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# *Listeria monocytogenes* Multidrug Resistance Transporters and Cyclic Di-AMP, Which Contribute to Type I Interferon Induction, Play a Role in Cell Wall Stress

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**The intracellular bacterial pathogen *Listeria monocytogenes* activates a robust type I interferon response upon infection. This response is partially dependent on the multidrug resistance (MDR) transporter MdrM and relies on cyclic-di-AMP (c-di-AMP) secretion, yet the functions of MdrM and cyclic-di-AMP that lead to this response are unknown. Here we report that it is not MdrM alone but a cohort of MDR transporters that together contribute to type I interferon induction during infection. In a search for a physiological function of these transporters, we revealed that they play a role in cell wall stress responses. A mutant with deletion of four transporter genes (Δ*mdrMTAC*) was found to be sensitive to sublethal concentrations of vancomycin due to an inability to produce and shed peptidoglycan under this stress. Remarkably, c-di-AMP is involved in this phenotype, as overexpression of the c-di-AMP phosphodiesterase (PdeA) resulted in increased susceptibility of the Δ*mdrMTAC* mutant to vancomycin, whereas overexpression of the c-di-AMP diadenylate cyclase (DacA) reduced susceptibility to this drug. These observations suggest a physiological association between c-di-AMP and the MDR transporters and support the model that MDR transporters mediate c-di-AMP secretion to regulate peptidoglycan synthesis in response to cell wall stress.**

*Listeria monocytogenes* is a Gram-positive, facultative, intracellular pathogen that invades a wide range of mammalian cells (1). Following internalization, the bacteria escape to the cell cytosol by secreting several virulence factors, primarily the pore-forming hemolysin, listeriolysin O (LLO). Once in the cytosol, *L. monocytogenes* replicates and spreads from cell to cell by recruiting host actin filaments (1). During infection, *L. monocytogenes* triggers a robust type I interferon response, as manifested by enhanced expression and secretion of the cytokine beta interferon (IFN-β) (2). This response was shown to be independent of Toll-like receptors but reliant on several innate immune signaling molecules (i.e., STING, TBK-1, and IRF3) (3–6). Remarkably, the bacteria must be replicating in the macrophages' cytosol to elicit a type I interferon response, as phagosomally trapped bacteria, such as an LLO-negative mutant, do not induce this response (2). A previous study aimed at identifying *L. monocytogenes* determinants involved in IFN-β activation identified multidrug resistance (MDR) transporters as modulators of the type I interferon response *in vivo* (7). Specifically, overexpression in bacteria of two closely related MDR transporters, MdrM and MdrT, was found to trigger enhanced induction of IFN-β by infected macrophages. However, only deletion of the *mdrM* gene resulted in reduced levels of IFN-β secreted by infected macrophages (7). This observation indicated that MdrM plays an active role during bacterial cytosolic growth that leads to induction of the type I interferon response.

MdrM and MdrT belong to the major facilitator superfamily (MFS) of MDR transporters and are closely related to the well-characterized MDR transporter, QacA, of *Staphylococcus aureus* (8). MDR transporters, such as QacA, are notorious for their ability to confer resistance to a wide variety of toxic compounds and drugs, including antibiotics, by utilizing proton motive force to actively extrude these compounds outside the cell (9). Accordingly, MdrM and MdrT were shown to be transcriptionally induced upon bacterial exposure to rhodamine 6G (R6G) and tetraphenylphosphonium (TPP), both well-known substrates of MDRs, and to confer resistance to cholic acid (7, 10). Nevertheless, none of these classical MDR functions could explain the observed role of these proteins in activating the innate immune system, implying they might possess distinct physiological roles during infection.

It was recently proposed that MdrM and MdrT transporters extrude cyclic-di-AMP (c-di-AMP) during *L. monocytogenes* intracellular growth, which in turn activates infected macrophages to elicit the IFN-β response (11, 12). Indeed, c-di-AMP activates a robust type I interferon response when added exogenously; however, a physiological association between c-di-AMP and the MDR transporters was not established. Notably, several reports had indicated that c-di-AMP serves as a second messenger molecule that influences central cellular processes of bacteria: e.g., genome surveillance, response to cell wall stresses, and, more recently, peptidoglycan homeostasis (13–17). In bacteria, c-di-AMP is synthesized by diadenylate cyclase (DAC) using ATP as a substrate and, conversely, linearized to 5′-pApA by a specific c-di-AMP phosphodiesterase (PDE) (15). While it was shown that the level of c-di-AMP is largely dependent on the expression levels of DAC

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**TABLE 1** Genes similar to *mdrM* in *L. monocytogenes* strain 10403S based on protein sequence

| *L. monocytogenes* 10403S gene no. | *L. monocytogenes* EGDe gene identifier no. | % identity, % similarity of amino acid sequence | Intracellular induction[a] | Gene name |
|---|---|---|---|---|
| LMRG_02976.6 | lmo1617 | | Yes | *mdrM* |
| LMRG_02679.6 | lmo2588 | 45, 65 | Yes | *mdrT* |
| LMRG_00200.6 | lmo0519 | 35, 60 | Yes | *mdrA* |
| LMRG_02080.6 | lmo0981 | 25, 46 | No | |
| LMRG_01853.6 | lmo2845 | 23, 44 | Yes | *mdrB* |
| LMRG_01880.6 | lmo2818 | 22, 41 | Yes | *mdrC* |
| LMRG_02296.6 | lmo0872 | 20, 38 | Yes | *mdrD* |
| LMRG_01872.6 | lmo2826 | 16, 33 | Yes | *mdrE* |

