AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Tolerance of *Listeria monocytogenes* to Quaternary Ammonium Sanitizers Is Mediated by a Novel Efflux Pump Encoded by *emrE*

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A novel genomic island (LGI1) was discovered in *Listeria monocytogenes* isolates responsible for the deadliest listeriosis outbreak in Canada, in 2008. To investigate the functional role of LGI1, the outbreak strain 08-5578 was exposed to food chain-relevant stresses, and the expression of 16 LGI1 genes was measured. LGI1 genes with putative efflux (*L. monocytogenes emrE* [$emrE_{Lm}$]), regulatory (*lmo1851*), and adhesion (*sel1*) functions were deleted, and the mutants were exposed to acid (HCl), cold (4°C), salt (10 to 20% NaCl), and quaternary ammonium-based sanitizers (QACs). Deletion of *lmo1851* had no effect on the *L. monocytogenes* stress response, and deletion of *sel1* did not influence Caco-2 and HeLa cell adherence/invasion, whereas deletion of *emrE* resulted in increased susceptibility to QACs ($P < 0.05$) but had no effect on the MICs of gentamicin, chloramphenicol, ciprofloxacin, erythromycin, tetracycline, acriflavine, and triclosan. In the presence of the QAC benzalkonium chloride (BAC; 5 μg/ml), 14/16 LGI1 genes were induced, and *lmo1861* (putative repressor gene) was constitutively expressed at 4°C, 37°C, and 52°C and in the presence of UV exposure (0 to 30 min). Following 1 h of exposure to BAC (10 μg/ml), upregulation of *emrE* (49.6-fold), *lmo1851* (2.3-fold), *lmo1861* (82.4-fold), and *sigB* (4.1-fold) occurred. Reserpine visibly suppressed the growth of the Δ$emrE_{Lm}$ strain, indicating that QAC tolerance is due at least partially to efflux activity. These data suggest that a minimal function of LGI1 is to increase the tolerance of *L. monocytogenes* to QACs via $emrE_{Lm}$. Since QACs are commonly used in the food industry, there is a concern that *L. monocytogenes* strains possessing *emrE* will have an increased ability to survive this stress and thus to persist in food processing environments.

Listeria monocytogenes is a foodborne pathogen capable of causing severe human disease in at-risk populations. It is especially known for causing complications during pregnancy and severe infections in unborn fetuses, neonates, and elderly and immunocompromised individuals, for whom 20 to 40% of infections can lead to death (1). In addition to being widespread in the natural environment, *L. monocytogenes* is frequently associated with food processing environments and is most problematic in ready-to-eat (RTE) foods. Challenges in controlling these ubiquitous bacteria in RTE products and their processing environments are associated with the ability of *L. monocytogenes* to form biofilms, to grow at temperatures below refrigeration temperatures, to resist antimicrobial compounds, and to tolerate acidic and high-salt conditions (2–7). However, there is a large variation among strains when it comes to stress survival and the ability to adapt, grow, and persist in the food chain, as well as the potential to cause human illness (8, 9).

Studies have shown that point mutations and premature stop codons in *inlA* (10–15) and *prfA* (9, 15, 16) that lead to formation of truncated InlA and PrfA proteins and attenuated virulence occur in 35 to 45% of *L. monocytogenes* strains found in food processing environments. Similarly, *L. monocytogenes* isolates originating from human clinical isolates, foods, and processing environments differ in their relative capacity to adapt to cold temperatures, though precise mechanisms that result in different adaptation rates remain elusive (12, 17–19). Various degrees of tolerance and resistance of *L. monocytogenes* to antimicrobials have also been noted, due to modifications of the cell membrane that reduce permeability, the acquisition of genes conferring resistance, and/or the function of several efflux pumps (20–24).

In Canada, a specific clone has predominated in outbreaks and sporadic cases of listeriosis across the country for more than 2 decades (25). This clone belongs to serotype 1/2a, clonal complex 8 (CC8; includes sequence type 8 [ST8], ST120, ST232, ST289, ST292, ST387, and ST551), and more specifically, it includes single-locus variants of multilocus sequence type 120 and pulsed-field gel electrophoresis (PFGE) profile LMACI.0001/LMAAI.0001 (25). Examination of 1,061 *L. monocytogenes* isolates collected from 1995 to 2010 revealed the presence of this clone in 22.3% of isolates, with nationwide distribution believed to have occurred by the mid-1990s (25). This particular PFGE clone was linked to RTE deli meats implicated in the nationwide outbreak of listeriosis in 2008 (26). Isolates associated with this outbreak also possessed a previously unreported genomic island, LGI1 (26). Subsequent testing of the 71 human clinical *L. monocytogenes* isolates collected in Canada between 1988 and 2010 revealed the presence of LGI1 in 61% of isolates (25). Notably, all of the isolates possessing LGI1 belonged to CC8 and, with the exception of one serotype 3a isolate, were exclusively of serotype 1/2a (25). A small survey of retail

Received 16 November 2015 **Accepted** 18 November 2015

Accepted manuscript posted online 20 November 2015

Citation Kovacevic J, Ziegler J, Wałecka-Zacharska E, Reimer A, Kitts DD, Gilmour MW. 2016. Tolerance of *Listeria monocytogenes* to quaternary ammonium sanitizers is mediated by a novel efflux pump encoded by *emrE*. Appl Environ Microbiol 82:939–953. doi:10.1128/AEM.03741-15.

Editor: H. L. Drake

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03741-15.

RTE foods in the Vancouver metropolitan area (British Columbia, Canada) also found LGI1 in a serotype 1/2a and ST120 isolate recovered from an RTE fish product (27); however, it was absent from 58 *L. monocytogenes* serotype 1/2a isolates, including CC8 isolates, recovered from clinical samples in Switzerland (28). Presently, the worldwide prevalence of the island among *L. monocytogenes* isolates recovered from the food chain and from clinical samples and its role are not known.

The 50-kb LGI1 element carries a combination of putative antimicrobial resistance, stress response, and virulence genes, thereby possibly enhancing the capacity of *L. monocytogenes* to survive in the food chain and to cause human listeriosis (26). The presence of genes that are typically involved in stress responses, such as those encoding a two-component signal transduction system possessing a response regulator (locus 1851) and a sensor histidine kinase (locus 1852), a putative small multidrug-resistant (SMR) efflux pump (encoded by *L. monocytogenes emrE*[$emrE_{Lm}$]; locus 1862), and a putative small RNA polymerase sigma-24 subunit (locus 1859), indicates that strains possessing LGI1 may be better equipped to survive environmental and/or food processing stresses (26). It is also tempting to speculate that the island contributes to virulence, considering that it was found in a number of clinical isolates examined over more than 2 decades. The presence of genes homologous to type IV secretion system genes (e.g., *vriB4*, *virD4*, *cpa*, and *tad*), as well as a putative adhesin gene (i.e., *sel1*), further supports this notion, albeit evidence of increased virulence due to LGI1 activity is currently lacking. In fact, the functions of genes located on LGI1 and their contributions to fitness and/or virulence of *L. monocytogenes* have not yet been reported. The purpose of the present study was to explore whether LGI1 contributes to increased tolerance of *L. monocytogenes* to food-related stresses. The *L. monocytogenes* 08-5578 strain, responsible for a nationwide listeriosis outbreak in Canada in 2008, was exposed to a variety of stresses (e.g., hot and cold temperatures, UV light, and sanitizers), and the expression of selected LGI1 genes was measured. The roles of specific LGI1 genes were investigated further by use of gene deletions (e.g., deletions of $emrE_{Lm}$, encoding a putative efflux pump; *lmo1851*, encoding a response regulator [locus 1851]; and *sel1*, encoding a putative adhesin [locus 1861]) and the exposure of *L. monocytogenes* 08-5578 and its isogenic mutants to diverse stresses encountered in the food chain (e.g., acid, cold, and saline conditions). The contribution of *sel1* to adherence to human cell lines (Caco-2 and HeLa) was also examined in further defining the mechanism of virulence.

MATERIALS AND METHODS

Bacterial isolates and media. *Listeria monocytogenes* strains and plasmids used in this study are listed in Table 1. All LGI1 deletion mutants were generated in *L. monocytogenes* 08-5578, a clinical strain responsible for the Canadian deli meat listeriosis outbreak in 2008 (26). This strain does not possess genes encoding previously described SMR efflux pump systems in *L. monocytogenes* (e.g., *bcrABC* and *qacH*) (26). Additionally, when phenotypic changes in mutants were observed, other strains possessing LGI1 ($CC8^+$ $LGI1^+$; $n = 8$) (25), strains from CC8 that do not possess LGI1 ($n = 4$), and strains belonging to serotype 1/2a but not to CC8 and not possessing LGI1 ($n = 2$) were exposed to identical conditions to investigate whether the same growth behavior was seen across unrelated strains possessing LGI1 (Table 1). *Listeria monocytogenes* EGD-SmR (29) and 81-0861 (25) were used as LGI1 serotype 1/2a and 4b negative controls, respectively.

Strains and transformants were stored long-term at −80°C in tryptic soy broth (TSB; Acumedia, Neogen, Lansing, MI) supplemented with 20% (wt/vol) glycerol (*L. monocytogenes*) or in Luria-Bertani (LB; Difco, Becton Dickinson, Sparks, MD) broth with 20% glycerol (*Escherichia coli*). Prior to use, the strains were streaked from frozen stocks onto tryptic soy agar (TSA; Acumedia) supplemented with 0.6% yeast extract (YE; Thermo Fisher Scientific, Ottawa, ON, Canada) or onto LB agar, followed by 24 h of incubation at 37°C. With the exception of specific sanitizer stress survival studies, which were performed in TSB, brain heart infusion broth (BHI; Difco) was used to grow strains prior to stress experiments. Specific conditions are described below for each stress treatment. Recovery of survivors following exposure to stress conditions was performed on TSA-YE, with incubation at 37°C for 24 to 48 h.

