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Listeria monocytogenes Biofilm-Associated Protein (BapL) May Contribute to Surface Attachment of *L. monocytogenes* but Is Absent from Many Field Isolates▽

Suzanne J. Jordan,[1] Stefano Perni,[4] Sarah Glenn,[1] Isabel Fernandes,[2] Manuela Barbosa,[2] Manuela Sol,[2] Rogerio P. Tenreiro,[3] Lelia Chambel,[3] Belarmino Barata,[3] Isabel Zilhao,[3] Timothy G. Aldsworth,[4] Andreia Adriao,[5] M. Leonor Faleiro,[5] Gilbert Shama,[4] and Peter W. Andrew[1]*

Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, United Kingdom[1]; INETI-DTIA, EdificoS, Estrada do Paco Lumiar, 221649-038 Lisbon, Portugal[2]; Instituto de Ciencia Aplicada e Tecnologia, Campus da FCUL, Campo Grande, 1749-016 Lisbon, Portugal[3]; Department of Chemical Engineering, University of Loughborough, Loughborough LE11 3TU, United Kingdom[4]; and Universidade do Algarve, FERN, IBB-CBME, Campus de Gambelas, 8000-117 Faro, Portugal[5]

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***Listeria monocytogenes* is a food-borne pathogen capable of adhering to a range of surfaces utilized within the food industry, including stainless steel. The factors required for the attachment of this ubiquitous organism to abiotic surfaces are still relatively unknown. In silico analysis of the *L. monocytogenes* EGD genome identified a putative cell wall-anchored protein (Lmo0435 [BapL]), which had similarity to proteins involved in biofilm formation by staphylococci. An insertion mutation was constructed in *L. monocytogenes* to determine the influence of this protein on attachment to abiotic surfaces. The results show that the protein may contribute to the surface adherence of strains that possess BapL, but it is not an essential requirement for all *L. monocytogenes* strains. Several BapL-negative field isolates demonstrated an ability to adhere to abiotic surfaces equivalent to that of BapL-positive strains. BapL is not required for the virulence of *L. monocytogenes* in mice.**

Listeria monocytogenes is a food-borne pathogen that causes serious illness, including meningitis, septicemia, and stillbirth, with a mortality rate of up to 30% (37). More recently, there have been reports of listerial gastroenteritis following the consumption of several different food types (16, 34). A number of studies have demonstrated that this organism is able to persist in the food-processing environment for several months and even up to 10 years (23, 29). One of the major causes for concern about *L. monocytogenes* in these environments is its ability to attach to many different surfaces (2). Indeed, there is recent evidence to show that listerial biofilms formed inside the lumens of stainless steel tubes are able to withstand the shears generated by high-Reynolds-number flows (31). Biofilms, including those produced by *L. monocytogenes*, are more resistant to detergents and disinfectants (33) and also are a potential source of contamination within food-processing plants; hence, they pose a risk to the maintenance of product safety (27). Consequently, there is considerable interest in determining the mechanisms of attachment and biofilm formation.

Our in silico analysis of the genome sequence of *L. monocytogenes* identified an open reading frame (lmo0435) for a protein with similarity to biofilm-associated proteins (Bap) believed to be important for the binding of staphylococci to abiotic surfaces (10). This Bap protein also has been implicated in the virulence of *Staphylococcus aureus* (10, 11). Thus, the aim of the current study was to establish if this protein (Lmo0435 [BapL]) of *L. monocytogenes* influenced biofilm formation and virulence and to determine the prevalence of the lmo0435 (*bapL*) gene within a selection of field isolates.

MATERIALS AND METHODS

Bacterial strains and plasmids. A list of the *L. monocytogenes* isolates and plasmids used in this study is given in Table 1. The strains were cultured in tryptone soya broth (TSB; Oxoid) or brain heart infusion agar (Oxoid) with shaking at 37°C unless otherwise stated. *Escherichia coli* JM109 used for the other cloning procedures was grown in Luria-Bertani broth (35) with shaking at 37°C. When required, agar (L11; Oxoid) at a concentration of 1.5% (wt/vol) was added to the broth to create a solid medium.