[a] Based on microarray analysis (23).

and PDE enzymes (15, 18), the mechanism coordinating the activity of these enzymes is not known. The prevalence of DAC domains among bacteria and archaea strengthens the premise that this c-di-AMP is fundamentally involved in microbial physiology (13). The *L. monocytogenes* genome contains both c-di-AMP *dac* and *pde* genes (*dacA* [lmo2120] and *pdeA* [lmo0052]). The *dacA* gene was shown to be essential for growth and to be the gene responsible for c-di-AMP production, while *pdeA* was shown to degrade c-di-AMP (11, 18).

In the present study, we aimed to identify a physiological association between *L. monocytogenes* MDR transporters and c-di-AMP. We discovered that MDR transporters play a role in the *L. monocytogenes* response to cell wall stress and found that this MDR function was linked to c-di-AMP production. More generally, this report furthers the understanding of the molecular mechanism whereby intracellular *L. monocytogenes* cells trigger type I interferon responses during infection.

## MATERIALS AND METHODS

**Bacterial strains, cells, growth media, and reagents.** *L. monocytogenes* strain 10403S was used as the wild-type (WT) strain and as the parental strain for all mutants generated in this work (Table 1). The *Escherichia coli* XL-1 Blue (Stratagene) and DH12 strains were used for vector propagation. *E. coli* strain SM-10 (19) was used for conjugative plasmid delivery to *L. monocytogenes*. *L. monocytogenes* strains were grown in brain heart infusion (BHI) (BD) medium or minimal medium (20) at 37°C, and *E. coli* strains were grown in Luria-Bertani (LB) medium (BD) at 37°C. For infection experiments, *L. monocytogenes* bacteria were grown overnight in BHI at 30°C without agitation. IPTG (isopropyl-β-D-1-thiogalactopyranoside) was purchased from Bio-Lab, Ltd. (Israel), penicillin G, vancomycin hydrochloride, and mutanolysin were purchased from Sigma, and c-di-AMP and c-di-GMP were purchased from the Biolog Institute (Germany). Primary bone marrow-derived (BMD) macrophages were isolated from 6- to 8-week-old female C57BL/6 mice (Harlan Laboratories, Ltd., Israel) and cultured as described previously (21). RAW264 macrophages were grown and maintained in Dulbecco's modified Eagle's medium (DMEM)-based media.

**Generation of *L. monocytogenes* in-frame deletion mutants.** Deletion mutants were generated by standard techniques using the pKSV7oriT vector, as described in reference 7. Plasmid pLIV2-*mdrM* was used for generation of 6×His-tagged MdrM and the F58V mutant (for primers, see Table S1C in the supplemental material).

**Protein analysis by Western blotting.** Overnight cultures were diluted 1:100 and grown to an optical density at 600 nm ($OD_{600}$) of 1 U and then supplemented with 0.25 mM IPTG when indicated. Bacteria were harvested, treated with 50 U of mutanolysin for 1 h, and sonicated in a mixture of 20 mM Tris-HCl (pH 8), 0.5 M NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After removal of cell debris, membranes were collected by ultracentrifugation. Membrane fractions (50 μg of protein) were then subjected to 12.5% SDS-PAGE and blotted for His tag detection using India HisProbe-horseradish peroxidase (HRP) (Pierce) and the ECL enhanced chemiluminescence reagent.

**Bacterial growth curves.** Overnight cultures were adjusted to an $OD_{600}$ of 0.03 in 20 ml fresh BHI broth, supplemented when indicated with 0.25 mM IPTG, 0.08 μg $ml^{-1}$ penicillin G, 1 μg $ml^{-1}$ vancomycin, or 3 μg $ml^{-1}$ c-di-AMP. For bacterial RNA extraction, bacteria were grown at 37°C to an $OD_{600}$ of 0.4 and then supplemented with 3 μg $ml^{-1}$ lincomycin, 50 μM rhodamine 6 G (R6G), 1 μg $ml^{-1}$ vancomycin, and 0.08 μg $ml^{-1}$ penicillin G for 2 h or centrifuged and resuspended in: minimal medium (pH 5) (lactic acid), minimal medium with 10 mM $H_2O_2$, or defined minimal medium (pH 5) with 10 mM $H_2O_2$ for 30 min. For microscopy, bacteria were grown similarly and supplemented with 1 μg $ml^{-1}$ vancomycin for 2 h. Growth curves in the presence of drugs were performed in a Synergy HT Biotek plate reader at 37°C with continuous shaking and monitoring of the $OD_{600}$ every 15 min for 24 h. Of note, bacterial growth in the plate reader is different from that in flasks with respect to the OD levels that are measured. In each experiment, growth conditions are indicated.

