under an epifluorescence microscope equipped with an excitation filter of 330 to 380 nm, an emission filter of 435 to 485 nm, and a dichroic mirror of 400 nm. Images of biofilms grown in DTSB were captured with a Nikon Eclipse E600 epifluorescent microscope (Southern Micro Instruments, Marietta, Ga.) equipped with a Magnafire CCD camera (Southern Micro Instruments) and had an area of 593,350 μm^2. Images of biofilms grown in TSB were captured with a Nikon eclipse TE300 (Southern Micro Instruments) equipped with a RTE/CCD-1300-Y/HS CCD (Princeton Instruments, Trenton, N.J.) and had an area of 148,874 μm^2. Ten images were captured for each coupon. Grayscale images were then converted to black and white by thresholding (Adobe Pho-

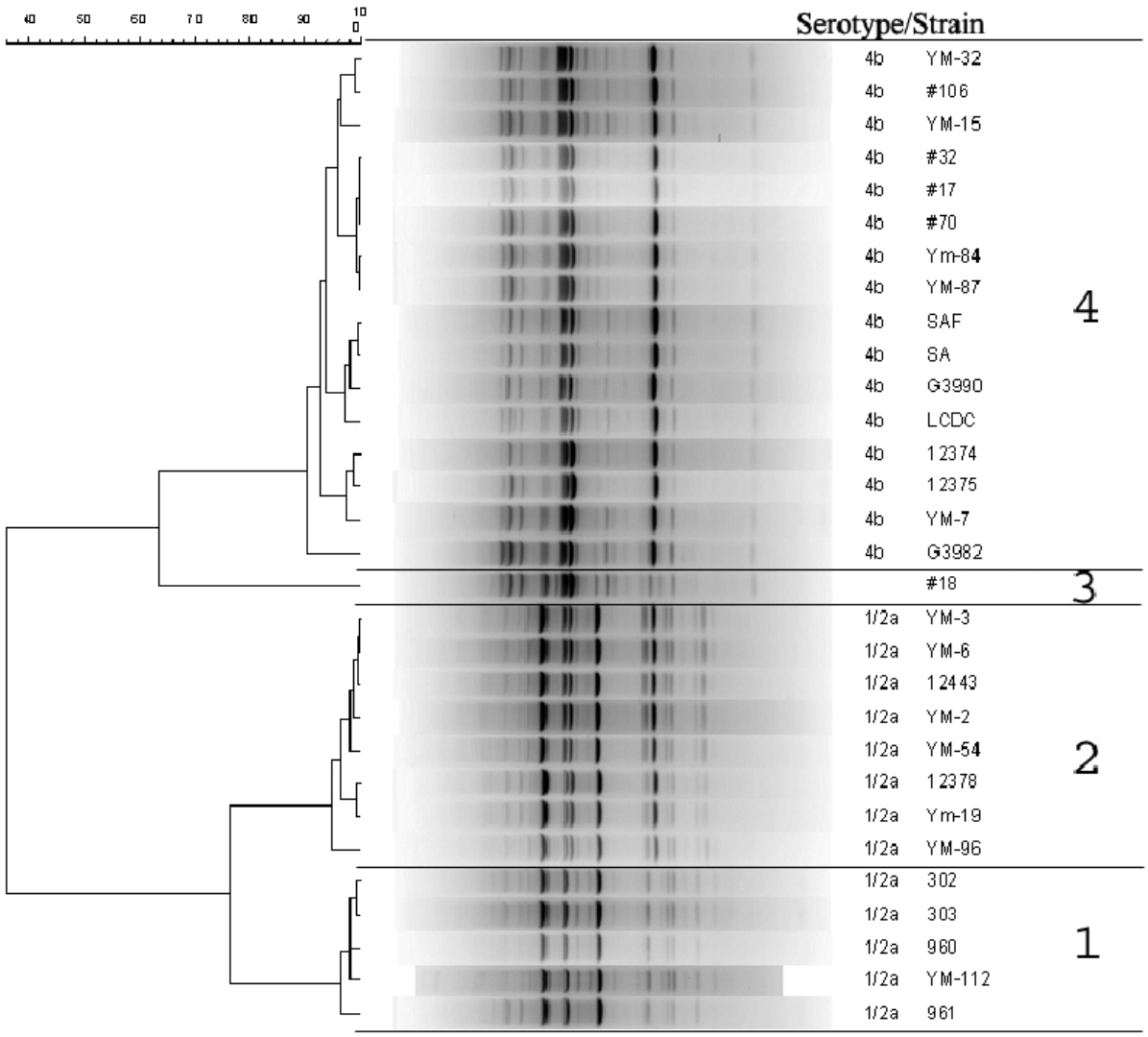

FIGURE 1. *Pearson UPGMA analysis of rep-PRO$_{Dt}$ fingerprints of* L. monocytogenes *strains with serotype. Optimization = 0.3. Large bold numbers indicate genetic groupings.*

toshop, Adobe Systems, San Jose, Calif.). Each image was thresholded such that the stained cells were white and the background was black. Black and white pixels were counted using Image Tool (Health Science Center, University of Texas, San Antonio). The amount of biofilm accumulation was reported as percent coverage based on the percentage of white pixels *(2, 33).*

Data analysis. Data were analyzed using a 2×31 factorial design with three replications (30 strains + negative control). An analysis of variance (ANOVA) and Duncan's multiple range test using general linear models (SAS Institute, Cary, N.C.) were used to determine significant effects and interactions