In silico analysis. Sequence data were submitted to BLASTP (1) available on the EGD genome sequence webpage (http://genolist.pasteur.fr/ListiList). Searches to determine the potential subcellular location and the functional regions of the query peptides were performed using PSORT-b (17) and Interproscan, respectively, on the ExPASy proteomics server. The EGD chromosome was viewed on ARTEMIS software version 6 (Sanger Centre, Cambridge, United Kingdom) to determine the location of the query sequences and the identity of the surrounding genes.

DNA manipulation and transformation. Standard DNA manipulation and electroporation protocols were used for *E. coli* as detailed by Sambrook et al. (35). Plasmid and chromosomal DNA was extracted from *Listeria* as outlined by Birnboim and Doly (3) and Dillard and Yother (12), respectively, with a few modifications. During the plasmid extraction, cell suspensions were incubated at 37°C for 15 min in the presence of 5 mg/ml lysozyme (Sigma) and 100 U/ml mutanolysin (Sigma) prior to alkaline lysis.

Competent cells of *L. monocytogenes* were prepared and electroporated as described by Park and Stewart (30). Following the 3-h recovery period, the cell

* Corresponding author. Mailing address: Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, United Kingdom. Phone: 44 116 252 2941. Fax: 44 116 252 5030. E-mail: pwa@le.ac.uk.
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TABLE 1. Strains and plasmids

Strain or plasmid	Serotype	Origin	Source or reference[a]
Strains			
10403s	1/2a	Clinical	32
EGD	1/2a	Clinical	18
ScottA	4b	Clinical	14
SLU2157	4b	Clinical	13
SLU1922	1/2a	Clinical	25
C52	1/2a	Clinical	24
C681	4b	Cheese	INETI-DTIA
C882	4b	Cheese	INETI-DTIA
C895	1/2a	Cheese	INETI-DTIA
C897	1/2a	Cheese	INETI-DTIA
A9	4b	Cheese-making dairy	6
G8	4b	Cheese-making dairy	6
SA11	4b	Cheese-making dairy	6
T8	4b	Cheese-making dairy	6
CLIP 6.04	3a	Seafood product	C. Jacquet
CLIP 8.04	1/2c	Seafood product	C. Jacquet
CLIP 7.04	1/2b	Meat product	C. Jacquet
Plasmids			
pAUL-A			36
pGEM-T Easy		Promega	
pSJ004		This study	

suspensions were plated onto brain heart infusion agar supplemented with the appropriate antibiotics.

Construction of the *bapL* mutant. A 919-bp fragment was amplified by PCR from a cell suspension of *L. monocytogenes* 10403s prepared according to the protocol detailed by Bubert et al. (5), using the primers sj007 and sj008 (Table 2). The fragment was cloned into a pGEM-T Easy vector (Promega) and excised with KpnI and HindIII for insertion into pAUL-A cut with the same enzymes. The resulting plasmid, pSJ004, was transformed into competent cells of *L. monocytogenes* 10403s as described above. The successful transformation was confirmed by PCR using primers binding to the insert (sj007) and the vector (UR). Transformants harboring the plasmid were incubated in TSB at 40°C in the presence of 10 mg ml^{-1} erythromycin prior to plating onto agar. The success of the pSJ004 insertion into the chromosome was determined by performing PCR on the chromosomal DNA of potential integrants using sj008 and UR (Table 2). The selected mutant was designated *L. monocytogenes* SJ78.

Attachment assay. A modified version of the protocol described previously (39) was used. Overnight cultures of the mutant and its parent were diluted 1:80 into microtiter wells (Greiner polystyrene, 655161) containing 200 μl fresh TSB supplemented with the appropriate antibiotics and prewarmed to 37°C. Only alternate wells of the microtiter tray were inoculated; the outer wells were not inoculated because this was found to be necessary to obtain a normal data set. These cultures were subsequently incubated at 37°C for 6 h to allow growth and attachment to occur.