***L. monocytogenes* intracellular growth in cells.** Intracellular growth curves were performed as described previously (22). Briefly, $2 \times 10^6$ cells were seeded on a petri dish with glass coverslips and infected with $8 \times 10^6$ bacteria. At 0.5 h postinfection (p.i.), cells were washed, and at 1 h p.i., gentamicin was added. At each time point, cells from 3 coverslips were lysed and CFU were counted. For bacterial gene expression of intracellularly grown *L. monocytogenes* cells, $25 \times 10^6$ BMD macrophages were infected with $1 \times 10^8$ bacteria and lysed in 20 ml of ice-cold water at 6 h p.i., and the released bacteria were collected on 0.45-μm-pore hydroxyapatite (HA) filters (Millipore, catalog no. HAWP04700).

**Gene expression analysis.** RNA was purified from bacteria in mid-log-phase growth in BHI or from infected cells using standard phenol-chloroform extraction methods. RNA from intracellularly grown bacteria was amplified using the MessageAmp II (Ambion) bacterial RNA amplification kit according to the manufacturer's instructions. RNA of infected macrophages was extracted using TRIzol reagent according to standard protocols. In all cases, 1 μg of RNA was reverse transcribed to cDNA using an Applied Biosystems high-capacity reverse transcription kit. Real-time quantitative PCR (RT-qPCR) was performed on 10 ng of cDNA using SYBR green with the Step-One Plus RT-PCR system (Applied Biosystems). The transcription of bacterial genes was normalized using 16S rRNA or the *rpoB* gene, and that of macrophage cytokines was normalized using the glyceraldehyde 3-phosphate dehydrogenase gene (*gpdh*). Statistical analysis was performed using the StepOne V2.1 software. Error bars represent 95% confidence intervals; in a case where the error bars of two samples do not overlap, the *P* value is $\ll 0.01$. Primer sequences are described in Table S1A and B in the supplemental material. The complete intracellular expression profile of *L. monocytogenes* 10403S was published separately (23).

**β-Galactosidase MUG assay for *mdrC* transcription.** Overnight cultures of WT *L. monocytogenes* pPL2-$P_{mdrC}$*lacZ* and Δ*mdrMTA* pPL2-$P_{mdrC}$*lacZ* cells were adjusted to an $OD_{600}$ of 0.05. Cultures were grown in 96-well black plates (200 μl) with a clear bottom to an $OD_{600}$ of ~0.4 at 37°C. Next, the plates were centrifuged for 10 min at 3,800 rpm, supernatants were aspirated, and the cells were washed twice with phosphate-buffered saline (PBS). Two hundred microliters of ABT buffer (60 mM $K_2HPO_4$, 40 mM $KH_2PO_4$, 100 mM NaCl, 0.1% Triton X-100 [pH 7]), supplemented with 80 μg $ml^{-1}$ of MUG substrate (4-methylumbelliferyl-β-D-galactopyranoside) (Sigma), was added to each well. Plates were shaken for 30 s and incubated at room temperature for 1 h in the dark. Following incubation, the optical density (600 nm) and the fluorescence intensity (excitation, 360 nm; emission, 460 nm) were measured using a Synergy HT Biotek plate reader. β-Galactosidase activity was normalized

to the sample's OD (24, 25). The experiment was performed in triplicate and was repeated three times independently.

**TEM.** Bacteria were grown as described above with and without vancomycin treatment. For negative staining, PBS-washed bacteria were adsorbed on Formvar carbon-coated grids and stained with 2% aqueous uranyl acetate. For transmission electron microscopy (TEM) sections, a bacterial pellet from 20 ml of culture was fixed in 2.5% glutaraldehyde in PBS at 4°C for 20 h, washed three times with PBS, and postfixed in 1% $OsO_4$ in PBS at 4°C for 2 h. Dehydration was carried out in graded ethanol and embedding in glycid ether. Thin sections were mounted on Formvar carbon-coated grids and stained with uranyl acetate and lead citrate. All images were acquired using a Jeol 1200 EX transmission electron microscope (Jeol, Japan). Measurements of cell wall thickness were performed from three independent biological repeats; a total of 35 frames were taken for each strain and condition.

**Mouse infection.** *L. monocytogenes* bacteria were grown in BHI medium at 30°C overnight. Six- to 8-week-old C57BL/6 female mice (Harlan Laboratories, Ltd., Israel) were infected via tail vein injections with $4 \times 10^4$ washed bacteria (5 mice in each group). Spleens and livers were harvested 72 h p.i. and homogenized in 0.2% saponin, and bacterial CFU were determined by plating. The experiment was repeated twice.

**Measurement of peptidoglycan synthesis rate.** Overnight bacterial culture was diluted 1:100 into 10 ml of BHI, grown to an $OD_{600}$ of 0.4, and supplemented with 20 μM *N*-acetylglucosamine and 10 μl of 1 μCi $\mu l^{-1}$ of $^{14}$C-*N*-acetylglucosamine (American Radiolabeled Chemicals). The culture then was divided into two parts, and 0.8 μg $ml^{-1}$ of vancomycin was added to one of them. One-hundred-microliter aliquots from cultures incubated without agitation at 37°C were withdrawn in triplicate every 30 min and added to 100 μl of boiling 8% SDS, and the mixture was incubated for 5 min at 95°C. Cell wall was collected on 0.45-μm-pore-size membrane filters (Millipore, catalog no. HAWP02500), washed with 15 ml of water, and counted using 5 ml of EcoLite(+) liquid scintillation cocktail with a PerkinElmer TriCarb 3110TR β-counter.