Antimicrobial agents and MIC determinations. Antimicrobial agents used in the study included the following: (i) the antibiotics chloramphenicol (CHL; 2.5, 5, 10, 15, and 20 μg/ml), ciprofloxacin (CIP; 2.5, 5, 10, 15, and 20 μg/ml), erythromycin (ERY; 1, 2.5, 5, 10, and 15 μg/ml), gentamicin (GEN; 1, 2.5, 5, and 10 μg/ml), and tetracycline (TET; 2.5, 5, 10, 15, and 30 μg/ml); (ii) quaternary ammonium compounds (QACs), such as 10% E-San (2.5, 5, 10, 15, and 20 μl/ml), a sanitizer containing 5% *N*-alkyl dimethyl benzyl ammonium chloride (Epsilon Chemicals Ltd., Edmonton, AB, Canada), and a benzalkonium chloride (BAC; 5, 10, 20, 25, and 30 μg/ml) with alkyl distribution from C_8H_{17} to $C_{16}H_{33}$ (Acros Organics, NJ); (iii) acriflavine (12, 16, 20, and 24 μg/ml; Sigma-Aldrich Canada Co., Oakville, ON, Canada), a cationic dye used in enrichment media during isolation of *Listeria* spp.; and (iv) triclosan (Irgasan; 5-chloro-2-[2,4-dichlorophenoxy] phenol) (1, 2, 4, and 8 μg/ml; Sigma-Aldrich), a broad-spectrum antimicrobial agent that inhibits enoyl acyl carrier protein reductase in fatty acid synthesis (30). Antibiotic stock solutions were prepared according to the manufacturer's instructions and were stored at −20°C for up to 2 months. Other antimicrobial agents were stored according to the manufacturer's recommendations (i.e., at 4°C or at room temperature). Working solutions of water-soluble agents (1,000 μg/ml) were prepared by diluting the concentrated sanitizers in sterile deionized water on the day of the experiment, and these solutions were stored at 4°C and used within 3 h of preparation. CIP was dissolved in dimethyl sulfoxide (DMSO) and sterile deionized water (1:9 [vol/vol]), whereas triclosan was dissolved in 70% ethanol. A working solution of reserpine (10,000 μg/ml; Sigma-Aldrich), an efflux inhibitor (31), was prepared in DMSO and added to bacterial cultures to a final concentration of 20 μg/ml. The highest volumes of DMSO used for dissolving CIP and reserpine and of 70% ethanol used for dissolving triclosan were applied as controls to check for a diluent effect.

MICs were determined using a slightly modified agar dilution method (e.g., for antibiotics and QACs) described by Elhanafi et al. (23) and a broth microdilution protocol (e.g., for acriflavine and triclosan) described by Kovacevic et al. (32). Briefly, for the agar dilution method, strains were grown on Mueller-Hinton agar (MHA-B) (1.2% agar; Difco) supplemented with 5% defibrinated sheep blood (Alere Inc., Ottawa, ON, Canada) and incubated at 37°C overnight. Two colonies were transferred to 200 μl of Mueller-Hinton broth (MHB; Difco), and 5 μl of suspension was spotted in duplicate onto MHA-B plates containing appropriate concentrations of antimicrobials/other compounds. Following 48 h of incubation at 30°C, MICs were determined as the lowest assessed concentrations that prevented confluent growth. For the broth microdilution method, plates were incubated at 30°C in a SpectraMax M2 plate reader (SoftMax Pro 6.3 software; Molecular Devices, Sunnyvale, CA), with the optical density at 600 nm (OD_{600}) measured at 30-min intervals for 24 h. All MIC experiments were performed at least three times.

Growth in the presence of sublethal concentrations of antimicrobials. Growth of *L. monocytogenes* in the presence of sublethal concentrations of E-San (0.8 and 1.6 μl/ml) and BAC (1 and 2 μg/ml), with or without reserpine (20 μg/ml), was assessed using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) at 30°C. Briefly, single colonies were inoculated into 5 ml of TSB and incubated at 30°C with shaking

TABLE 1 Bacterial strains and plasmids used in the present study

Strain or plasmid	Description[c]	Presence of LGI1[a]	Reference or source
Strains used for mutant constructs			
L. monocytogenes strains			
08-5578	Wild-type clinical strain (Ontario); serotype 1/2a, ST120, CC8	+	26
08-5578 Δ*lmo1851*	08-5578 with 408-bp in-frame deletion within the *lmo1851* gene	Δ*lmo1851*	This study
08-5578 Δ*emrE*	08-5578 with 240-bp in-frame deletion within the *emrE* gene	Δ*emrE*	This study
08-5578 Δ*sel1*	08-5578 with 2,598-bp in-frame deletion within the *sel1* gene	Δ*sel1*	This study
E. coli strains			
DH5α	DH5α with pKSV7	−	This study
One Shot TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	−	Invitrogen
TOP10 Δ*lmo1851*	TOP10 carrying pKSV7 with 08-5578 *lmo1851* insert with deletion	Δ*lmo1851*	This study
TOP10 Δ*emrE*	TOP10 carrying pKSV7 with 08-5578 *emrE* insert with deletion	Δ*emrE*	This study
TOP10 Δ*sel1*	TOP10 carrying pKSV7 with 08-5578 *sel1* insert with deletion	Δ*sel1*	This study
Plasmids used for mutant constructs			
pKSV7	Gram-positive and Gram-negative temperature-sensitive shuttle vector; CHL^r (*L. monocytogenes*) AMP^r (*E. coli*); multiple-cloning sites; *lacZ* β-*laccat* pE194ts[b]		37
pKSV7:Δ*emrE*	pKSV7 with 08-5578 *emrE* insert with deletion		This study
pKSV7:Δ*lmo1851*	pKSV7 with 08-5578 *lmo1851* insert with deletion		This study
pKSV7:Δ*sel1*	pKSV7 with 08-5578 *sel1* insert with deletion		This study
Control strains			
EGD-SmR	EGD derivative resistant to streptomycin; serotype 1/2a, non-CC8	−	29
81-0861	Isolated from coleslaw (Nova Scotia); serotype 4b, ST1, CC1	−	25
Other *L. monocytogenes* strains used in stress experiments			
01-1465	Clinical human blood isolate (Ontario); serotype 1/2a, ST120, CC8	+	25
01-2417	Clinical human blood isolate (British Columbia); serotype 1/2a, ST120, CC8	+	25
01-7210	Liverwurst sausage isolate (British Columbia); serotype 1/2a, ST120, CC8	+	25
02-4056	Clinical human blood isolate (Ontario); serotype 1/2a, ST311, CC155	−	25
03-0402	Clinical human blood isolate (Alberta); serotype 1/2a, ST120, CC8	+	25
06-6956	Clinical human blood isolate (Quebec); serotype 1/2a, ST37, CC37	−	25
08-6040	RTE meat isolate (Ontario); serotype 1/2a, ST120, CC8	+	25
95-0093	Clinical human blood isolate (Alberta); serotype 1/2a, ST120, CC8	+	25
95-0151	Clinical human blood isolate (Ontario); serotype 1/2a, ST120, CC8	+	25
99-3046	Clinical human blood isolate (Ontario); serotype 1/2a, ST120, CC8	−	25
01-5373	Clinical human blood isolate (Ontario); serotype 1/2a, ST120, CC8	−	25
03-5833	Clinical human blood isolate (Alberta); serotype 1/2a, ST120, CC8	−	This study
08-5375	Clinical human blood isolate (Ontario); serotype 1/2a, ST120, CC8	−	25
LR39-1	RTE fish isolate (British Columbia); serotype 1/2a, ST120, CC8	+	27

[a] Presence (+) or absence (−) of *Listeria* genomic island 1 (LGI1).
[b] Thermosensitive replication origin of plasmid pE194.
[c] CC8, clonal complex 8; based on multilocus sequence typing.

(200 rpm). Following 16 h of incubation, cultures were diluted 1:100 in TSB containing appropriate concentrations of test compounds. Aliquots (200 μl) for each strain and treatment were transferred in duplicate into a 96-well plate. The OD_{600} levels were monitored at 30-min intervals for 24 h. The OD_{600} data were fitted to growth curves to obtain the lag-phase duration (LPD), maximum growth rate (MGR), and maximum density, using the DMFit 3.0 Excel add-in program (ComBase; Computational Microbiology Research Group, Institute of Food Research, Colney, Norwich, United Kingdom), based on the models of Baranyi and Roberts (33). Experiments were performed at least three times. With each run, blank controls containing TSB only or TSB with appropriate concentrations of the tested antimicrobial were included, and their values were subtracted from those for the strains containing the respective treatments. The correspondence between the OD_{600} values and viable cell counts was examined by plating onto TSA-YE at seven time points (i.e., 0, 1, 3, 5.5, 8, 10, and 24 h) representing the early logarithmic, late logarithmic, and late stationary growth phases at 30°C.

Acid stress survival. Survival of *L. monocytogenes* 08-5578 and its LGI1 mutant derivatives under acidic conditions was assessed according to protocols described by Ells and Truelstrup Hansen (34) and Oliver et al. (35). Briefly, BHI (50 ml) was adjusted to pHs of 4.5, 3.5, and 2.5 (Pinnacle pH meter; Nova Analytics Corporation) with 6 N HCl, and 9.9-ml samples were distributed into sterile tubes (i.e., five for each pH). Overnight cultures (10 μl) grown in BHI at 30°C (with shaking at 200 rpm) were inoculated into 5 ml of fresh BHI. Following incubation for 16 h at 30°C, 2 ml of culture was collected, centrifuged at 6,000 × *g* for 10 min at room temperature, washed twice in 0.1% peptone water (2 ml), resuspended in 2 ml of 0.1% peptone water, and added to pH-adjusted BHI to obtain 10^7 to 10^8 CFU/ml (counts were confirmed by enumeration on TSA-YE plates). Cultures were vortexed and incubated at 30°C with shaking (200

TABLE 2 Oligonucleotide primers used for mutant construction[a]

Gene target and primer name	PCR conditions	Oligonucleotide sequence (5′-3′)[b]	Product size (bp) (primers used)	Digestion enzyme
emrE	95°C for 2 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1.7 min; 72°C for 10 min		1,588 (AD)	
SOE-A		CCC CTG CAG AGA CCC TCG GCT TTG CGT CC	881 (AB)	PstI
SOE-B		GCA GGG GTT GTA GGC CTG AAC		
SOE-C		*GTT CAG GCC TAC AAC CCC TGC* AAG TTC AAG TAC GAT AGC GAC	707 (CD)	
SOE-D		CCC GGT ACC GAT GGC GTG AAA ACG GCG GC		KpnI
emrE-XF		GCC ACA AAA GGG CAG GTT		
emrE-XR		TAA AGC TCT CCC GCA GTA CC		
lmo1851	95°C for 2 min; 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1.3 min; 72°C for 2 min		1,083 (AD)	
SOE-A		CCC CTG CAG ATC CAT TAG AGC ATC AAT TTG	537 (AB)	PstI
SOE-B		TTA CTA AAA GAA ATC AGT TCT		
SOE-C		*AGA ACT GAT TTC TTT TAG TAA* ATT AGC CAC TTC ATC TTC TAT	546 (CD)	
SOE-D		CCC GGT ACC CAT TAT AGC AAC TTG ATT GTG		KpnI
sel1	95°C for 2 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min; 72°C for 10 min		3,603 (AD)	
SOE-A		GG TCT AGA GCT GCT TGA TGA GGT ATG C	501 (AB)	XbaI
SOE-B		GCA TTC CAC ATT GAC CGC		
SOE-C		*GCG GTC AAT GTG GAA TGC* CGG TAA CAG TAG CTT GCT ATC ATC	504 (CD)	
SOE-D		GG GGT ACC ACA TGA GCC TAT CAG AAT TAA CCC		KpnI
sel1-XF		CAT CTA CAC CGA CAA ATA CCG CA		
sel1-XR		GCA ATC TTG TGC GAG TCT TTC		

[a] All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).
[b] Endonuclease restriction sites are underlined, and regions complementary to SOE-B primers are italicized.

rpm). A 100-μl aliquot was removed immediately ($t = 0$) and at 1, 2, 4, 6, 8, 10, and 24 h, diluted in buffered peptone water (BPW; Acumedia), and spread onto TSA-YE plates, in duplicate. Plates were incubated at 37°C, and counts were recorded after 24 h. Experiments were repeated three times.