based on $\alpha = 0.05$ for Duncan's test and $\alpha < 0.05$ for the ANOVA. Biofilm accumulation data were transformed (log[percent area + 1]), to correct for violations of the normality and constancy of variance assumptions in the ANOVA model.

RESULTS

Planktonic growth. Based on optical density, all strains exhibited similar growth kinetics in TSB and DTSB. Maximum cell density was achieved in 10 h in TSB and in 11 h in DTSB. The final optical density of strains grown in DTSB was approximately 10-fold lower than for strains grown in TSB (data not shown).

Genetic subtyping. The rep-PRO$_{Dt}$ primer differentiated the strains into four groups at a similarity coefficient of 90% (Fig. 1). Groups 1 and 2 contained serotype 1/2a strains, and group 4 contained serotype 4b strains. Group 3 consisted only of strain 18. The rep-PRO$_{G5}$ primer differentiated the strains into six groups at a similarity coefficient of 80%: groups a, b, c, d, e, f, and g in Figure 2. This primer differentiated the group 1 strains into two groups, a and c. Group 2 strains were further differentiated into groups b and d. The single strain (18) in group 3 was combined with 4b strains in group g. Strain 12375 was placed into a separate group e. The remaining group 4 strains were divided into groups f and g. Both primers (rep-PRO$_{Dt}$ and rep-PRO$_{G5}$) differentiated the strains by serotype. This separation occurs at 38% dissimilarity for primer rep-PRO$_{Dt}$ and at 15% dissimilarity for rep-PRO$_{G5}$ (Figs. 1 and 2). Combining the group designations from each primer into an alphanumeric combination yielded eight dis-