**Peptidoglycan extraction and muropeptide analysis.** Cell wall and peptidoglycan were purified as described previously (26). Muropeptides were generated from highly purified cell wall and peptidoglycan samples by mutanolysin and then reduced using sodium borohydride. Muropeptide separation was performed by high-performance liquid chromatography (HPLC) as previously described for *L. monocytogenes* (27, 28). For activation of cytokines by cell wall samples, lyophilized cell wall extracts were resuspended at a concentration of 1.5 mg $ml^{-1}$, and then the pH was adjusted to 7.5 with NaOH, and 20 μl was added to $2 \times 10^6$ BMD macrophages in 2.5 ml medium. After 6 h, macrophage RNA was harvested and analyzed for cytokine induction.

## RESULTS

**A functional MdrM transporter is required to trigger macrophages to elicit the IFN-β response.** As mentioned, *L. monocytogenes* bacteria overexpressing the MdrM transporter have been shown to trigger infected macrophages to express enhanced IFN-β levels (7). Initially, we wanted to validate that this enhancement of the IFN-β response requires MdrM to be functional. To this end, an *mdrM* mutant was generated that harbored a mutation that inactivates function but preserves expression. In the *S. aureus* QacA transporter, substitution for tyrosine at position 63 with valine resulted in a nonfunctional transporter and enhanced sensitivity of the bacteria to a wide variety of drugs (29). Therefore, using site-directed mutagenesis, the corresponding phenylalanine in MdrM at position 58, F58, was substituted with valine. The resulting *mdrM-F58V* gene construct was tagged with histidine at the 3′ end and cloned into the integrative pLIV2 vector under an IPTG-inducible promoter to generate pLIV2-*mdrM-F58V-6His*. This plasmid or a control plasmid containing the His-tagged native *mdrM* gene (pLIV2-*mdrM-6His*) was conjugated to a Δ*mdrM* mutant, and the expression levels of the native and mutated MdrM were compared by Western blotting. Indeed, both the native MdrM and MdrM-F58V proteins were expressed and found in the membrane fraction at similar levels upon IPTG addition (Fig. 1A). Next, the ability of the MdrM and MdrM-F58V proteins to confer resistance to R6G was tested. As expected, the Δ*mdrM* mutant was more sensitive to R6G than the wild-type (WT) bacteria were, and introduction of the native *mdrM* gene (via pLIV2-*mdrM-6His* with IPTG induction) rescued the sensitivity. However, introduction of MdrM-F58V did not restore full growth, providing support that the F58V mutation does interfere with MdrM's transport function (Fig. 1B). Next, the capacity of this mutant to enhance the IFN-β response was tested. To this end, macrophages were infected with the Δ*mdrM* mutant harboring the pLIV2 plasmid expressing the native or the mutated MdrM. As shown in Fig. 1C, all strains grew to a similar extent intracellularly (Fig. 1C). Notably, only bacteria overexpressing the native MdrM induced an enhanced IFN-β response, while bacteria overexpressing the mutated MdrM did not (Fig. 1D). These results indicate that MdrM's function is required for induction of an IFN-β response during infection.

**MdrM transporter and several MDR homologs are transcriptionally induced during intracellular growth.** MDR transporters are known to exhibit functional redundancy due to overlapping substrate specificity (9, 30). Since MdrM was shown to be responsible for a third of the IFN-β induction by infected macrophages (7), we examined if additional transporters are involved in mediation of the IFN-β response. A search of the *L. monocytogenes* strain 10403S genome for *mdrM* homologs revealed several genes encoding putative MDR transporters, among them the previously identified gene *mdrT* (Table 1). On this list, the LMRG_00200.6 gene (an ortholog of lmo0519 in EGD-e), named here *mdrA*, was highly similar to *mdrM* and *mdrT*, with 60% similarity and 45% identity in protein sequence, whereas the other protein genes exhibited only 33 to 44% sequence similarity (Table 1). To gain insight into the potential requirement for these transporters during *L. monocytogenes* infection, we analyzed their transcription levels during intracellular growth in macrophages. Our previous transcriptomic data from intracellularly grown bacteria indicated that all of these transporters are induced during infection of macrophages, except for LMRG_02080.6 (using microarray analysis [23]) (Table 1). We thus further compared the transcription levels of the induced MDR transporters by real-time quantitative PCR (RT-qPCR) analysis. As shown in Fig. 2, all of the transporters were transcriptionally upregulated during intracellular growth in macrophages, at least 4-fold, over their levels in BHI. These results suggest that each of the transporters might play an active role during *L. monocytogenes* infection.