Cold growth evaluation. Cold growth adaptation of *L. monocytogenes* and its LGI1 mutant derivatives was evaluated according to the protocol described by Kovacevic et al. (12).

Salt stress survival. To assess the growth and survival of the isolates at different salt concentrations, *L. monocytogenes* 08-5578 and its LGI1 mutant derivative strains were exposed to NaCl solutions (5 to 20% [wt/vol]; Fisher Scientific) according to the protocol described by Ells and Truelstrup Hansen (34), with slight modifications. Briefly, 10-μl aliquots of overnight cultures grown in BHI at 30°C with shaking (200 rpm) were inoculated into 5 ml of fresh BHI. Following incubation for 16 h at 30°C (with shaking at 200 rpm), cultures were centrifuged (3,000 × *g*, 10 min) at room temperature, washed twice in 0.1% peptone water, and diluted to 10^7 to 10^8 CFU/ml in BHI containing different concentrations of NaCl. Aliquots (200 μl) for each strain and treatment were transferred in duplicate into a 96-well plate. OD_{600} levels were monitored in a SpectraMax plate reader (Molecular Devices) at 30°C at 30-min intervals for 24 h. In parallel, cultures exposed to 10 and 20% NaCl were vortexed and incubated at 30°C with shaking (200 rpm). A 100-μl aliquot was removed immediately ($t = 0$) and after 1, 2, 4, 6, 8, 10, and 24 h and then serially diluted in BPW and spread onto TSA-YE plates, in duplicate. Plates were incubated at 37°C, and counts were recorded after 24 h. Experiments were repeated three times.

Construction and selection of deletion mutants. Nonpolar deletion mutants of putative efflux pump (*emrE* homolog; locus tag LM5578_RS09350 replaced the old locus tag LM5578_1862), two-component regulator (*lmo1851*; LM5578_1851), and adhesin (*sel1*; LM5578_1866) genes were generated in *L. monocytogenes* 08-5578 (GenBank accession number CP001602) by using the allelic exchange protocol described by Camilli et al. (36). A list of oligonucleotide primers, thermocycling conditions, and restriction endonucleases used is provided in Table 2. *Pfu* Turbo CX DNA polymerase (2.5 U) (Agilent Technologies Inc., Mississauga, ON, Canada) was used according to the manufacturer's instructions for all PCRs, with 0.4 μM (each) oligonucleotide primers and with *L. monocytogenes* 08-5578 genomic DNA, isolated by use of a DNeasy blood and tissue kit (Qiagen), as the template. PCR fragments (e.g., SOE-AB and SOE-CD) were purified using a QIAquick PCR purification kit (Qiagen, Toronto, ON, Canada) and subsequently used as templates in a PCR with SOE-A and SOE-D primers. The resulting SOE-AD PCR product was electrophoresed (Bio-Rad horizontal electrophoresis system) in a 1% agarose gel (Fisher Scientific), and ethidium bromide-stained bands were visualized using Image Master VSD (Amersham Pharmacia Biotech, Uppsala, Sweden) to confirm the presence of a single band of the appropriate size. When more than one band was present, the band of the appropriate size was cut out from the agarose gel and further purified using a QIAquick gel extraction kit (Qiagen). The SOE-AD PCR product and the suicide shuttle vector pKSV7 (Cornell University, Ithaca, NY) (37) were purified by use of a QIAquick PCR purification kit (Qiagen), digested with appropriate endonucleases (FastDigest; Fisher Scientific) (Table 2), and confirmed by running in a 1% agarose gel stained with ethidium bromide (Image Master VSD). Once confirmed, products were purified once more, and the SOE-AD PCR product was ligated (T4 ligase; Thermo Scientific) into pKSV7. The vector containing the gene of interest was first inserted into One Shot TOP10 chemically competent *E. coli* cells

(Invitrogen, Carlsbad, CA) via electroporation (11 kV/cm; 5-ms time constant) (Bio-Rad Gene Pulser; Bio-Rad, Hercules, CA) and subsequently electroporated into *L. monocytogenes* (11 kV/cm; 5-ms time constant). *Escherichia coli* transformants were selected on LB agar plates containing 100 μg/ml of ampicillin (AMP_{100}; Sigma-Aldrich). Plasmids were obtained from *E. coli* by using a GeneJET plasmid miniprep kit (Thermo Scientific), sequenced at the Nucleic Acid Protein Service Unit (NAPS) of the University of British Columbia with NAPS-prepared primers (−21M13 and M13R) to confirm the absence of nucleotide deletions and polymorphisms, and subsequently electroporated into 100 μl of competent *L. monocytogenes* 08-5578 cells. Following passaging and screening for vector excision, allelic exchange was confirmed by PCR amplification with SOE-A and SOE-D primers. Mutants were sequenced at NAPS (University of British Columbia) by use of SOE-A and SOE-D primers (*lmo1851*) or XF and XR primers ($emrE_{Lm}$ and *sel1*).

Preparation of *L. monocytogenes* competent cells. *Listeria monocytogenes* 08-5578 cells were first grown overnight in BHI at 37°C with shaking (200 rpm). Fresh BHI (50 ml) was inoculated with the overnight culture (500 μl) and subsequently incubated at 37°C with shaking until the culture reached an OD_{600} of 0.2 (iMark! microplate absorbance reader; Bio-Rad, Hercules, CA). Penicillin G (50 μl of a 100-mg/ml stock; Sigma-Aldrich, Oakville, ON, Canada) was added to the culture, and cells were incubated for an additional 2 h at 37°C with shaking. Following incubation, the culture was chilled on ice for 15 min, transferred to four centrifuge tubes, and centrifuged (5,939 × *g*; Eppendorf 5415 R centrifuge) for 1 min. The supernatant was discarded, and 1.2 ml of HEPES (1 mM; Sigma-Aldrich) with sucrose (0.5 M; Sigma-Aldrich) and glycerol (10% [wt/vol]; Fisher Scientific) was added to each tube. The contents of the tubes were mixed by gentle pipetting up and down five times. Tubes were centrifuged (5,939 × *g*) and supernatants discarded. Pellets were rehydrated with 100 μl of HEPES-sucrose-glycerol solution and either used immediately following preparation or stored at −80°C until use.

RNA isolation and cDNA preparation. Total RNAs were recovered from cultures under the various stress conditions tested, including cold, heat, UV, and BAC stress treatments and normal laboratory conditions as a control treatment, for TaqMan-based semiquantitative real-time PCR (sqRT-PCR) assay. RNA was isolated from each sample by using an RNeasy minikit with RNAprotect reagent (Qiagen) according to the manufacturer's instructions. Recovered RNA was treated with Ambion Turbo DNA-free DNase (Ambion Inc., Austin, TX) according to the manufacturer's double-digestion protocol to eliminate any genomic DNA contamination. Samples were immediately placed at −80°C or converted to cDNA by using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen), following the manufacturer's instructions. The same amount of total RNA of each sample was also subjected to the cDNA synthesis reaction without the reverse transcriptase enzyme. This provided control samples to assess the potential residual DNA contamination of each sample.

For quantitative real-time PCR (qRT-PCR) experiments to examine the expression levels of selected genes during BAC exposure, *L. monocytogenes* 08-5578 was grown in 25 ml of TSB at 30°C with shaking (200 rpm). Following 14 h of incubation, 5 ml was used as a control (no treatment), whereas BAC was added to another 5 ml of culture, to a final concentration of 10 μg/ml. Following 1 h of incubation at 30°C with shaking, cultures were used directly for RNA extraction, using an RNA PowerSoil total RNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Recovered RNA samples were treated with RTS DNase (Mo Bio) and immediately placed at −80°C or converted to cDNA by using a QuantiTect reverse transcription kit (Qiagen, Toronto, ON, Canada). RNAs were quantified and checked for quality by spectrophotometry (NanoDrop ND1000; Thermo Scientific, Toronto, ON, Canada) and gel electrophoresis.

Exposure to food chain-relevant stresses to assess LGI1 gene expression. *Listeria monocytogenes* 08-5578 was exposed to various stress conditions representative of those encountered in the food chain. Briefly, single colonies grown overnight at 37°C on TSA-YE plates were inoculated into BHI. Cultures were incubated at 37°C with shaking (250 rpm) for 18 h to reach the stationary growth phase, and total RNAs were isolated to represent the normal laboratory growth conditions. In the cold stress model, the stationary-phase cultures were pelleted by centrifugation (5,939 × *g*) and resuspended in fresh BHI. Each culture was further subdivided into two aliquots, which were incubated at 4°C and 35°C for 4 h, and total RNAs were then extracted. To assess expression in the heat stress model, the stationary-phase cultures were pelleted by centrifugation (5,939 × *g*), resuspended in fresh BHI, and subdivided into two aliquots, which were incubated for 4 h at 52°C and 35°C. Following individual treatments, total RNAs were isolated from the cultures. To assess gene expression following DNA damage with UV light, the stationary-phase cultures were exposed to UV light for 0, 0.17, 1, 5, 10, 15, and 30 min, and RNA was extracted at each time point. Cultures were also exposed to QACs by growing cells in BHI containing 0, 1, 5, and 10 μg/ml BAC at 37°C for 18 h, followed by RNA extraction. All experiments were performed in duplicate. Following each stress treatment, the expression of 16 genes (Table 3) within LGI1, representing various functional units and operons within LGI1, was measured using TaqMan sqRT-PCR. Reactions were performed using ABI TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, with 0.4 μM (each) oligonucleotide primers (Table 3), 0.2 μM TaqMan probe, and 5 μl cDNA template per 25-μl reaction mixture. Thermocycling conditions included denaturation at 95°C for 8 min and 35 cycles of 95°C for 10 s and 60°C for 30 s, using a Cepheid SmartCycler (Cepheid, Sunnyvale, CA). Two housekeeping genes, an rRNA gene and *bglA*, were used to ensure standardization of the cDNA samples based upon the crossing point (CP) values as computed by the SmartCycler software (Cepheid) (38). The cDNA was diluted if necessary to obtain standardized concentrations. LGI1 genes were determined to be expressed based on the CP values computed by the SmartCycler software (Cepheid).