The attached cells were washed with sterile phosphate-buffered saline (PBS) both prior to staining (with 0.1% [vol/vol] crystal violet) and in dye recovery (in an 80:20 [vol/vol] ethanol:acetone mixture). The eluted stain was quantified by absorbance at 595 nm (MRX enzyme-linked immunosorbent assay plate reader; Dynatech Labs).

An estimate of the total number of cells for the mutant and wild type was done from representative wells in a duplicate tray, prepared as described above. Following agitation to remove attached cells from the well surface, 100-μl samples were taken for analysis. The samples were serially diluted in PBS and plated onto tryptone soy agar using the Miles and Misra technique (28). These plates were incubated overnight at 37°C prior to enumeration.

The attachment data were evaluated using the Tukey-Kramer test and GraphPad InStat version 3.0a for the Macintosh (GraphPad Software, San Diego, CA [www.graphpad.com]).

Detachment assay. The detachment assay relies on the generation of quantifiable shear forces by the flow of a liquid between two parallel plates in order to remove cells attached to one of the plates. The shears are greatest at the center and decrease radially outwards. A radial flow chamber of the type originally described by Fowler and McKay (15) was used with a modified protocol to quantify the removal of attached *L. monocytogenes* to stainless steel discs. *Listeria monocytogenes* cultures were prepared by inoculating a single colony into 100 ml TSB and incubating the cultures statically at 30°C for 24 h. The cultures were aseptically poured into sterile petri dishes prior to the addition of a sterile stainless steel disc (diameter, 5 cm), which was immersed in each of the cultures for 2 h. Following their removal from the cell suspensions, the discs were mounted into a radial flow chamber (LH Engineering, Stoke Poges, United Kingdom) and exposed to a flow rate of 3 dm^3/min of distilled water for 3 min. The flow was delivered from a pressurized tank at 3 bars. After exposure to fluid shear flow, the discs were removed from the flow chamber, washed in PBS, fixed in 2% (vol/vol) glutaraldehyde (buffered with PBS) overnight, and washed three times in PBS for 15 min. The attached cells were stained with a live/dead stain (BacLite; Molecular Probes) and visualized with an epifluorescence microscope (Optiphot-2; Nikon). Starting from the center of the disc, the microscope stage was moved until the edge of the clearance zone was detected; this yielded the critical radius immediately beyond which the strength of the attachment of the cells just exceeded the removal forces generated by the flow. Knowledge of the critical radius enabled the shear force at that location to be calculated using the equation $\tau_0(r) = 3Q\mu/\pi rh^2$, where Q is the flow rate (m/s), h is the distance between the plates (m), μ is the fluid viscosity (Pa · s), and r is the radius (m) (15).

Four determinations of the critical radius (at an angular rotation of 90°) were recorded for each disc. For each strain, three independent cultures were used and three separate determinations of the critical radius were made.

In order to enumerate the number of cells attached, a second disc was prepared as above without exposure to the fluid flow. Twenty images of the biofilm were captured randomly across the disc surface with a digital camera (Coolpix 900; Nikon), and the cell count was done using Scion Image software (Scion Corporation, Frederick, MD).

PCR screen for the presence of the lmo0435 gene. Samples of the cell suspension were prepared in 100 μl of 1× PCR buffer (ABGene) as outlined before (5). The reactions were done in a total of 50 μl, containing 20 pmol of primers, 0.2 mM deoxynucleoside triphosphate mix (Bioline, United Kingdom), 1.5 mM $MgCl_2$ (ABGene), and 2.5 U *Taq* polymerase (ABGene). Following an initial denaturation step of 5 min at 95°C, the PCR was performed with 35 cycles of 95°C for 1 min, annealing at an appropriate temperature (Table 2) for 1 min, followed by an extension for the required time at 72°C (Table 2) and a final cycle of 72°C for 5 min (DYAD PCR engine; MJ Research). Two PCR screens were performed using the primer pairs sj007/sj008 and sj009/sj010 (Table 2). Correct amplification was verified by electrophoresis of the products, together with the