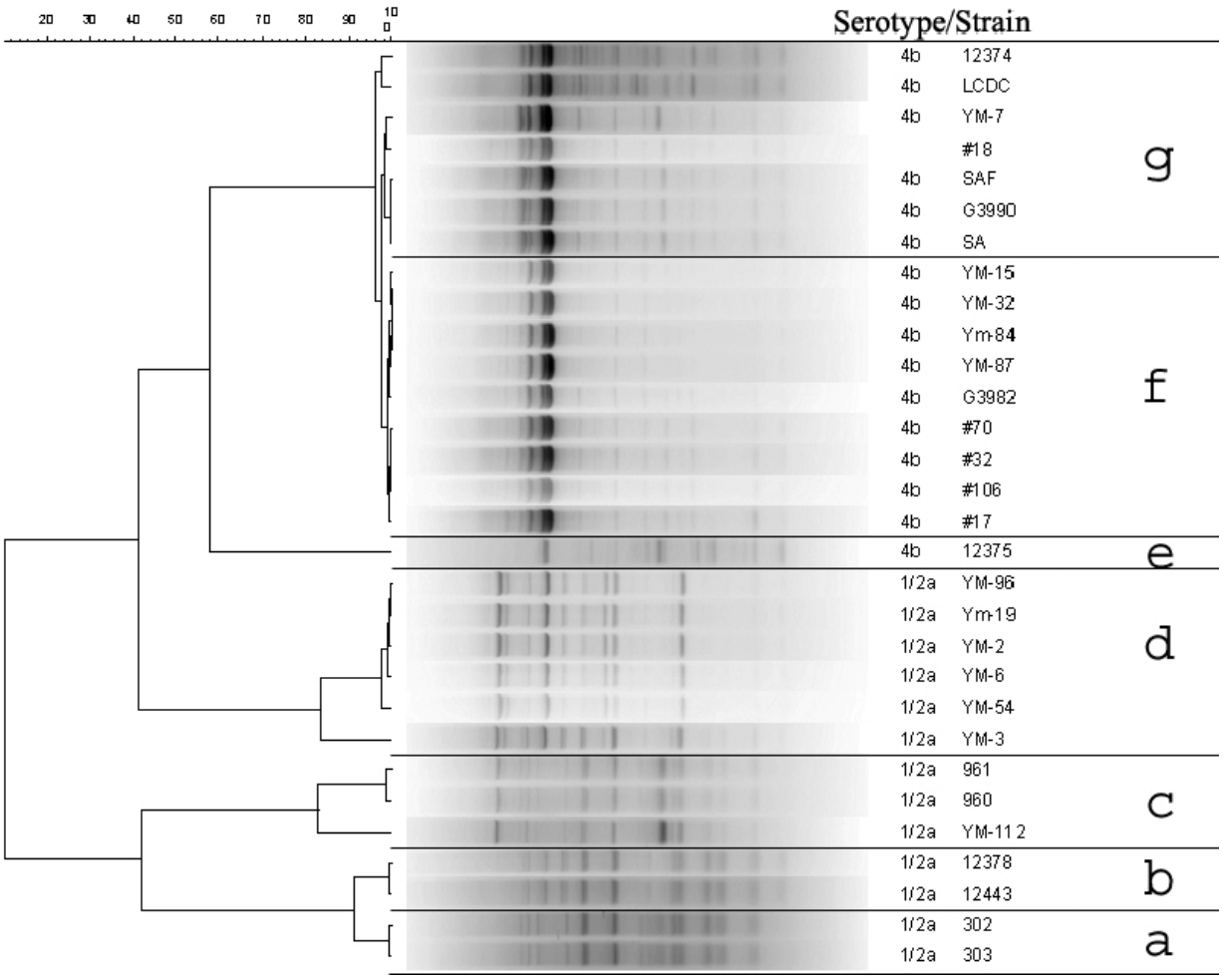

FIGURE 2. *Pearson UPGMA analysis of rep-PRO$_{G5}$ fingerprints of* L. monocytogenes *strains, including serotype. Optimization = 0.3. Large bold letters indicate fingerprint type.*

tinct subtypes. The composite dendrogram with the combined group designations is presented in Figure 3. This dendrogram is correlated roughly with the combined grouping designations. The only notable exception was the separation of strain G3982 from the other members of subtype 4f.