Exposure to benzalkonium chloride to assess LGI1 gene expression. qRT-PCR assays to examine transcript levels of two known efflux pump genes in *Listeria* (*lde* and *mdrL*), *sigB*, and LGI1-carried *emrE*, *lmo1851*, and *lmo1861* were performed following 1 h of exposure of *L. monocytogenes* 08-5578 to BAC (10 μg/ml) at 30°C. A list of primers used and their relative efficiencies are provided in Table 4. Primers were designed using Geneious 5.4 software (Biomatters Ltd., Auckland, New Zealand) and were optimized to achieve specific target gene amplification (a product with a single melting peak) and PCR efficiencies between 97 and 105% (Table 4). cDNA templates derived from *L. monocytogenes* 08-5578 treated with BAC (10 μg/ml) for 30 min at 30°C were used for PCR optimization and amplification efficiency evaluation. Reactions were carried out in a final reaction volume of 20 μl containing 1 μl cDNA template, 10 μl SsoAdvanced SYBR Green supermix (Bio-Rad), and 0.25 μM (each) forward and reverse oligonucleotides (Table 4). Thermocycling conditions included initial denaturation at 95°C for 3 min followed by 39 cycles of 95°C for 10 s, 56°C for 5 s, and 72°C for 12 s, melting curve measurement (65 to 95°C in 0.5°C increments for 5 s), and cooling (4°C), using a CFX96 Touch real-time PCR detection system (Bio-Rad). Target gene transcript levels were quantified using CFX Manager 3.1 software (Bio-Rad). Relative changes in expression levels for genes of interest relative to those of the control grown in TSB without sanitizer treatment were normalized against the housekeeping 16S rRNA gene, encoding the RNA component of the smaller subunit of the bacterial ribosome (38). Cycle threshold standard deviations (SD) for all genes were ≤0.3. Gene expression fold changes reported here represent the means and SD for three independent assays with each sample run in duplicate.

Adhesion and invasion assays. The adhesion and invasion efficiencies of *L. monocytogenes* 08-5578 (wild type [WT]) and its Δ*sel1* mutant were assessed according to the protocol described by Kovacevic et al. (12), using the TC-7 subclone of Caco-2 cells (39) and HeLa (ATCC CCL-2) cells (1×10^5 cells per well; passages 5 to 20). Bacterial cultures grown statically overnight in BHI (5 ml) at 30°C were pelleted by centrifugation (5,939 ×

TABLE 3 Oligonucleotides used for TaqMan sqRT-PCR LGI1 gene expression experiments

08-5578 LGI1 target gene	Sequence (5′–3′)			Length of PCR product (bp)
	Forward primer	TaqMan probe	Reverse primer	
bglA[a]	AGCTGCTGCTGCAAACCAAT	CGAAGGCGCTTACAACGTCGATGG	AGTAACATCTTGAACGGAAAGTCCTT	73
rRNA gene[a]	CTTCCGCAATGGACGAAAGT	TGACGGAGCAACGCCGCGT	TTACGATCCGAAAACCTTCTTCA	66
lmo1901	TGATCCGCCGTATTACGAAAC	AGGGTCACTACGCTGTGGTGTTCCGA	AAGCCGTGCATGATCTTCCT	70
lmo1891	ACGGCAGGCAGTGGTTATG	TGCCAACACAGGTGAGCAGGAAGA	AAGGCAGTTCCTTTTCCTCAGA	67
lmo1889	GGCGGGAAAACCGAGAAG	AACACTTTGACAGCCCAGAACACGCC	TGCGGCGTACCACATTGA	70
lmo1885	CGGCGATGTGGTGGAATAC	TGTCGGGCTTGGAGGTAACAGTT	GAGCCAGCGGAGCATTTC	65
lmo1883	TTCACCTCACTATGCTCCACTACTG	CTATGCGGTTGGTGACGGGAATGTAGA	CATTCCACCACCGCCATT	72
lmo1882	CCGCTTTTGCAGCAGGAA	AGGCGATGTTGCCGGAGCGATT	GGGATGCAGTCGTCCATGTC	66
lmo1876	CACTTACCGCTGCGTTTGG	CGCTCCGAGAATACCCGACTGCTACA	TGTCGAAGAATTGCCTGCTCTT	69
lmo1873	ACAGCATTCGTATGAAAGCCATT	TTTACGCTTTGTTTTTCGGCACGGAA	TCGTCAGCGTTCATTTTGAGA	73
lmo1866	CACGTCATTAGTCTTCGCAGAGA	ATGCTGCCCGCCTTGGATATGACA	ATTAAATCCTGCCATTGCTTTCC	74
lmo1865	AGTTCCTATCCCTGCGGAAGA	CATCCACAGCTTATGCTTCGGGATGAA	TTGGGATACTTGCCCGCATA	70
lmo1864	ACCGGGAGATGCTGACAATT	CTGATGAATACGGTGGCAAGCTGCC	CTGCGAAAATCATCACACGATT	69
lmo1863	TCTCCCGCAGTACCGTGAA	CGTGCCATTGCTGACCTTGAA	CCTTGGAGAGATGGCCAGTCT	63
lmo1862	AAATGTCTGATGGACTTACAAAGCTATT	CCAAGTGCAGGGATGTTCATAGCATTTATCCTA	GCACTAACCGGTATTTTTTTTAATGC	109
lmo1861	GGAAATAGAAGCTTTCGCCAGTAT	CGCAGGAACAACGTAGGGCTATATCCG	GGAACTCCCGAGAAAAGAAAATG	79
lmo1859	AAGAGCGCGAAGCTGAAAGATA	AAGGAAGTGCATTCATCAATTTGAGCTTTCC	CCTCATCTTGGAATCGTTCCA	78
lmo1857	CACTGCCGCCAAGAAAAAAA	CCAGCTTCTTGACAGTAATGGTTTTGACCGA	GCAACTCTGTCCGAGCAATTT	77

[a] Housekeeping gene not carried on LGI1. The relevant primers were described by Tasara and Stephan (38).

g at 22°C; Eppendorf 5415 R centrifuge), washed once, resuspended in 1× Dulbecco's phosphate-buffered saline (DPBS; HyClone) with magnesium and calcium, and adjusted to an OD_{600} of 0.5 (Genesys 10UV system; Thermo Spectronic, Rochester, NY). Prior to infection, bacterial cultures were diluted in Dulbecco's modified Eagle's medium (DMEM) to approximately 5×10^6 CFU/ml as assessed by plating on TSA-YE plates. Bacterial suspensions (0.5 ml) were added to Caco-2 and HeLa cells and incubated at 37°C for 30 min and 1 h to allow bacterial adherence and entry, respectively. Infected cells were washed three times with DPBS and then lysed with sterile ice-cold water for 10 min at 37°C (adhesion) or overlaid with fresh prewarmed DMEM containing gentamicin (50 mg/liter) and incubated at 37°C for 45 min (invasion). Following gentamicin treatment, the cell monolayers were washed three times with DPBS and lysed with sterile ice-cold water for 10 min at 37°C. The number of viable bacteria was quantified by spreading a direct inoculum of lysed cells and serial dilutions (10^{-1} to 10^{-4}) in DPBS onto TSA-YE plates, which were incubated at 37°C for 24 to 48 h. Adhesion was reported as the average $\log_{10}$ CFU per milliliter, where the starting inoculum and recovered cells for each strain were normalized to those of the 08-5578 strain. The invasion efficiency was reported as the percentage of the inoculum recovered by enumeration of intracellular bacteria, normalized to the value for the 08-5578 strain (set at 100%). *Listeria monocytogenes* BUG5 (Tn*1545*-induced *inlA* mutant of EGD-SmR) (29) and 10403S were used as other serotype 1/2a controls. Assays for each isolate were carried out in duplicate and repeated at least three times.

Statistical analysis. Data analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA). One-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test was used to compare maximum OD_{600} values for parent and mutant strains exposed to salt stress, as well as adhesion and invasion efficiencies among strains. The unpaired two-tailed *t* test was used to compare LPD, MGR, and maximum OD_{600} values between the parent strain and its Δ*emrE* mutant. The Mann-Whitney test was used to compare LPD, MGR, and maximum OD_{600} values between strains possessing LGI1 and those without LGI1. For all analyses, differences were considered significant if the *P* values were <0.05.

RESULTS

LGI1 and *emrE* genetic content. LGI1 is a large, 49.8-kbp contiguous region (39.9% GC content) first described by Gilmour et al. (26) for two Canadian clinical isolates (08-5578 and 08-5923). In *L. monocytogenes* 08-5578 (accession number CP001602.2), it is inserted at coordinates 1836435 to 1886209 and includes 54 coding sequences (LM5578_1850 to LM5578_1903) oriented in the same direction. The bordering coding sequences LM5578_1849 and LM5578_1904 were 98% identical to contiguous EGDe coding sequences *lmo1702* and *lmo1703*, respectively (26). Compared to other bacterial genomes in the NCBI database, both homology and an organization of coding sequences within LGI1 similar to that for regions present in several environmental firmicutes were observed. These firmicutes included *Clostridium saccharolyticum* WM1 (accession number CP002109.1; 61% nucleotide [nt] coverage, with 73% identity), *Clostridium kluyveri* NBRC 12016 (accession number AP009049.1) and DSM 555 (accession number CP000673.1; 57% nt coverage, with 76% identity), and *Desulfitobacterium hafniense* Y51 (accession number AP008230.1; 50% nt coverage, with 70% identity) (see Fig. S1 in the supplemental material). However, the entire LGI1 sequence was not present within these genomes.

The genetic content of $emrE_{Lm}$ (36.4% GC content) was closest to those of genes encoding a cationic/cationic drug transporter found in *Desulfitobacterium dehalogenans* ATCC 51507 (100% coverage, with 74% nt and 77% amino acid [aa] identity) and a small multidrug resistance protein in *Desulfitobacterium hafniense*

TABLE 4 Oligonucleotides used for qRT-PCR experiments[a]

Primer	Oligonucleotide sequence (5′-3′)	Product size (bp)	Primer efficiency (%)
16S rRNA-F[b]	TTA GCT AGT TGG TAG GGT	318	91.3
16S rRNA-R[b]	AAT CCG GAC AAC GCT TGC		
emrE-JKq-F	GTT GCT ATA GCG GTG ATT GGA GT	102	104.3
emrE-JKq-R	GTT CAG GCC TAC AAC CCC TG		
lde-JKq-F	TCC CAA TGG CTT TCG CAC AA	136	99.4
lde-JKq-R	ATT CGA CCT GCA ACC TCA CG		
lmo1861-JKq-F	GCT TAC AGA AGA AGG AGC GCA	101	99.6
lmo1861-JKq-R	CCC TAC GTT GTT CCT GCG G		
mdrL-JK2q-F	TCG AGC TGG TTG GGG TTT TG	96	97.1
mdrL-JK2q-R	ATC CCA ATT GCA TGG CCT GG		
*sigB*q-F[c]	TGT TGG TGG TAC GGA TG	221	100
*sigB*q-R[c]	CAT TCT GCA ACG CCT C		

[a] All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).
[b] Primers validated by Tasara and Stephan (38).
[c] Primers designed by Arguedas-Villa et al. (19).