TABLE 2. Details of PCR primers and conditions of use

Primer	Sequence	Source or reference	Annealing temp (°C)	Extension time
sj007	TAGGTACCTGCTGGTACTTCTGGCAAG	This study	45.0	1 min
sj008	ATGAAGCTCACCTGCTACGTCCTCC	This study	45.0	1 min
sj009	TGCTCCAGCGAAAATCAA	This study	45.0	30 s
sj010	TGCTTCCCAGTAATACAACG	This study	45.0	30 s
UR	CAGGAAACAGCTATGAC	Promega	N/A[a]	N/A
Bact 1	CCAACAGAAGCTGCAAAACC	M. L. Faleiro	60.0	45 s
Lis1B	TTATACGCGACCGAAGCCAA	5	60.0	45 s

[a] N/A, not applicable.

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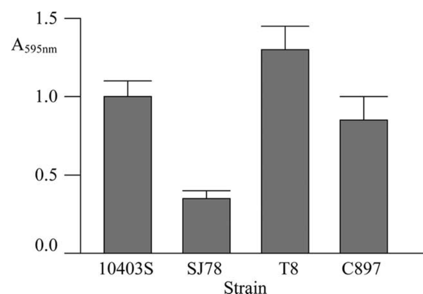

FIG. 1. Attachment of *L. monocytogenes* strains to polystyrene. Data are the means ± standard deviations of the results from six experiments.

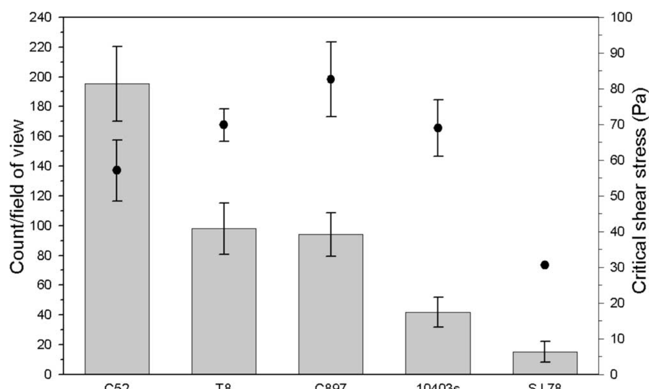

FIG. 2. Detachment of *L. monocytogenes* strains from stainless steel. The points show the average critical shear stress zone of clearance following the exposure of triplicate discs to a flow rate of 3 dm^3/min. The bar chart displays the mean number of cells per field of view on the control discs. Data are the means ± the standard deviations of 12 readings for the zone of clearance and 60 fields of view for the cell counts.

appropriate type of size ladder (NEB), through a 1.0 to 1.5% (wt/vol) agarose gel (Bioline) containing 1 μl of a 100-mg/ml ethidium bromide solution (Sigma).

Southern hybridization. Listerial chromosomal DNA was restricted with HindIII and transferred onto a nylon membrane (Hybond N+; Amersham Biosciences) by capillary transfer, and hybridization of the probe was done as detailed previously (35) with the following modifications: the membrane was incubated in a hybridization bottle containing Quikhyb solution (Stratagene) at 55°C for 30 min in a hybridization oven (Hybaid) prior to the addition of the radiolabeled probe.

The probe was created by PCR amplification with the primer pair sj009 and sj010, using colonies of *L. monocytogenes* 10403s as templates, as described in the previous section. Following purification using the Qiaquick PCR purification kit (Qiagen), the probe was sequenced prior to radiolabeling using the Ready-to-Go labeling kit (Pharmacia Biotech) as detailed in the manufacturer's protocol. Immediately before its addition to the hybridization solution, the probe was boiled for 5 min and subsequently cooled on ice for 2 min.