Effect of nutrient level and strains. Biofilm formation data are presented in Table 2. The range of biofilm accumulation in TSB was 3.3 to 41.7% of the coupon area covered. Biofilms at the upper end of this range exhibited well-developed multicell structures, whereas those at the low end of the range consisted of sporadic attachment of single cells or small clumps of cells. The range of biofilm accumulation in DTSB was 0.8 to 19% coverage. Biofilms at the upper end of this range consisted of microcolonies and attached cells, whereas those at the lower end consisted mostly of attached single cells and occasional small clumps. Biofilm accumulation was decreased for some strains when DTSB was used as the growth medium. All strains exhibited some attachment, as indicated by significant differences from the sterile control. The ANOVA indicated that the effects of nutrient level and strain were significant. The mean biofilm accumulation for all strains was greater when the strains were grown in TSB. The separation of means for biofilm accumulation in TSB is shown in Figure 4. Four strains were placed into two groupings of high biofilm accumulation, whereas the other strains produced a continuum of less biofilm accumulation. Strains grown in DTSB produced a range of biofilm accumulations with overlapping Duncan's groupings (Fig. 5). There was also a significant effect of the interaction of nutrient level and strain. Because of this interaction, the effect of nutrient level is different for the different strains: 11 strains had the same capacity to produce biofilms at both nutrient levels, 14 strains produced more biofilm in TSB than in DTSB, and 5 strains produced more biofilm in DTSB than in TSB (Table 2).

Association of biofilm production with subtype and serotype. Subtype and serotype were associated with the decrease in biofilm accumulation in DTSB. Table 2 shows the results of Duncan's multiple range test of mean biofilm accumulation by media type and strain. These data indicate that biofilm accumulation means at each nutrient level differ for a particular strain. Subtypes 4f, 4g, and 4e are serotype 4b, and Duncan's multiple range test indicates that all but four of these strains exhibit marked reductions in biofilm accumulation when grown on DTSB. Of these four strains, only strains YM-32 and Ym-84 were definitely unaffected by growth in DTSB. Although the difference was not statistically significant, biofilm accumulation of strain