DCB-2 (100% coverage, with 72% nt and 73% aa identity) and Y51 (100% coverage, with 71% nt and 72% aa identity) (see Fig. S2 and S3 in the supplemental material). Some homology with predicted multidrug resistance proteins in *Clostridium ljungdahlii* DSM 13528 (100% coverage, with 68% nt identity [see Fig. S2] and 58% aa identity) and *Bacillus subtilis* 168 (95% coverage, with 54% aa identity [see Fig. S3]) was also observed.

Growth in the presence of acid, cold, or salt stress. No differences were observed between the WT parent (08-5578) strain and the LGI1 deletion mutants when strains were grown in BHI adjusted to pH 2.5, 3.5, or 4.5. At pH 4.5, bacterial counts remained constant for all four strains, at approximately 7.6 $\log_{10}$ CFU/ml. When the pH was reduced to 3.5, bacterial counts started to decline after 10 h, with an overall decrease of approximately 1.5 $\log_{10}$ CFU/ml within 24 h. Exposure of cells to pH 2.5 resulted in the largest decrease in bacterial counts for all four strains after approximately 5 h, and no viable bacteria were recovered at 24 h. When exposed to 4°C, *L. monocytogenes* strain 08-5578 adapted rapidly, and it resumed growth approximately 2 to 2.5 h following cold exposure (Table 5). No differences in adaptation to a cold environment were observed between the parent strain and its LGI1 mutants when LPD, MGR, and maximum CFU/ml were measured at 4°C following a downshift from 37°C (Table 5). All four strains reached the stationary phase within approximately 3 weeks at 4°C. In the presence of salt, the *L. monocytogenes* Δ*lmo1851* mutant reached slightly higher maximum OD_{600} values than those of the parent and other mutant strains in BHI containing 5 and 10% NaCl; however, the differences were not statistically significant ($P > 0.05$). No growth was observed in the presence of 15 and 20% NaCl for the parent and mutant strains, based on OD_{600} measurements. Similarly, there was no increase or decrease in viable counts over 24 h in the presence of 15 and 20% NaCl.

MICs of antimicrobials. The MICs for *L. monocytogenes* 08-5578 were 20 μl/ml and 30 μg/ml for E-San and BAC, respectively. The same MICs were observed for the Δ*lmo1851* and Δ*sel1* mutants, whereas the Δ*emrE* mutant had two and three times lower MICs for E-San (10 μl/ml) and BAC (10 μg/ml), respectively. No differences were observed between the MICs for the WT parent strain and those for the deletion mutants for the tested antibiotics (Table 6). All strains were sensitive to CHL, ERY, GEN, and TET and exhibited reduced susceptibility to CIP. Similarly, no differences were observed in the MICs of acriflavine or triclosan (Table 6).

Growth in the presence of sublethal concentrations of sanitizers. When exposed to sublethal concentrations of QAC sanitizers, the Δ$emrE_{Lm}$ mutant showed impaired growth compared to that of the other mutants and the parent strain (Fig. 1). This effect was particularly pronounced at concentrations of 1.6 μl/ml and 2 μg/ml for E-San and BAC, respectively (Fig. 1).

A significantly longer LPD was observed for the Δ$emrE_{Lm}$

TABLE 5 Cold growth adaptation of *L. monocytogenes* 08-5578 and its LGI1 deletion mutants, based on the lag phase duration, growth rate, and maximum density reached during incubation at 4°C following a downshift from 37°C[a]

L. monocytogenes strain	LPD (h)	Growth rate (Δ$\log_{10}$ CFU/h)	Maximum density ($\log_{10}$ CFU/ml)
08-5578 (parent)	2.48 ± 0.74	0.47 ± 0.036	9.22 ± 0.13
08-5578 Δ*emrE*	1.55 ± 0.56	0.40 ± 0.0065	9.23 ± 0.19
08-5578 Δ*lmo1851*	2.68 ± 0.59	0.49 ± 0.073	8.99 ± 0.10
08-5578 Δ*sel1*	2.37 ± 0.17	0.43 ± 0.056	9.18 ± 0.010

[a] Data are means ± SD.

TABLE 6 MICs of different antimicrobials for the *L. monocytogenes* 08-5578 wild-type parent strain and its LGI $emrE_{Lm}$ mutant

Antimicrobial agent	Strain MIC (μg/ml or μl/ml)			
	08-5578	08-5578 Δ$emrE_{Lm}$	08-5578 Δ*lmo1851*	08-5578 Δ*sel1*
Antibiotics				
Chloramphenicol	15	15	15	15
Ciprofloxacin	5	5	5	5
Erythromycin	1[a]	1	1	1
Gentamicin	5	5	5	5
Tetracycline	5	5	5	5
Sanitizers				
Benzalkonium chloride	30	10	30	30
E-San	20	10	20	20
Other compounds				
Acriflavine	18	18	18	18
Triclosan	8	8	8	8

[a] Lowest concentration tested.

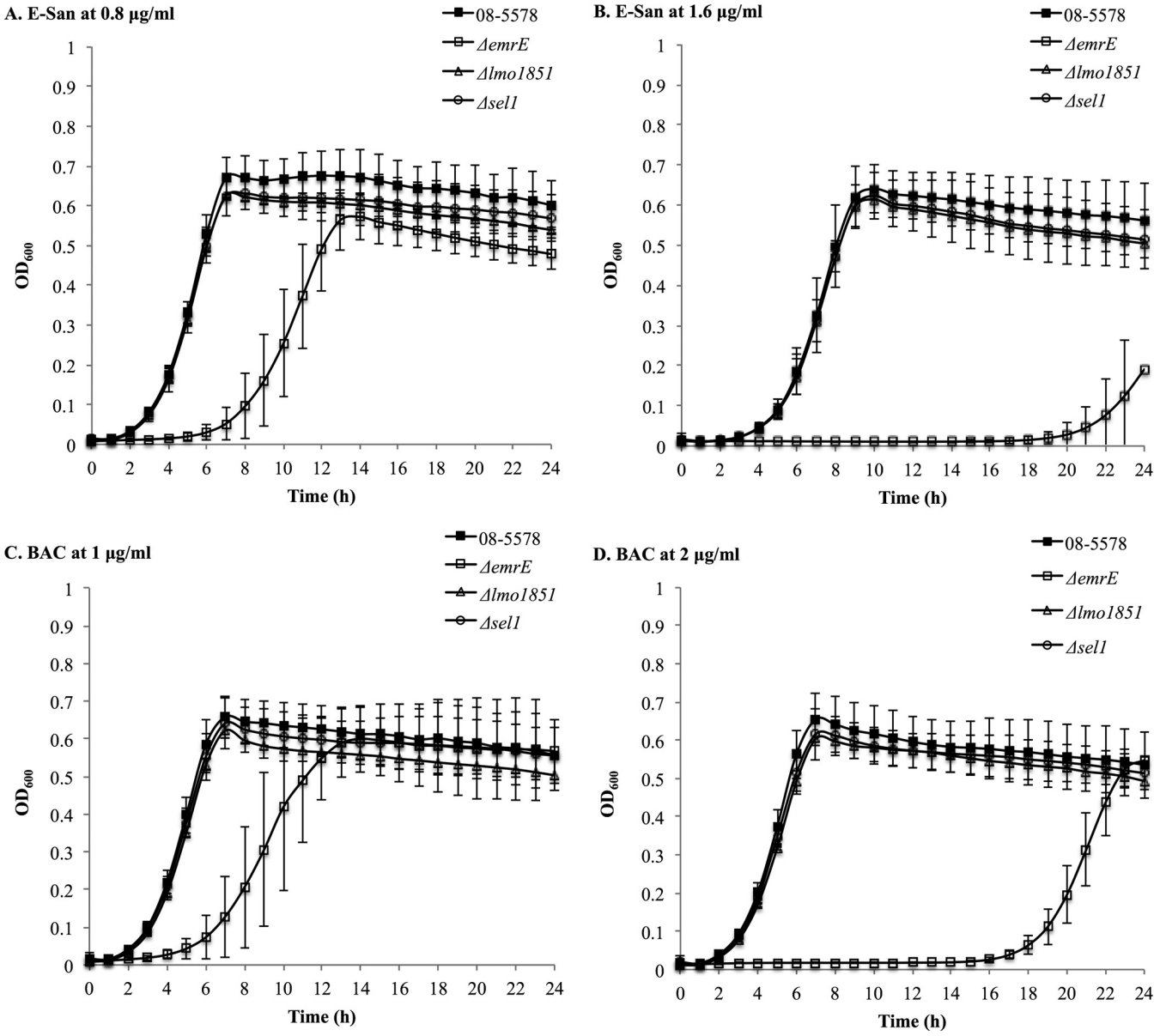

FIG 1 Growth of the *L. monocytogenes* 08-5578 WT strain (black squares) and its Δ$emrE_{Lm}$ (white squares), Δ*lmo1851* (white triangles), and Δ*sel1* (white circles) mutants in TSB with different concentrations of the E-San (A and B) and benzalkonium chloride (BAC) (C and D) sanitizers at 30°C. The data shown represent the mean OD_{600} values for five independent cultures.

strain than for the parent strain ($P < 0.0001$). In particular, it took the Δ$emrE_{Lm}$ strain 2.6 and 2.4 times longer to resume growth when exposed to E-San and BAC at 0.8 and 1 µg/ml, respectively. The LPD was also 4 and 6 times longer for the strain exposed to E-San and BAC at 1.6 and 2 µg/ml, respectively, than those of the parent strain (Table 7).

Similarly, the Δ$emrE_{Lm}$ mutant grew 1.3 times slower than the parent strain in the presence of E-San and BAC at 0.8 and 1 µg/ml, respectively, and 2.8 and 1.6 times slower when the sanitizer concentrations were increased to 1.6 and 2 µg/ml, respectively (Table 7). The Δ$emrE_{Lm}$ mutant also had significantly lower maximum OD_{600} values over 24 h in the presence of sanitizers than those of the parent strain (Table 7).

A significantly shorter LPD ($P < 0.05$) was seen for strains possessing LGI1 than for strains without LGI1 when strains were exposed to sublethal concentrations of E-San and BAC sanitizers (Fig. 2). Similarly, strains possessing LGI1 grew faster ($P < 0.001$) and reached higher maximum OD_{600} values ($P < 0.05$) than those of strains without LGI1 when the strains were grown in the presence of sublethal concentrations of E-San and BAC (Fig. 2).