After hybridization at 55°C overnight, the membrane was washed as before (35), apart from the third wash, which was done at 55°C. The membrane was exposed to X-ray film (Fuji Photo Film Company, Tokyo, Japan), which was developed in a Curix 60 film processor (Agfa Geveart).

Sequence analysis. Fragments were sequenced using the ABI Prism dye terminator cycle sequence ready reaction kit with AmpliTaq DNA polymerase FS, in conjunction with an Applied Biosystems 373 sequencing system, at the University of Leicester, Leicester, United Kingdom.

Virulence assay. Passaged stocks of SJ78 and its isogenic parent were prepared using female MF1 outbred mice (Harlan Olac), as detailed before (39), and stored in single-use aliquots containing 10% (vol/vol) glycerol at −70°C. The thawed stocks were prepared for infection by centrifugation, followed by resuspension in an appropriate volume of sterile PBS to obtain the required cell concentration. For virulence tests, groups of 10 mice were injected with approximately 5×10^5 viable bacteria in 100 μl into the tail vein. Determination of the dose administered was done by plating onto tryptone soy agar. Predetermined sets of five mice from each group were killed by cervical dislocation at 72 h postinfection. The livers and spleens were removed and homogenized, and the bacterial load was enumerated by colony counting (38).

RESULTS

In silico analysis. A BLASTP search with Bap from *S. aureus* against the EGD amino acid sequence (NC_003210) revealed that Lmo0435 has 34% similarity to this protein. Lmo0435 has 42% similarity to the Bap homolog in *Staphylococcus epidermidis* (Bhp; AAK29746). We designated lmo0435 as *bapL*. Lmo0435 (BapL) is predicted to have 2,013 amino acids, compared to 2,276 in Bap from *S. aureus*. These proteins have the classic C-terminal LPXTG cell wall anchor domain of a cell surface protein, as well as a signal peptide sequence. The gene *bapL* and its upstream open reading frames have transcriptional terminators, indicating that *bapL* is not cotranscribed as part of an operon in *L. monocytogenes*.

Construction of an lmo0435 mutation in strain 10403s. The mutation of lmo0435 was prepared by constructing a gene-specific mutagenesis vector, pSJ004, which was introduced into 10403s. The selection of potential integrants was by growth in erythromycin at 40°C in liquid medium. The integrants were subsequently quantified by plating onto solid medium. In order to confirm the mutation, PCR was done on the chromosomal DNA of the potential integrants using primers sj008 and UR. PCR with successful integrants amplified a product of 950 bp (data not shown). One recombinant mutant was selected and named *L. monocytogenes* SJ78.

Attachment assay. Figure 1 shows the results for the attachment assays with the *bapL* mutant SJ78, its parent (10403s), a dairy isolate (T8), and a cheese isolate (C897). The mutant SJ78 showed a 50% reduction in attachment compared to 10403s ($P < 0.001$). This reduction in attachment was independent of the growth of the mutant over the 6-h incubation period, as the total numbers of planktonic cells enumerated for the mutant and parent were the same (data not shown). C52 also showed a significantly lower level of attachment than that of 10403s ($P < 0.001$), also without differences in the numbers of planktonic cells (data not shown). The dairy and cheese isolates (T8 and C897) attached as well as 10403s (P of >0.05 for all comparisons). The planktonic cell counts for C897 and T8 were also equivalent to 10403s at the end of the assay period.