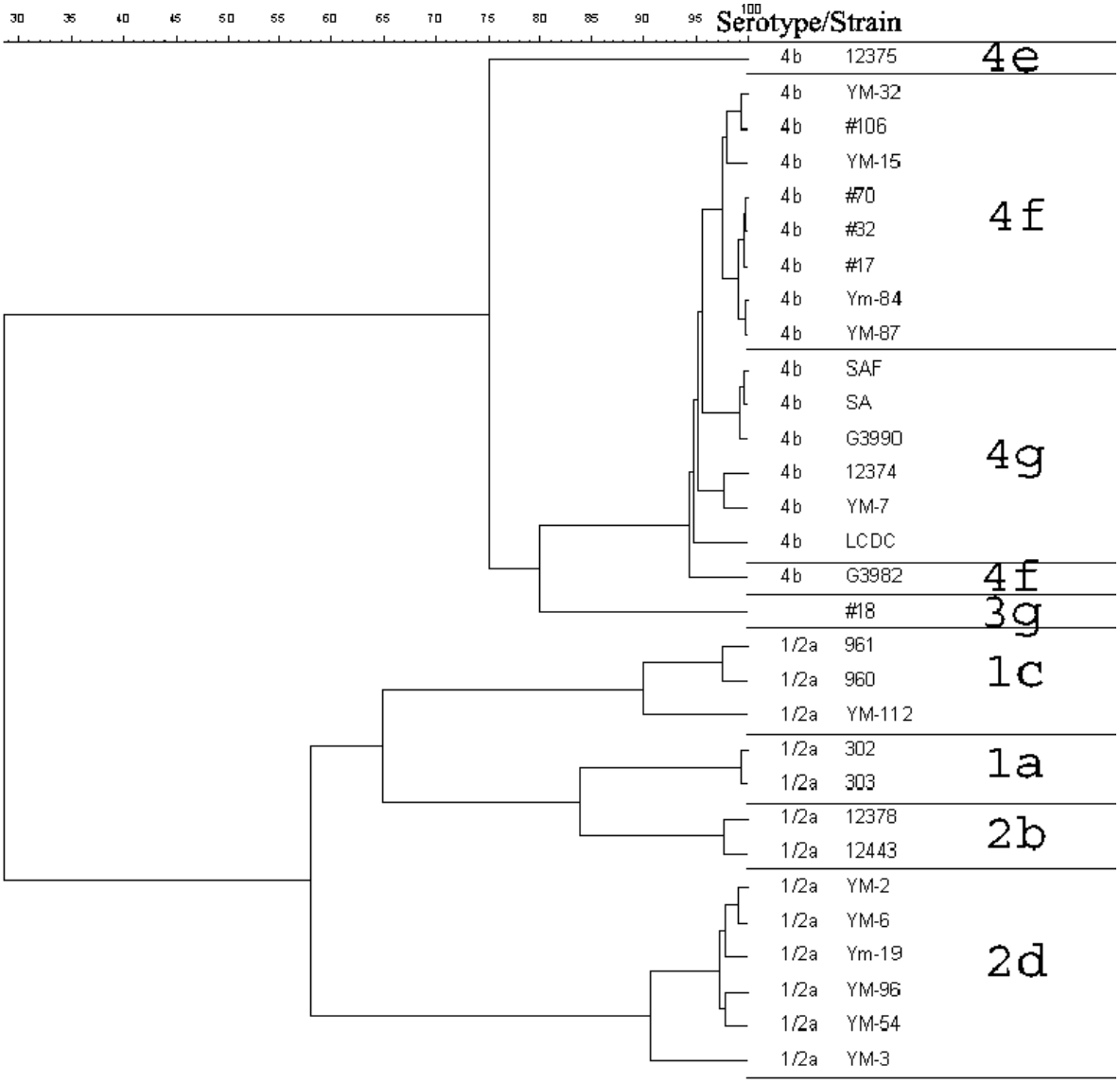

FIGURE 3. *Pearson UPGMA analysis of rep-PRO$_{G5}$ and rep-PRO$_{Dt}$ fingerprints of* L. monocytogenes *strains, includes serotype. rep-PRO$_{Dt}$ optimization = 0.3; rep-PRO$_{G5}$ optimization = 0.3. Large bold alphanumeric designations show the combined group assignments.*

70 was reduced by almost half by growth on DTSB. Strain 12374 formed no biofilm at either nutrient level, so biofilm accumulation could not be further reduced by growth on DTSB. Subtypes 2d, 2b, 1a, and 1c are all serotype 1/2a. Of these strains, only strain 12443 exhibited reduced biofilm accumulation in DTSB.

Subtypes of serotype 1/2a either were stimulated to produce biofilms by DTSB or produced biofilms equally well in both media, whereas the serotype 4b subtypes generally had less biofilm accumulation in DTSB. Serotype biofilm accumulation means were compared by ANOVA using Duncan's multiple range test. The biofilm accumulation mean for serotype 4b strains was greater than that for serotype 1/2a strains when they were grown in TSB. When the strains are grown in DTSB, the biofilm accumulation mean for serotype 1/2a strains was greater than that for serotype 4b strains.

Neither subtype nor serotype was associated with the amount of biofilm accumulation. Table 2 gives the Duncan's multiple range test of the biofilm accumulation data by subtype. These data illustrate that there is significant variation in the amount of biofilm produced for all of the subtypes that have more than one member for at least one of the nutrient levels tested. Strains SA and SAF are isolates of strain Scott A obtained from different labs. Both of these isolates produce more biofilm when grown in TSB; however SAF produced two to four times the amount of biofilm produced by SA.

DISCUSSION

The number of *L. monocytogenes* cells present in suspension during attachment does not greatly affect the amount of biofilm that is later formed. Djordjevic et al. *(9)* found only a slight correlation ($P = 0.03$) between initial inoculum concentration and biofilm growth when biofilms were grown for 20 h and no significant correlation at 40 h. There is also no relationship between planktonic growth rate and biofilm growth rate *(6, 9)*. Even the number of

TABLE 2. *Biofilm growth of genetic subtypes of* Listeria monocytogenes *in DTSB and TSB after 4 h of attachment and 24 h of incubation at 32°C*