Exposure to sanitizers in the presence of reserpine. The addition of reserpine, a known inhibitor of efflux pumps (31), to TSB containing a low concentration of E-San (0.8 µl/ml) and the two tested BAC concentrations (1 and 2 µg/ml) was observed to only marginally affect the growth of the WT 08-5578 strain (Fig. 3). However, at a higher concentration of E-San (1.6 µl/ml), the effect was more pronounced (Fig. 3B). When reserpine was added to Δ$emrE_{Lm}$ mutant cultures containing

TABLE 7 Average lag phase durations, maximum growth rates, and maximum optical density rates of *Listeria monocytogenes* 08-5578 and its Δ$emrE_{Lm}$ mutant exposed to sublethal concentrations of the QAC-based sanitizers E-San and BAC for 24 h at 30°C

Parameter and treatment	Value[a]	
	08-5578 (WT)	Δ$emrE_{Lm}$ mutant
Lag-phase duration (h)		
E-San		
0.8 μl/ml	3.31 ± 0.15	8.45 ± 1.34***
1.6 μl/ml	5.17 ± 0.50	21.02 ± 0.79****
BAC		
1 μg/ml	2.87 ± 0.39	6.80 ± 1.40**
2 μg/ml	3.16 ± 0.19	18.58 ± 0.68****
TSB	2.68 ± 0.62	2.79 ± 0.56
Maximum growth rate (increase in OD_{600}/h)		
E-San		
0.8 μl/ml	0.19 ± 0.03	0.15 ± 0.02*
1.6 μl/ml	0.17 ± 0.02	0.06 ± 0.04***
BAC		
1 μg/ml	0.19 ± 0.01	0.15 ± 0.01***
2 μg/ml	0.20 ± 0.02	0.12 ± 0.01***
TSB	0.19 ± 0.03	0.21 ± 0.03
Maximum OD_{600}		
E-San		
0.8 μl/ml	0.69 ± 0.07	0.60 ± 0.06*
1.6 μl/ml	0.65 ± 0.06	0.23 ± 0.18**
BAC		
1 μg/ml	0.68 ± 0.07	0.66 ± 0.11
2 μg/ml	0.66 ± 0.07	0.56 ± 0.04**
TSB	0.67 ± 0.06	0.73 ± 0.07

[a] Values represent mean values ± SD for five independent assays, with each sample and treatment measured in duplicate. Statistically significant values are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) (*P* values were obtained using the unpaired two-tailed *t* test).

sublethal concentrations of E-San and BAC, longer LPDs were observed at lower concentrations of BAC and E-San (Fig. 3A and C). At the higher concentration of BAC (2 μg/ml), growth was visibly suppressed, whereas the addition of reserpine to cultures with 1.6 μl/ml E-San completely inhibited the growth of the Δ$emrE_{Lm}$ mutant (Fig. 3B and D).

LGI1 gene expression in response to stresses encountered in the food chain. The expression of LGI1 was analyzed under various growth conditions by using sqRT-PCR with 16 LGI1 gene targets to identify the island's functional roles. Under normal laboratory growth conditions, when the cells were incubated in BHI at 37°C, only one gene (LM5578_1861), encoding a putative MarR family repressor, was expressed (Fig. 4). Similar results were obtained when the cells were subjected to heat or cold shock, and also when they were treated with UV light. In contrast, when the cells were grown at 37°C in BHI supplemented with 5 μg/ml BAC, 14 of the 16 LGI1 gene targets were induced (Fig. 4). This included the putative MarR family repressor gene (LM5578_1861). The two targets for which expression was not detected in the presence of BAC were LM5578_1883, encoding a putative surface/membrane protein, and LM5578_1863, encoding a putative protein involved in DNA processing (Fig. 4).

Gene expression in WT *L. monocytogenes* 08-5578 exposed to a sublethal concentration of BAC. To further explore gene expression in the presence of QACs, *L. monocytogenes* 08-5578 was exposed to BAC at 10 μg/ml for 1 h, and the expression profiles of LGI1 genes and other genes involved in stress responses were investigated. The largest change (82.4-fold) in the expression of LGI1 genes was seen for *lmo1861*, encoding a putative MarR family transcriptional regulator, followed by *emrE* (49.6-fold), encoding an SMR efflux pump. The expression of an LGI1-encoded putative response regulator (*lmo1851*) of a two-component transduction system also increased 2.3-fold, whereas the expression of *sigB*, encoding a major stress response regulator in *L. monocytogenes*, increased 4.1-fold. There was no change in the expression of *lde* and *mdrL*, encoding proteins that belong to the major facilitator superfamily (MFS) multidrug resistance efflux pumps.

Adhesion and invasion of TC-7 and HeLa cells by the Δ*sel1* mutant. There were no differences in the adhesion or invasion efficiencies of the Δ*sel1* and WT parent (08-5578) strains measured with the TC-7 and HeLa cell lines (Fig. 5). The numbers of adherent cells for the parent and Δ*sel1* deletion mutant strains were similar to those observed for the control strain 10403S (6.4 $\log_{10}$ CFU/ml). Adhesion efficiencies for all strains were higher for TC-7 cells than for HeLa cells, with the exception of the *inlA*-deficient mutant BUG5 (Fig. 5A and B). No differences in invasion efficiency were observed between the 10403S control strain, the 08-5578 parent strain, and the Δ*sel1* mutant by use of TC-7 cells (Fig. 5C). However, significantly ($P < 0.05$) fewer colonies of 08-5578 and its Δ*sel1* mutant than of the 10403S control strain invaded HeLa cells (Fig. 5D).

DISCUSSION

The roles of LGI1 in *L. monocytogenes* survival in the food chain and in virulence were investigated in the clinical outbreak strain *L. monocytogenes* 08-5578 and its LGI1 mutant derivatives. LGI1 was first reported for Canadian *L. monocytogenes* isolates that caused a nationwide listeriosis outbreak in 2008 (26). Since it appears to be absent from all other genomes of *L. monocytogenes* that are available in the NCBI database, as of August 2015 (see Fig. S1 in the supplemental material), it is highly likely that LGI1 is specific to Canadian isolates. The presence of a number of putative antimicrobial resistance, stress response, and virulence genes on LGI1 indicates that it may be important for *L. monocytogenes* survival in the food chain and for human listeriosis (26). To investigate this, we deleted three genes located on LGI1, with putative stress response (i.e., the Δ*emrE* and Δ*lmo1851* strains) and virulence (i.e., the Δ*sel1* strain) functions, and exposed the resultant isolates to various conditions relevant to the predicted functions of the deleted genes. Overall, *L. monocytogenes* 08-5578 possessed high tolerance to acidic, antimicrobial (Table 6), cold (Table 5), and high-salt conditions. Both marked adhesion and invasion of the Caco-2 TC-7 and HeLa cell lines (Fig. 5) were observed, which is consistent with the high mortality rate (40%) and a number of previously reported invasive listeriosis cases (26).

LGI1 was tightly regulated, with multiple gene targets induced, when strains were grown in the presence of the QAC sanitizer BAC (Fig. 4), but this was not detected with other stresses. When LGI1 mutant strains were exposed to stress conditions, the putative role of the response regulator of a two-component signal transduction system (*lmo1851*) underlying the increased tolerance to the tested stressors could not be confirmed. Similarly, deletion of the *sel1*

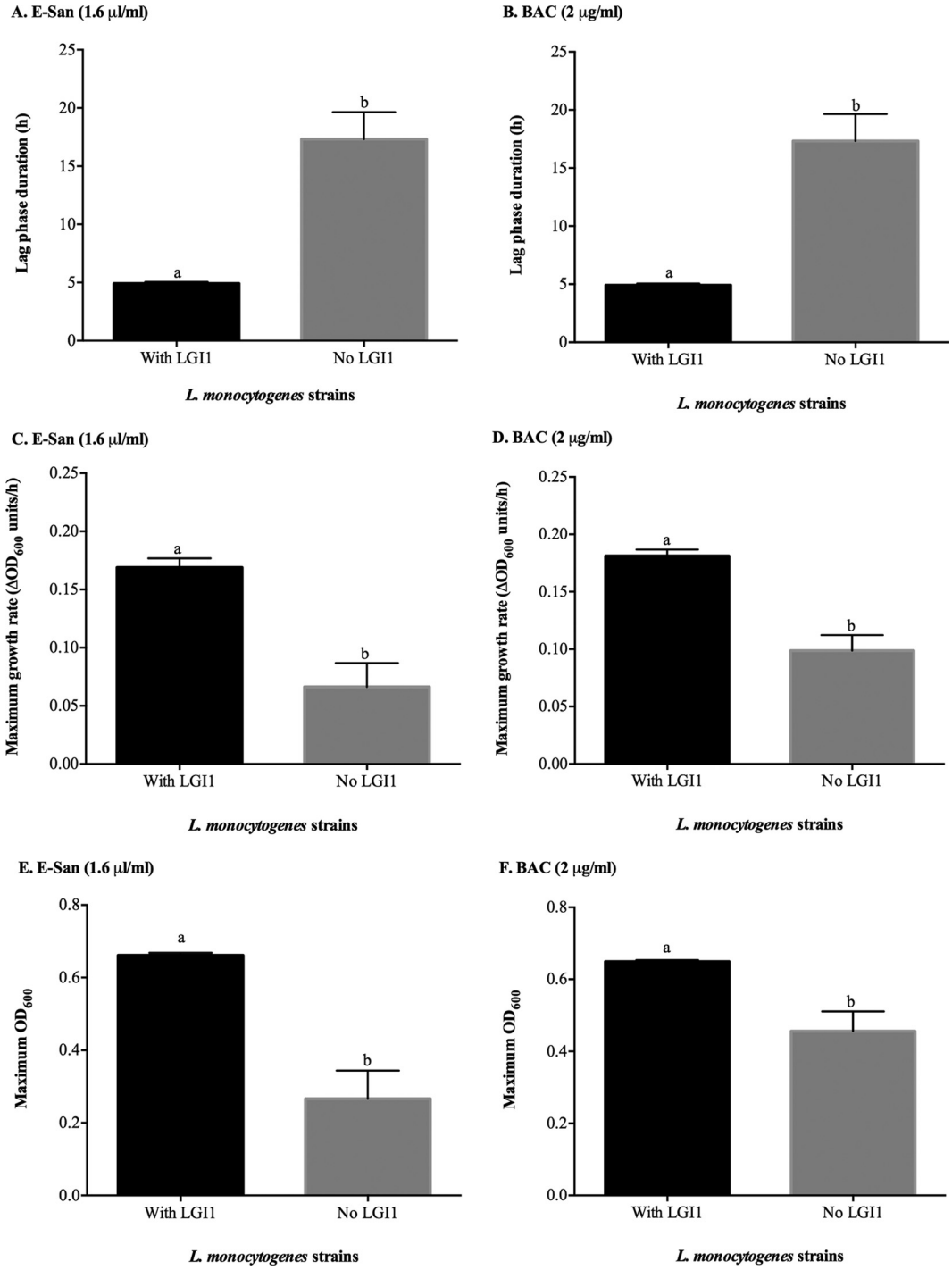

FIG 2 Mean lag-phase duration (h), maximum growth rate (ΔOD_{600}/h), and maximum OD_{600} values for *L. monocytogenes* isolates possessing LGI1 ($n = 9$) and isolates without LGI1 ($n = 8$) grown in the presence of sublethal concentrations of E-San (1.6 μl/ml) (A, C, and E) and benzalkonium chloride (BAC) (2 μg/ml) (B, D, and F) at 30°C for 24 h. Bars represent mean values, and error bars indicate standard errors of the means. Different letters above the bars represent significant differences ($P < 0.05$) between the groups, determined using the Mann-Whitney test.

gene, encoding a putative adhesin, did not affect *L. monocytogenes* adhesion to and invasion of the TC-7 and HeLa cell lines. These results suggest that *sel1* does not affect the virulence potential of *L. monocytogenes* under the conditions tested herein and that this gene does not contribute to the increased adherence and invasion of *L. monocytogenes in vitro*. In contrast, deletion of the *emrE* gene, encoding an SMR efflux pump, resulted in susceptibility to QAC-based sanitizers.