**A set of MdrM-like transporters control most of the type I interferon response to *L. monocytogenes* infection and virulence.** To examine whether MdrM homologs contribute to IFN-β induction during infection, we generated a series of in-frame deletion mutants harboring single or multiple (double, triple, quadruple, and quintuple) MDR gene deletions (Table 2). All of the MDR mutants grew similarly to WT bacteria both in BHI broth and intracellularly in macrophages, except for the *mdrMTAD* mutant, which exhibited a moderate intracellular growth defect (see Fig. S1A and B in the supplemental material). The IFN-β response elicited by macrophages after infection with each of the mutants was evaluated using RT-qPCR analysis of IFN-β transcript levels.

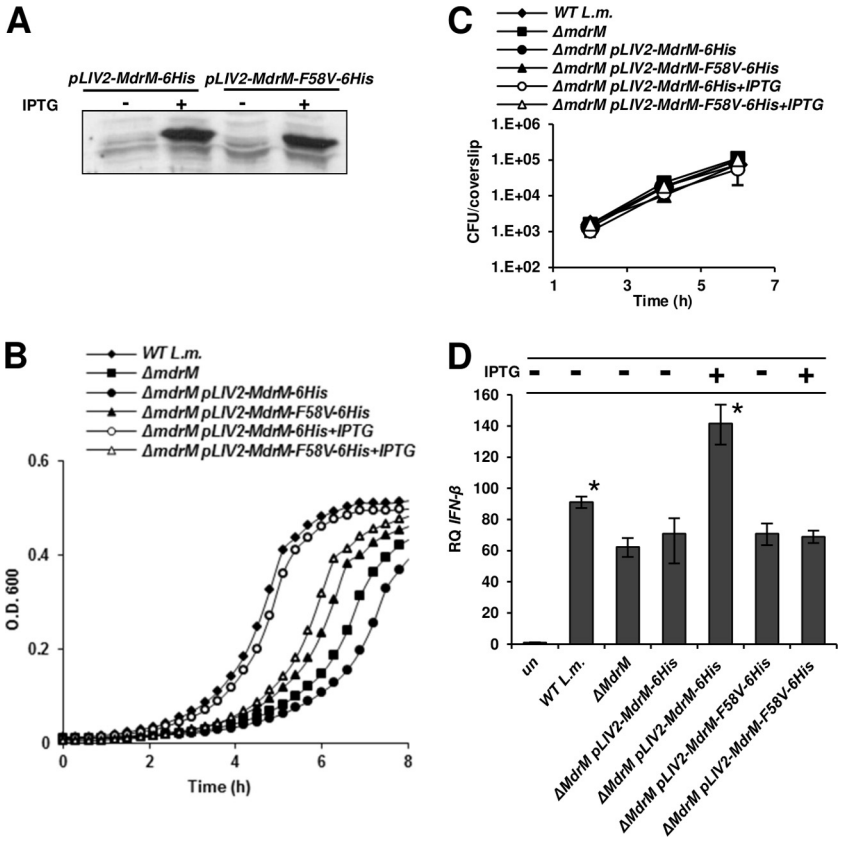


**FIG 1** MdrM transporter function is required for activation of the IFN-β response during macrophage infection. (A) Western blot analysis of bacterial membrane fraction probed for 6×His-tagged MdrM and MdrM-F58V (using HisProbe-HPR) expressed from the IPTG-inducible vector pLIV2, with and without IPTG. (B) Growth in the presence of R6G (3.5 μM) of WT *L. monocytogenes* (*L.m.*), the Δ*mdrM* mutant, and the Δ*mdrM* mutant harboring pLIV2 expressing MdrM-6×His or MdrM-F58V-6×His, with and without IPTG (1 mM) in BHI. The experiment was performed in a 96-well format in a Synergy HT Biotek plate reader. Growth curves from one representative experiment are shown. Error bars representing the standard deviation of a triplicate sample are hidden by the symbols. (C) Intracellular growth curves of WT *L. monocytogenes*, the Δ*mdrM* mutant, or the Δ*mdrM* mutant harboring pLIV2 expressing MdrM-6×His or MdrM-F58V-6×His, with and without IPTG, in RAW264 macrophages. Representative growth curves are shown. Error bars represent standard deviations of 3 biological repeats. (D) RT-qPCR analysis of IFN-β transcriptional levels in macrophages infected with WT *L. monocytogenes*, the Δ*mdrM* mutant, or the Δ*mdrM* mutant harboring pLIV2 expressing MdrM-6×His or MdrM-F58V-6×His, with and without IPTG, at 6 h postinfection (p.i.). Transcription levels are represented as relative quantity (RQ) compared to levels in uninfected cells (un). The data are an average of 3 independent experiments. Error bars represent 95% confidence intervals. *, $P < 0.01$ compared to the rest of the samples. The data in panels A to C are representative of at least 3 independent biological repeats.