Strain	% area covered by biofilm growth in[a]: TSB	DTSB	Medium that produced the most biofilm[b]	rep-PRO$_{Dt/G5}$ subtype	Serotype
YM-3	10.9 (A)	13.3 (BC)	Both	2d	1/2a
YM-54	7.4 (BC)	11.86 (ABC)	DTSB	2d	1/2a
YM-96	8.7 (ABC)	13.3 (AB)	DTSB	2d	1/2a
Ym-19	8.3 (ABC)	17 (A)	DTSB	2d	1/2a
YM-6	10.7 (AB)	10 (C)	Both	2d	1/2a
YM-2	8.2 (ABC)	10 (BC)	Both	2d	1/2a
12378	11.8 (A)	19 (A)	DTSB	2b	1/2a
12443	8.9 (B)	2 (B)	TSB	2b	1/2a
302	5.7 (A)	9.9 (A)	Both	1a	1/2a
303	3.3 (B)	7.7 (A)	DTSB	1a	1/2a
961	4.3 (A)	6.6 (B)	Both	1c	1/2a
960	4.8 (A)	8.2 (B)	Both	1c	1/2a
YM-112	5.6 (A)	11.6 (A)	Both	1c	1/2a
18	19.5	0.8	TSB	3g	
G3982	21.3 (A)	1.47 (CD)	TSB	4f	4b
YM-32	7.32 (B)	8.6 (A)	Both	4f	4b
106	6.7 (B)	2 (C)	TSB	4f	4b
YM-15	6.5 (BC)	1.3 (CD)	TSB	4f	4b
70	6.3 (C)	3.8 (B)	Both	4f	4b
32	5.2 (BC)	0.6 (D)	TSB	4f	4b
17	4.3 (C)	1.28 (BCD)	TSB	4f	4b
Ym-84	7.4 (B)	10 (AB)	Both	4f	4b
YM-87	7.6 (B)	2.5 (C)	TSB	4f	4b
SAF	9.7 (C)	2.74 (B)	TSB	4g	4b
SA	21.8 (B)	11 (A)	TSB	4g	4b
G3990	41.7 (A)	3.75 (B)	TSB	4g	4b
12374	3.8 (E)	2.8 (B)	Both	4g	4b
YM-7	9.9 (C)	3.8 (B)	TSB	4g	4b
LCDC	5.95 (D)	1.4 (C)	TSB	4g	4b
12375	5.53	1	TSB	4e	4b

[a] Values are the mean percentage of the area of coupons covered with biofilm. Letters are groups determined from the results of Duncan's multiple range test of the strain means by genotype ($\alpha = 0.05$).

[b] Results of Duncan's multiple range test of the biofilm accumulation means by media type and strain ($\alpha = 0.05$).

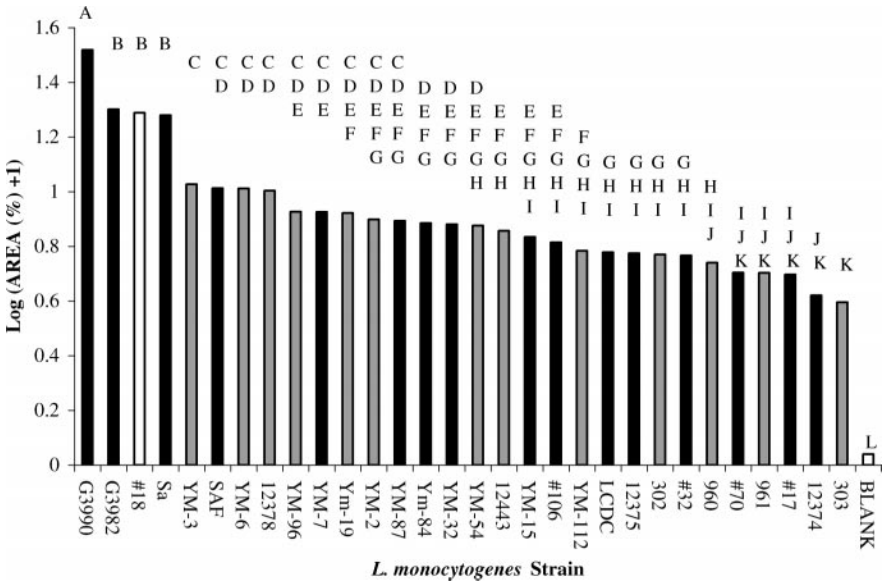

FIGURE 4. *Biofilm accumulation (log[percent area + 1]) of various strains of* L. monocytogenes *on stainless steel coupons in TSB after 4 h of attachment and 24 h of incubation at 32°C. Letters above each bar indicate the Duncan's multiple range test groupings ($\alpha = 0.05$). Bars with the same letter are not significantly different. Serotype 4b strains are in black, and serotype 1/2a strains are in gray. An open bar indicates that serotype is not applicable.*