Further analyses revealed that the MICs of two different QAC-based sanitizers against the Δ*emrE* mutant were up to 3 times lower than those for the WT parent strain (Table 6). When the $\Delta emrE_{Lm}$ mutant was grown in the presence of sublethal concentrations of the

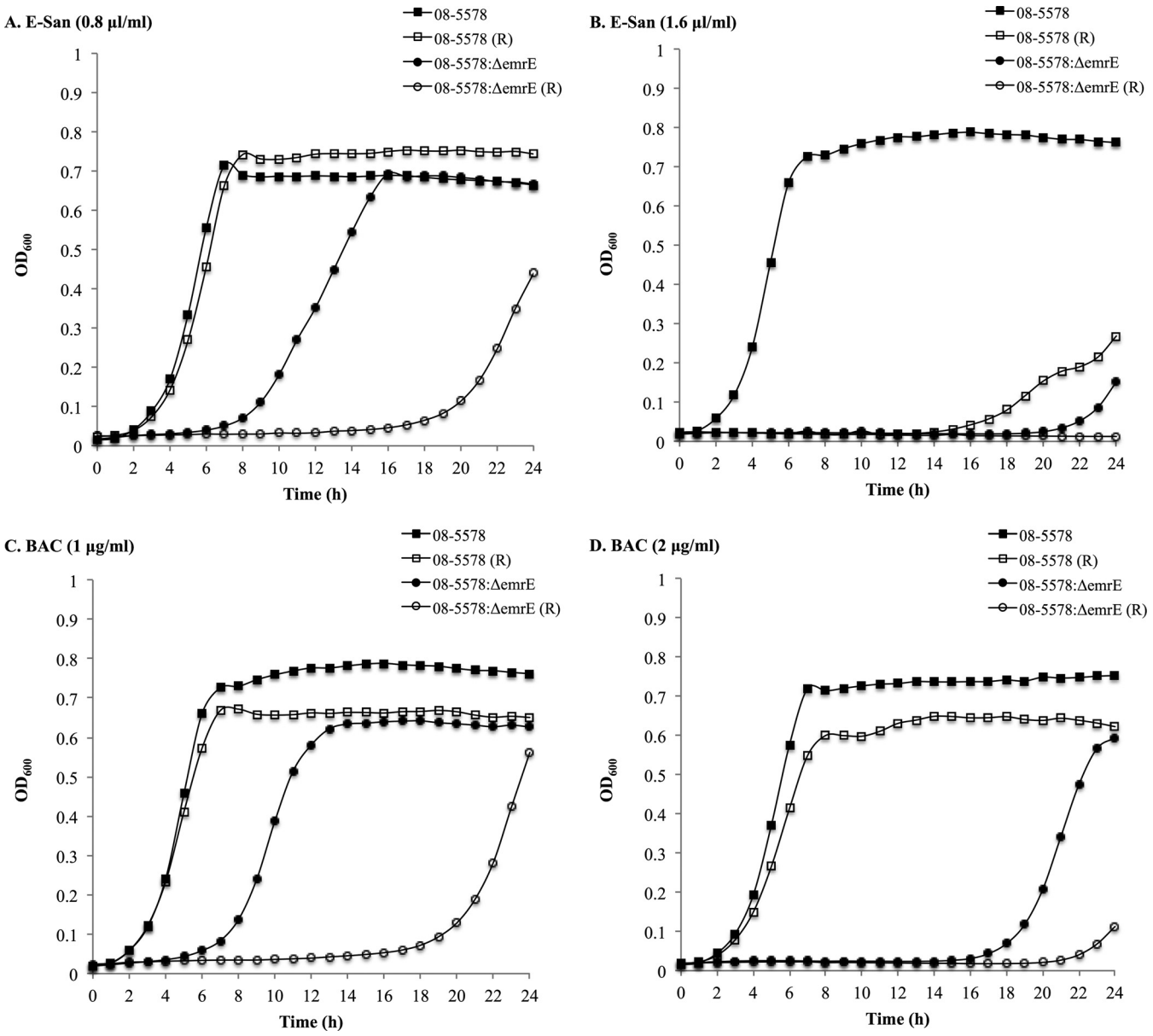

FIG 3 Growth of *L. monocytogenes* 08-5578 (WT) and its Δ$emrE_{Lm}$ mutant in the presence of E-San at 0.8 µl/ml (A) and 1.6 µl/ml (B) or benzalkonium chloride (BAC) at 1 µg/ml (C) and 2 µg/ml (D) at 30°C, with (white symbols) and without (black symbols) reserpine (R; 20 µg/ml). The data shown represent the mean OD_{600} values for three independent assays. Standard deviations ranged from 0.0010 to 0.24.

BAC and E-San sanitizers, we noted longer lag phases, lower growth rates, and overall lower maximum cell densities (OD_{600}) than those of the parent strain (Table 7). Further evidence that LGI1 improves the survival of *L. monocytogenes* in the presence of QACs was demonstrated with eight additional clinical and food chain strains possessing LGI1, as these strains exhibited faster adaptation and higher growth rates than those of genetically similar strains without LGI1 (Fig. 2). The addition of reserpine, a known efflux pump inhibitor (31), impaired the growth of both the WT and Δ*emrE* mutant strains in the presence of different concentrations of QAC sanitizers, thus confirming that increased QAC tolerance is due at least in part to a modified efflux activity. However, definite proof of the role of *emrE* and the level of its contribution to QAC tolerance would require complementation of the mutants.

The role of *emrE* in QAC tolerance was also apparent from the significant upregulation of *emrE* in the presence of the BAC sanitizer. However, the upregulation of other LGI1-carried genes suggests that they may also partially contribute to QAC tolerance. Under the same conditions, increased expression of the *lmo1861* gene, encoding a putative MarR family regulator, was also observed. It is tempting to speculate that *lmo1861* encodes a repressor of the SMR efflux protein, as the MarR family regulator has been shown to negatively regulate MdrM, a multidrug efflux transporter important in activation of the host cytosolic surveillance system, in *L. monocytogenes* (40). However, additional experiments are required to confirm this possibility. The expression of *mdrL*, a gene responsible for the production of an MdrL chromosomal efflux pump belonging to the MFS transporters in *L.*

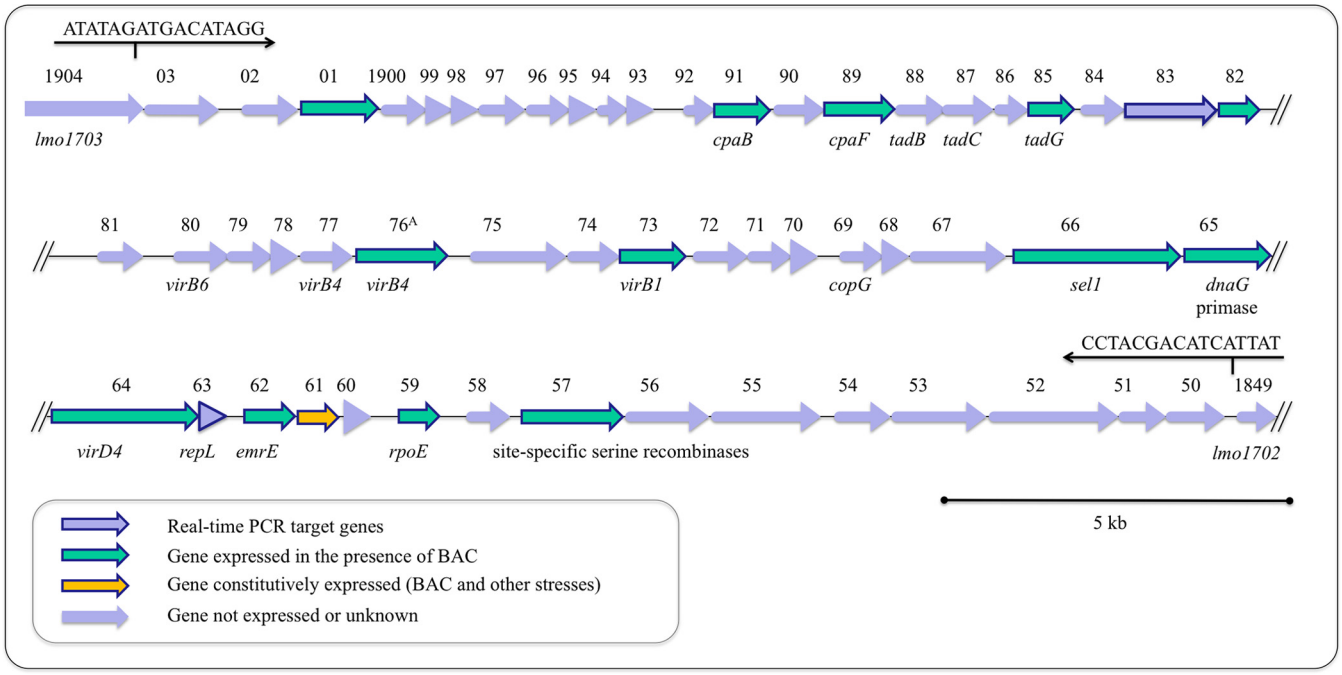

FIG 4 Expression profile of selected LGI1 genes when *L. monocytogenes* 08-5578 was grown in BHI supplemented with 5 μg/ml benzalkonium chloride at 37°C for 18 h.

monocytogenes, was not upregulated in the current study. This result is not surprising, as *mdrL* was previously shown to be overexpressed only in *L. monocytogenes* strains that were experimentally adapted to BAC, not in naturally resistant WT strains (21), such as those tested in the current study. The findings obtained here also confirm that *lde*, encoding an additional efflux pump belonging to the MFS efflux transporters, has no role in *L. monocytogenes* resistance to QAC-based sanitizers. This conclusion was expected, as the Lde efflux pump has been linked primarily to quinolone resistance and, to some degree, is believed to contribute to the tolerance of *L. monocytogenes* to dyes, such as ethidium bromide and acridine orange (31, 41).