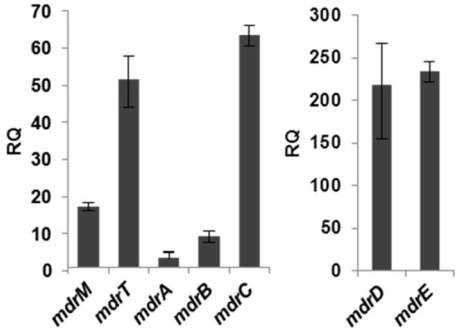


**FIG 2** Transcription of MdrM transporter homologs is induced during *L. monocytogenes* intracellular growth. Transcription analysis of *mdrM*-like transporter genes during *L. monocytogenes* 10403S intracellular growth in BMD macrophages at 6 h p.i. in comparison to their levels during exponential growth in BHI medium using RT-qPCR analysis. Transcription levels are represented as relative quantity (RQ) compared to the transcription levels during growth in BHI. If the transcript levels are equal under both conditions, RQ = 1. Transcription levels were normalized to the levels of 16S rRNA as a reference gene. The data are representative of 3 independent biological repeats ($n = 3$). Error bars represent 95% confidence intervals (as described in Materials and Methods).

Overall, we observed that the greater the number of transporters that were deleted, the lower the IFN-β levels that were expressed by infected cells (Fig. 3A). Notably, macrophages infected with the Δ*mdrMTAC* quadruple mutant (with the *mdrM*, *mdrT*, *mdrA*, and *mdrC* genes deleted) exhibited the lowest IFN-β level among the tested mutants, approximately 15% the amount of IFN-β relative to macrophages infected with WT bacteria. Infection with the Δ*mdrMTAC* mutant was also observed to induce macrophages to transcribe low levels of IL-6 but normal levels IL-1α, indicating that the action of these transporters primarily affects the induction of the type I interferon response (in which both IL-6 and IFN-β are included) (Fig. 3B). The latter observation corroborated the previous characterization of the Δ*mdrM* mutant showing it to particularly modulate the type I interferon response rather than general proinflammatory responses (7). In summary, this analysis revealed that several MDR transporters, homologs of MdrM, are functionally involved in the activation of the type I interferon response during *L. monocytogenes* infection.

Since the Δ*mdrMTAC* mutant grew like WT bacteria in macrophages (see Fig. S1B in the supplemental material) yet triggered

**TABLE 2** Bacterial strains used in this study

| Strain | Genotype | Source or reference |
|---|---|---|
| *Listeria monocytogenes* | | |
| 10403S (WT) | Str$^r$ | D. A. Portnoy, lab stock |
| Δ*mdrM* mutant | Δ*mdrM* | 7 |
| Δ*mdrA* mutant | Δ*mdrA* | This study |
| Δ*mdrC* mutant | Δ*mdrC* | This study |
| Δ*mdrB* mutant | Δ*mdrB* | This study |
| Δ*mdrD* mutant | Δ*mdrD* | This study |
| Δ*mdrE* mutant | Δ*mdrE* | This study |
| Δ*mdrMC* mutant | Δ*mdrM* Δ*mdrC* | This study |
| Δ*mdrMTA* mutant | Δ*mdrM* Δ*mdrT* Δ*mdrA* | This study |
| Δ*mdrMTAC* mutant | Δ*mdrM* Δ*mdrT* Δ*mdrA* Δ*mdrC* | This study |
| Δ*mdrMTAB* mutant | Δ*mdrM* Δ*mdrT* Δ*mdrA* Δ*mdrB* | This study |
| Δ*mdrMTAD* mutant | Δ*mdrM* Δ*mdrT* Δ*mdrA* Δ*mdrD* | This study |
| Δ*mdrMTAE* mutant | Δ*mdrM* Δ*mdrT* Δ*mdrA* Δ*mdrE* | This study |
| Δ*mdrMTABC* mutant | Δ*mdrM* Δ*mdrT* Δ*mdrA* Δ*mdrB* Δ*mdrC* | This study |
| Δ*mdrM* mutant (pLIV2-*mdrM-6His*) | Δ*mdrM* pLIV2 expressing MdrM-6×His | 7 |
| Δ*mdrM* mutant (pLIV2-*mdrM-F58V-6His*) | Δ*mdrM* pLIV2 expressing MdrM-F58V-6×His | This study |
| Δ*mdrMTAC* mutant (pLIV2-*mdrM-6His*) | Δ*mdrMTAC* pLIV2 expressing MdrM-6×His | This study |
| Δ*mdrMTAC* mutant (pLIV2-*mdrT*) | Δ*mdrMTAC* pLIV2-*mdrT* | This study |
| Δ*mdrMTAC* mutant (pLIV2-*mdrA*) | Δ*mdrMTAC* pLIV2-*mdrA* | This study |
| Δ*mdrMTAC* mutant (pLIV2-*mdrC*) | Δ*mdrMTAC* pLIV2-*mdrC* | This study |
| WT (pLIV2-*pdeA*) | pLIV2-*pdeA* | This study |
| Δ*mdrMTAC* mutant (pLIV2-*pdeA*) | Δ*mdrM* Δ*mdrT* Δ*mdrA* Δ*mdrC* pLIV2-*pdeA* | This study |
| WT (pLIV2-*dacA*) | pLIV2-*dacA* | This study |
| Δ*mdrMTAC* mutant (pLIV2-*dacA*) | Δ*mdrM* Δ*mdrT* Δ*mdrA* Δ*mdrC* pLIV2-*dacA* | This study |
| Δ*marR* mutant | Δ*marR* (LMRG_01348.6 [lmo1618]) | 7 |
| WT (pPL2-P$_{mdrC}$*lacZ*) | 10403S harboring integrative plasmid pPL2 containing *lacZ* gene under *mdrC* promoter | This study |
| Δ*mdrMTA* mutant (pPL2-P$_{mdrC}$*lacZ*) | 10403S Δ*mdrMTA* harboring integrative plasmid pPL2 containing *lacZ* gene under *mdrC* promoter | This study |
| *Escherichia coli* | | |
| DH12s | ϕ80d*lacZ*ΔM15 *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *araD139* Δ(*ara leu*)*7697* Δ(*lacX74*) *galU galK rpsL* (Str$^r$) *nupG recA1*/[F′ *proAB*$^+$ *lacI*$^q$*Z*ΔM15] | |
| XL-1b | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*$^q$*Z*ΔM15 Tn*10* (Tet$^r$)] | Stratagene |
| SM-10 | Conjugation donor; F$^-$ *thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44* (Muc$^+$) λ$^-$ [RP4-2(Tc::Mu)] Km$^r$ Tra$^+$ | 54 |