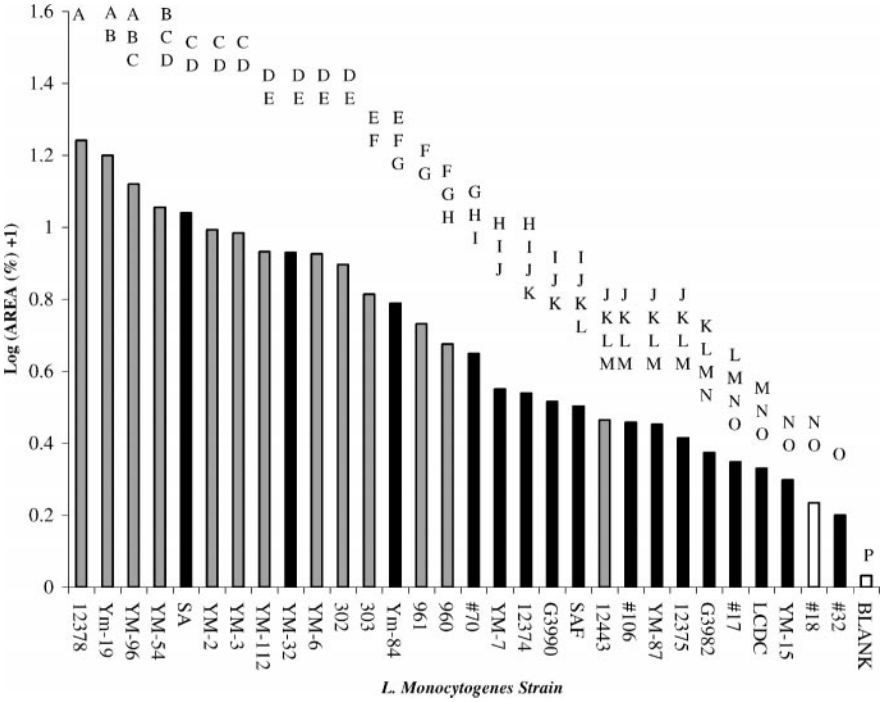

FIGURE 5. *Biofilm accumulation (log[percent area + 1]) of various strains of* L. monocytogenes *on stainless steel coupons in a 1:10 dilution of TSB after 4 h of attachment and 24 h of incubation at 32°C. Letters above each bar indicate the Duncan's multiple range test groupings (α = 0.05). Bars with the same letter are not significantly different. Serotype 4b strains are in black, and serotype 1/2a strains are in gray. An open bar indicates that serotype is not applicable.*

initial attached cells had no effect on the final amount of biofilm formed *(6, 14)*. In the present study, all strains exhibited similar growth kinetics and reached the same optical density at the time of use. In a similar study, Djordjevic et al. *(9)* also found that 31 strains of *L. monocytogenes* exhibited similar growth kinetics in modified Welshimer's broth *(24)*. Thus, precise standardization of inoculum concentration before each experiment is unnecessary. Washing of inocula could change surface characteristics that influence attachment.

This study indicates that rep-PCR subtyping of *L. monocytogenes* produces results that are consistent with other genetic subtyping methods. Piffaretti et al. *(23)* also found that serotype 4b strains of *L. monocytogenes* could be differentiated from serotype 1/2a strains using multilocus enzyme electrophoresis. In other studies involving a variety of genotyping methods, serotype 1/2a also could be differentiated from serotype 4b *(1, 5, 10, 11, 18, 20, 25, 26, 31, 32)*.