Detachment assay. This assay was conducted to compare the levels of adherence of *L. monocytogenes* cells already attached to stainless steel discs. The radial flow device used here ensured that the discs were subjected to a constant liquid flow for a fixed period of time, thus enabling the surface shear force as a function of radius to be precisely determined. Locating the radius at which the cells were detached from the discs gave the critical shear force that marginally exceeded the forces of attachment of the bacteria. The values of critical shear stress are shown in Fig. 2. As can be seen, SJ78 required a shear detachment force of 30 Pa, less than half ($P < 0.01$) of that required to remove the isogenic parent 10403s (68 Pa). In order to

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TABLE 3. Summary of the PCR assays to detect the presence of lmo0435 in the collection of *L. monocytogenes* isolates[a]

Strain	Serotype	PCR screen 1[b]	PCR screen 2[c]	Control screen
10403s	1/2a	+	+	+
EGD	1/2a	+	+	+
ScottA	4b	−	−	+
SLU2157	4b	−	−	+
SLU1922	1/2a	−	−	+
C52	1/2a	−	−	+
C681	4b	−	−	+
C882	4b	−	−	+
C895	1/2a	−	−	+
C897	1/2a	−	−	+
A9	4b	−	−	+
G8	4b	−	−	+
SA11	4b	−	−	+
T8	4b	−	−	+
CLIP 6.04	3a	+	+	+
CLIP 8.04	1/2c	+	+	+
CLIP 7.04	1/2b	−	−	+

[a] + denotes presence and − denotes absence.
[b] PCR with primers sj007/sj008.
[c] PCR with primers sj009/sj0010.

determine if the difference was also reflected in a reduced ability to adhere to the stainless steel, counts were performed on the number of attached cells after the 2-h contact time. The *bapL* mutant had 50% less cells attached per field of view ($P < 0.01$).

The cheese isolate C897 and dairy isolate T8 showed significantly greater adherence to stainless steel than 10403s ($P < 0.01$), with at least twice the number of attached cells per field of view. However, this did not correlate with the strength of attachment quantified by the critical shear data; neither of the two isolates required a significantly different ($P > 0.05$) detachment shear force than 10403s.

C52, on the other hand, had a significantly higher number of surface-attached cells than the other strains used in this assay ($P < 0.01$). The strength of C52's adherence, however, was significantly less than that of the other wild-type isolates, as the critical shear force was lower than that of 10403s, C897, and T8 ($P < 0.01$).

Virulence assay. There were no significant differences ($P > 0.05$) in the numbers of *L. monocytogenes* 10403s and the mutant SJ78 recovered from the livers and spleens of mice 72 h after infection (10403s, 9.09 ± 0.82 $\log_{10}$ CFU in the spleen and 7.87 ± 0.86 $\log_{10}$ CFU in the liver; SJ78, 8.99 ± 0.51 $\log_{10}$ CFU in the spleen and 7.94 ± 0.45 $\log_{10}$ CFU in the liver; $n = 5$).

Presence of *bapL* (lmo0435) in *L. monocytogenes* strains. Two different PCR assays were done to determine the presence of lmo0435 in a range of *L. monocytogenes* isolates from different sources. In the first screen, using the primer pair sj007 and sj008, the expected product size was 919 bp. A second screen used the primers sj009 and sj010, and the expected product size was 343 bp. Positive control reactions were done with primers Bact 1 and Lis1B (Table 2) for the *iap* gene.

Table 3 displays a summary of both of the PCR screens. All 17 isolates were positive for the control *iap* fragment, but only four, EGD, 10403s, CLIP 6.04, and CLIP 8.04, possessed the *bapL* gene. The amplified products for the four positive isolates were of the expected size in both PCR screens. The strains also were screened for the presence of *bapL* by Southern hybridization, using the PCR product amplified by sj009 and sj010 as the probe. This probe was to the central region of *bapL*. Each of the isolates found positive by PCR showed a single band of the same size after hybridization, whereas the PCR-negative isolates were negative by hybridization (data not shown).