a reduced type I interferon response, we examined if this phenotype influenced virulence in mice. Young female C57BL/6 mice were injected intravenously with Δ*mdrMTAC* mutant or WT bacteria (total of 10 mice for each strain). Seventy-two hours postinfection (p.i.), a log decrease in the number of bacterial CFU was observed in the livers and spleens of the Δ*mdrMTAC* strain-infected mice compared to that observed in mice infected with WT bacteria (Fig. 3C). These results further support the premise that the MdrM-like transporters are active *in vivo* and play a role in promoting *L. monocytogenes* virulence.

**MdrM-like transporters are expressed and required during cell wall stress.** It was still not clear to us what was activating the MdrM-like transporters during intracellular growth. We reasoned that some physiological process was inducing the transporters' function *in vivo*. Therefore, we searched for physiological conditions that might require the MdrM-like transporters' activity. To this end, the transcription profile of four transporter genes was measured using RT-qPCR analysis under a set of *in vitro* conditions that mimic different physiological environments. In these studies, we focused on the four transporters MdrM, MdrT, MdrA, and MdrC (MTAC transporters), as together these were responsible for most of the IFN-β induction during infection of macrophages. The conditions involved cell wall stresses (growth in the presence of vancomycin or penicillin G), acidic pH (near 5), oxidative stress (using hydrogen peroxide), and growth in minimal media, all representing conditions that likely exist within the phagosome compartment. In addition, growth in the presence of glucose-1P and charcoal was tested as these conditions are known to activate PrfA, the master regulator of *L. monocytogenes* virulence (31). Lincomycin and R6G served as positive controls for MDR substrates known to induce expression of MDR transporters (7, 8). The *hly* gene (encoding LLO toxin) was used as a reporter for the induction of virulence genes.

To summarize the RT-qPCR results, the data are presented as a heat map (Fig. 4A). In general, we observed that while the transporter genes were largely induced by lincomycin and R6G, they were downregulated under all conditions that triggered *hly* expression (Fig. 4A). These findings indicate that the transporters

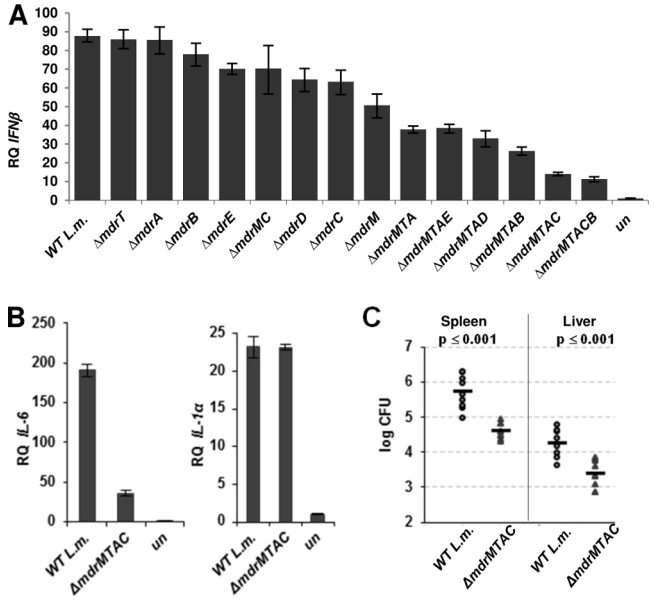


**FIG 3** MdrM-like transporters are responsible for most of the type I interferon induction upon *L. monocytogenes* infection. (A) RT-qPCR analysis of IFN-β transcriptional levels in BMD macrophages infected with WT *L. monocytogenes* or MDR deletion mutants. (B) RT-qPCR analysis of IL-6 and IL-1α induction in BMD macrophages infected with WT *L. monocytogenes* in comparison to the Δ*mdrMTAC* mutant. Transcription levels are represented as relative quantity (RQ) compared to uninfected cells (un). The data in panels A and B represent at least 3 biological repeats. Error bars represent 95% confidence intervals (as described in Materials and Methods). (C) Intravenous infection of C57BL/6 mice with WT *L. monocytogenes* and the Δ*mdrMTAC* mutant. Bacterial CFU were numerated at 72 h p.i. from livers and spleens taken from 10 infected mice for each strain. The results are means of 2 independent experiments in which 5 mice were infected in each group. Horizontal bars represent the mean. The *P* value was calculated using Student's *t* test.