In WT *L. monocytogenes* strains, the presence of a plasmid-borne or chromosomally carried *bcrABC* cassette or a transposon (Tn*6188*)-based QacH efflux pump has been shown to increase tolerance to QAC-based sanitizers in outbreak, clinical, and food chain *L. monocytogenes* isolates (22, 23, 42). However, QAC sanitizer resistance in *L. monocytogenes* due to the activity of the SMR EmrE homolog described here has not been reported before. Efflux pumps belonging to the SMR family of proteins are typically 100 to 140 amino acids long and often confer resistance to aminoglycosides, fluoroquinolones, dyes, and QAC (43–45). The first *emrE* (i.e., *E. coli* multidrug resistance E)-encoded efflux pump described was linked to resistance of *E. coli* to TET, ERY, and sulfadiazine (46). Similar SMR pumps have also been described for *Mycobacterium smegmatis* (e.g., Mmr), *Pseudomonas aeruginosa* (e.g., $EmrE_{Pae}$), and *Staphylococcus* spp. (e.g., QacC/D and QacH); however, the substrates of each efflux pump vary depending on the pump and the bacterial species (44, 45, 47). Li et al. (48) demonstrated that the *emrE* homolog in *P. aeruginosa* contributed to resistance to ethidium bromide, acriflavine, and aminoglycosides (i.e., amikacin, GEN, kanamycin, neomycin, and tobramycin), albeit resistance to aminoglycosides was observed only in low-ionic-strength medium. In *M. smegmatis*, an in-frame deletion in *emrE* resulted in decreased MICs of ethidium bromide, acriflavine, CIP, and norfloxacin but had no effect on CHL, ERY, GEN, and TET MIC values (49). In *L. monocytogenes*, *emrE* did not appear to contribute to resistance to GEN, CHL, CIP, TET, and triclosan. It also did not play a role in *L. monocytogenes* tolerance to acriflavine dye. This result is not unexpected, since *L. monocytogenes emrE* did not possess any similarity to other well-characterized SMR efflux pump genes from Gram-negative and Gram-positive bacteria. In fact, its genetic content is closest to that of a small multidrug resistance protein in *Desulfitobacterium hafniense* (72% similarity) and a cationic/cationic drug transporter found in *Desulfitobacterium dehalogenans* (74% similarity). *Desulfitobacterium* spp. are anaerobic, motile, Gram-positive, rod-shaped bacteria that often reside in environments contaminated by halogenated organic compounds (50). Some homology (66 to 68%) also exists among EmrE of *L. monocytogenes*, a predicted multidrug resistance protein in *Clostridium ljungdahlii*, and QAC resistance proteins observed in *Bacillus thuringiensis* serovar *kurstaki* and *Bacillus cereus* strains. The presence of these bacteria in soil and effluents, which are also natural environments for *L. monocytogenes*, may result in sharing of the genetic material that confers survival under harsh conditions, though more research is needed to explore this possibility.

Resistance to QACs is a particular concern in regard to *L. monocytogenes*, as these compounds are often used as sanitizers in food processing facilities due to their noncorrosive properties. Even though the risk of selecting for sanitizer-resistant microorganisms when sanitizers are used at the concentrations recommended by manufacturers is low, it should not be overlooked that inadequate cleaning and sanitation practices can result in exposure of *L. monocytogenes* to sublethal concentrations of sanitizers, which in turn can lead to selection pressure for progeny possessing

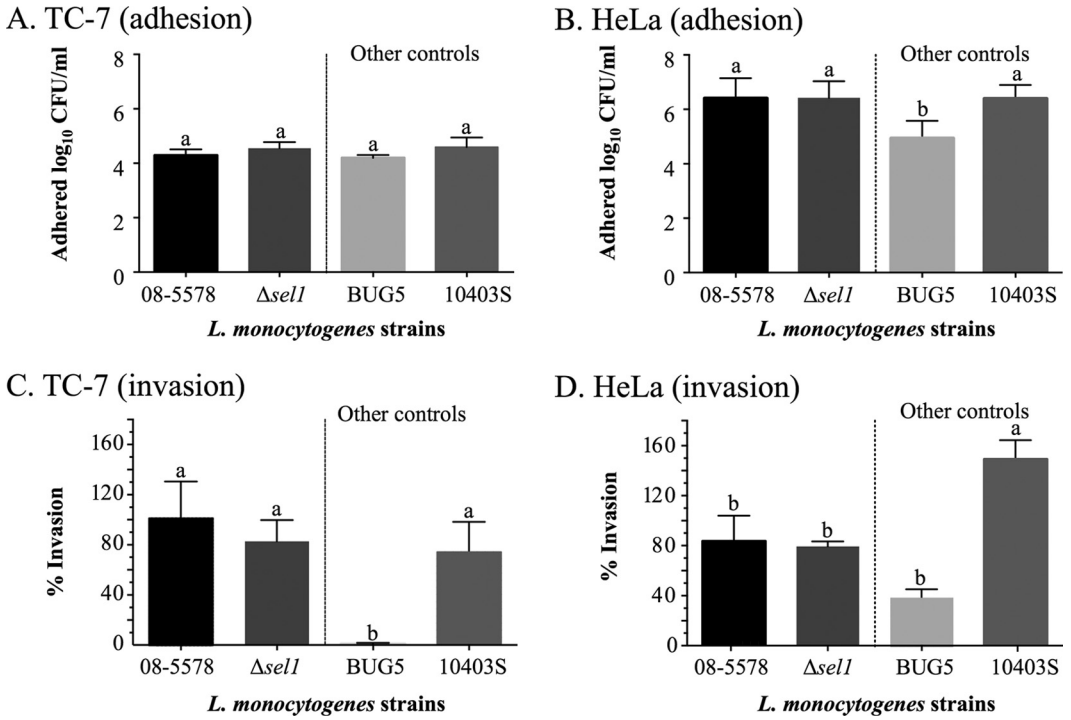

FIG 5 Adhesion and invasion efficiencies (% bacteria recovered relative to the initial inoculum, normalized to the 08-5578 strain) of the *L. monocytogenes* WT strain (08-5578) and its isogenic mutant possessing a deletion in the *sel1* gene, located on LGI1, compared to those of the clinical isolate 10403S and a Tn*1545*-induced noninvasive *inlA* mutant of EGD-SmR (BUG5), using the TC-7 (A and C) and HeLa (B and D) cell lines. Assays for each isolate were carried out in duplicate and repeated four times. Bars show mean adhesion ($\log_{10}$ CFU/ml) and invasion efficiencies (normalized to the 08-5578 strain), and error bars indicate standard errors of the means. Different symbols above the bars indicate significantly different adhesion or invasion efficiencies ($P < 0.05$; one-way ANOVA with Dunnett's multiple-comparison test).

increasing sanitizer tolerance (51). This is especially likely to occur if equipment and niches that are difficult to clean and sanitize are encountered, allowing bacteria to persist in food processing environments (52, 53). A number of listeriosis outbreaks implicating isolates with increased tolerance to sanitizers have been noted (23, 42, 52). The *L. monocytogenes* 08-5578 isolate characterized in the current study was implicated in one of the largest listeriosis outbreaks in Canada, with the source of contamination suspected to be a large commercial slicer harboring the bacterium (54). The presence of LGI1-carried *emrE* likely contributed to survival of this isolate in the food processing environment.

An additional concern with isolates possessing efflux pumps that enhance *L. monocytogenes* tolerance to QAC-based sanitizers is the potential for these isolates to develop enhanced tolerance to antibiotics due to similar mechanisms of action (45). Rakic-Martinez et al. (51) demonstrated that *L. monocytogenes* strains selected on sublethal concentrations of CIP (2 μg/ml) or BAC (10 μg/ml) exhibited higher MICs not only of these agents but also of several other toxic compounds, including GEN, the dye ethidium bromide, and the chemotherapeutic drug tetraphenylphosphonium chloride. While the research performed in this study did not show that *emrE* increased tolerance to antibiotics relevant to listeriosis treatment (e.g., aminoglycosides), the adaptation to high concentrations of QAC sanitizers and the antibiotic coselection phenomenon were not investigated. In future research, it would be interesting to investigate whether the coselection phenomenon between QACs and antibiotics can occur via the $EmrE_{Lm}$ efflux pump. While the coselection phenomenon occurring in *L. monocytogenes* is not well understood, a growing body of evidence suggests that pressures occurring at food processing facilities may contribute to the selection of isolates with enhanced tolerance to different antimicrobials (32, 55). As only a small number of antibiotics were tested in the experiments described here, future studies should include a larger panel of antimicrobials comprising different classes of antibiotics and sanitizer compounds.

Conclusions. Collectively, our data demonstrate a role for LGI1-carried *emrE* in QAC tolerance, as evidenced by the following: (i) adaptation and growth of *L. monocytogenes* strains possessing LGI1-carried *emrE* are significantly improved in the presence of QACs compared to the adaptation and growth of genetically similar strains without LGI1, (ii) the expression of *emrE* and several other genes on LGI1 is induced in the presence of BAC, and (iii) deletion of $emrE_{Lm}$ results in reduced MICs and impaired isolate growth and survival in the presence of QACs. While, to date, there has been no evidence that proper use of sanitizers in food processing will lead to the development of resistant microorganisms, exposure of microorganisms to concentrations below the recommended levels required to eradicate *L. monocytogenes* is not an unlikely scenario. Since QACs are commonly used in the food industry, the presence of LGI1-carried $emrE_{Lm}$ may provide a survival advantage and lead to the persistence of *L. monocytogenes* in food processing environments, though more work is required to fully understand the mechanisms afforded by LGI1 that underlie persistence. In particular, further studies are needed to determine the extent of the contribution of $EmrE_{Lm}$ to QAC tolerance, the role of this efflux pump in resistance to other antimicrobials

via the coselection phenomenon, the genetic regions involved in the regulation of *emrE*, and the potential contributions of additional LGI1-carried genes to antimicrobial resistance and/or virulence. The identification and characterization of $emrE_{Lm}$ provide evidence for a novel mechanism for *L. monocytogenes* to resist injury when exposed to sanitizers commonly encountered in food processing, reaffirming the resilient nature of this foodborne pathogen.

ACKNOWLEDGMENTS

This work was funded in part by the National Sciences and Engineering Research Council of Canada.

We acknowledge the Canadian National Microbiology Laboratory for donating bacterial isolates; Cornell University (Ithaca, NY) and Kendra Nightingale (Texas Tech University) for donating the pKSV7 vector and strain 10403S, respectively; and Monique Rousset (Centre de Recherche des Cordeliers, Paris, France) and Pascale Cossart (Institut Pasteur, France) for donating Caco-2 TC-7 cells and the EGD-SmR strain, respectively, via B. Brett Finlay. We thank Kristie Keeney and B. Brett Finlay for their guidance and critical reviews of this work and for financial support, Kevin Allen for providing laboratory space and financial support, and Lili R. Mesak and Jessica Chen for technical assistance.

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