DISCUSSION

The factors required for biofilm formation in *L. monocytogenes* are still relatively unknown, although some progress has been made in this area through the screening of insertion mutant libraries (19, 39) and proteomic studies (21, 40). Several genes have been identified, including *relA* that encodes (p)ppGpp synthetase (39), the superoxide dismutase gene (*sod*), and the gene encoding 30s ribosomal protein S2 (*rpsB*) (40), but because these do not code for surface proteins, they will not directly determine attachment. The present study utilized an in silico approach to identify the potential surface proteins that may be involved in the attachment of this organism to surfaces. Data mining of the EGD chromosome revealed that Bap, which plays a significant role in *S. aureus* biofilm formation, shared similarity to the putative listerial protein Lmo0435. A mutation was constructed in lmo0435 to establish if this gene was required for the attachment of *L. monocytogenes* to abiotic surfaces. Because we showed that it could contribute to the attachment of *L. monocytogenes* 10403s to abiotic surfaces (polystyrene and stainless steel), we designated lmo0435 as *bapL*.

The lmo0435 mutant of *L. monocytogenes* 10403s displayed a significantly reduced level of attachment to polystyrene and stainless steel surfaces than its isogenic parent, which was in agreement with its predicted function. The presence of an LPXTG motif, and a signal peptide sequence, strongly suggests that Lmo0435 is outside the cell, anchored to the peptidoglycan. In addition to its extracellular location, the protein has polycystic kidney disease (PKD) repeat regions responsible for the formation of an immunoglobulin G-like fold in the PKD1 protein. These regions are thought to be involved in cell adhesion through the protein-protein interaction as well as the protein-carbohydrate interactions by the PKD1 protein (22).

Many organisms, such as *Pseudomonas* spp. and *Staphylococcus* spp., form biofilms that possess a thick extracellular polysaccharide matrix surrounding the attached cells (7, 8). In *S. aureus* and *S. epidermidis*, polysaccharide adhesin synthesis is encoded by the intercellular adhesion (*ica*) locus (9, 20). Strains with mutations in this operon are unable to form strong biofilms on polystyrene (9); however, recent research has shown that Bap expression is able to restore biofilm production in an IcaA mutant (11). Microscopy images of *L. monocytogenes* suggest that attached cells produce a small quantity of extracellular material (26 and our unpublished data), which contains ruthenium-red-positive polysaccharides (4). In the absence of a thick extracellular polysaccharide, Lmo0435 may serve as part of the adhesive matrix in *L. monocytogenes*.

Given the apparent role of *bapL* in the attachment of 10403s, it was surprising that a screen of dairy, food, and

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clinical isolates revealed that only 4 out of 17 possessed the gene. It is particularly noteworthy that strains A9, G8, SA11, and T8 do not have *bapL*, because these were isolated from abiotic surfaces in cheese-making dairies. Furthermore, in the laboratory assay of attachment and detachment, the *bapL*-negative dairy and cheese strains tested (C897 and T8) adhered significantly better than the *bapL*-positive 10403s and EGD. In contrast, the *bapL*-negative C52 was significantly worse in the attachment assays than did 10403s. Thus, at least two mechanisms of persistent attachment to surfaces appear to have evolved in *L. monocytogenes*: BapL dependent and BapL independent. These mechanisms do not appear to be distributed in a serotype-specific manner. Although all of the 4b isolates tested were *bapL* negative, both *bapL*-positive and *bapL*-negative strains were found among the 1/2a isolates. Where present, the *bapL* gene determines not only the ability to attach to a surface but also the force necessary to remove the cells already bound (see Fig. 2). There is not, however, a general linkage between the number of *L. monocytogenes* cells adhering to stainless steel and the force required to detach them, because the *bapL*-negative strains, such as T8 and C897, required higher shear stresses for removal than the *bapL*-positive 10403s.

The results gained during this study indicate that factors involved in the attachment of *L. monocytogenes* to surfaces can be isolate specific and that caution should be used when trying to extrapolate results from a single isolate. The strain 10403s is frequently used for in vitro and in vivo studies, often as the only object of study. Extrapolation from the data for 10403s in this study would suggest that Lmo0435 was generally involved in biofilm formation by *L. monocytogenes*, yet it was absent in all of the cheese and dairy isolates tested. Surface attachment by *L. monocytogenes* clearly is a multifactorial process, and further work is required to determine the full repertoire of the molecules involved.

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