The results of this study further confirm that *L. monocytogenes* strains exhibit variation in biofilm forming behavior. Chae and Schraft *(6)* observed that the cell density of biofilms differed between strains of *L. monocytogenes*. Others have reported similar variation in biofilm production, with some *L. monocytogenes* strains producing significantly more biofilm than others *(3, 9, 14, 19)*. The rate of biofilm accumulation can be different among isolates, even if they eventually reach a similar biofilm cell density *(7)*. When growing as a biofilm, *L. monocytogenes* exhibits cycles of attachment and detachment, the periodicity of which varies with strain *(7, 8, 22)*. Blackman and Frank *(2)* found that the effect of temperature varied according to surface and medium. The ability of *L. monocytogenes* to produce biofilm clearly differs according to strain, growth temperatures, cultural conditions, and growth surface. Researchers cannot assume that relative biofilm production among strains will be the same from one condition to another or that the same conditions will yield maximum biofilm cell density for each strain.

The effects of nutrient levels on the development of *L. monocytogenes* strain Scott A biofilms have been previously studied. Our current research indicates that strain Scott A produces more biofilm in rich medium. This observation supports those of others who observed that Scott A also produces more biofilm in TSB than DTSB for the first 4 days *(22)* and in modified Welshimer's broth for 24 and 48 h *(19)*. These findings are consistent with those of Kim and Frank *(16)*, who reported that amino acids were important for the early development of *L. monocytogenes* Scott A biofilms. Although these observations are supported by the results of the current research, Jeong and Frank *(12)* found that biofilms of *L. monocytogenes* Scott A grown at 21°C in a 1:15 dilution of TSB exhibited cell populations 10-fold greater than those of biofilms grown in a 1:3 dilution of TSB. This stimulation of biofilm production has been suggested to be the result of the starvation stress response *(21)*. Experimentally, the reported medium used was half the concentration of the DTSB used currently, indicating that nutrient limitation greater than that used in the current research could enhance biofilm production by serotype 4b strains. However, Oh and Marshall *(22)* found that after 7 days the biofilm growth of *L. monocytogenes* Scott A on DTSB equaled the growth on TSB *(22)*. This finding raises the possibility that nutrient availability affects biofilms differently according to their age.

Factors other than nutrient level and strain may also influence biofilm production by *L. monocytogenes*. There are three main evolutionary lines of *L. monocytogenes (23, 26)*: lineages I, II, and III *(20, 32)*. Lineage I is composed of serotypes 4b, 1/2b, 3c, and 3b, lineage II is composed

of serotypes 1/2a, 1/2c, and 3a *(1, 20),* and lineage III is the most recently elucidated lineage and contains serotypes 4a and 4c *(20, 26).* In our current work, mean biofilm accumulation of the lineage I group was greater than that of the lineage II group when grown in TSB; however, when grown in DTSB the mean biofilm accumulation of the lineage II group was greater. Djordjevic et al. *(9),* in a similar study, found that lineage I isolates produced more biofilm than did lineage II isolates when biofilms were grown in modified Welshimer's broth. However, Borucki et al. *(3),* using a modified version of Djordjevic's procedure, found the opposite result. In an attempt to determine the reason for the discrepancy, the two methods in question were compared using a sample of the same isolates used by Djordjevic et al., again resulting in disagreement *(3).* In our lab, two isolates of the same strain (SA and SAF) differed in their ability to produce biofilm. There also was significant variation in biofilm formation among members of the same rep-PCR subtype, indicating no apparent linkage between rep-PCR subtypes and the multistep phenotypic process of biofilm formation.

In this study, we found an association of serotype and subtype with a decrease in biofilm accumulation in low-nutrient medium (DTSB), indicating that there are differences between the two serotypes regarding their response to their environment and that serotype 1/2a isolates may also produce biofilm under a greater variety of environmental conditions. Such physiological differences could account for the prevalence of *L. monocytogenes* serogroup 1/2 in processing plant environments *(15, 17),* where nutrients may be limited.

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