and the virulence genes are differentially regulated, suggesting that different signals may induce the MDR transporters *in vivo*. Notably, among the tested conditions, growth in the presence of vancomycin and penicillin G resulted in upregulation of most transporter genes, with the exception of *mdrC*, which was downregulated under these conditions (Fig. 4A and B). Vancomycin and penicillin G are both inhibitors of peptidoglycan (PGN) synthesis and operate extracellularly on the expanding PGN polymer by blocking PGN peptides from cross-linking. Vancomycin is a branched tricyclic glycosylated heptapeptide that targets the terminal D-alanyl-D-alanine moiety of PGN peptides, while penicillin G, a β-lactam antibiotic, is a structural analogue of D-alanyl-D-alanine that inhibits transpeptidation.

To examine more directly if the transporters play a role in the response to vancomycin and penicillin G treatments, transporter mutants and WT bacteria were grown in the presence of sublethal concentrations of these drugs (1 μg $ml^{-1}$ of vancomycin and 0.08 μg $ml^{-1}$ of penicillin G). Interestingly, the quadruple Δ*mdrMTAC* mutant was more susceptible to these drugs, whereas WT, Δ*mdrMTA*, and Δ*mdrC* bacteria grew similarly (Fig. 4C). The MICs of penicillin and vancomycin were determined as 0.08 μg $ml^{-1}$ and 1.5 μg $ml^{-1}$ for Δ*mdrMTAC* bacteria and 0.15 μg $ml^{-1}$ and 2 μg $ml^{-1}$ for WT bacteria, respectively. To assess the contribution of MdrC in the background of the Δ*mdrMTA* mutant, we analyzed its transcription level in Δ*mdrMTA* and WT bacteria using a translational fusion of the *lacZ* reporter gene to the *mdrC* promoter region. Notably, we observed that the transcription level of the *mdrC* gene in the Δ*mdrMTA* mutant was upregulated (3-fold) in comparison to its level in WT bacteria (see Fig. S2 in the supplemental material). These observations suggest that the Mdr transporters exhibit redundant activities and that they are, respectively, regulated in order to compensate for each other. Indeed, introduction in *trans* of a copy of each of the transporter genes into the Δ*mdrMTAC* mutant (using plasmid pLIV2 with the IPTG-inducible promoter) only partially complemented its growth ability under vancomycin treatment (see Fig. S3 in the supplemental material). Overall, these results indicate that the Mdr transporters play active and overlapping roles in the response to vancomycin and penicillin G. Importantly, since vancomycin and penicillin G operate extracellularly on the PGN polymer and are not expected to cross the cytoplasmic membrane to the bacterial cytosol (particularly vancomycin), a simple drug efflux mechanism cannot explain the increased sensitivity of the Δ*mdrMTAC* mutant to these drugs. In our subsequent studies, we used only vancomycin, since active efflux has never been reported as a mechanism of resistance for this drug.

**The Δ*mdrMTAC* mutant responds aberrantly to cell wall stress.** To gain insight into the functional role of the MDR transporters during vancomycin stress, we examined bacteria using transmission electron microscopy (TEM). Changes in cell wall morphology are expected upon inhibition of PGN synthesis, and therefore, we anticipated visual differences between Δ*mdrMTAC* and WT bacteria upon vancomycin treatment. Inspection of TEM images confirmed that when grown without vancomycin treatment, both bacterial strains look similar (Fig. 5A). However, 2 h subsequent to addition of a sublethal concentration of vancomycin, WT bacteria were surrounded by massive extracellular material that was largely lacking around the Δ*mdrMTAC* mutant (Fig. 5A). Further analysis of TEM sections revealed that, without vancomycin treatment, WT bacteria and the Δ*mdrMTAC* mutant exhibit a similar defined cell wall structure with an average thickness of 21 nm ($P = 0.1$, based on 50 measurements) (Fig. 5B). In contrast, under vancomycin treatment, WT bacteria and the Δ*mdrMTAC* mutant were found to be significantly different ($P < 0.001$, based on 100 measurements). Under these conditions, a large population of the WT bacteria exhibited a very thick cell wall layer of up to 63 nm (Fig. 5B). The cell wall thickness ranged from 25 to 63 nm (35-nm average), as opposed to the range of 18 to 26 nm (24-nm average) associated with Δ*mdrMTAC* mutant bacteria (Fig. 5B). Cell wall thickening in response to vancomycin stress was reported previously for *S. aureus* bacteria, which were shown to respond to vancomycin treatment by accumulating peptidoglycan to facilitate vancomycin trapping (drug titration) (32, 33). In accordance with this mechanism, WT *L. monocytogenes* cells that were observed to undergo cell wall thickening in the presence of vancomycin grew better than the Δ*mdrMTAC* mutant cells (Fig. 5C and 4C). These observations suggest that the Δ*mdrMTAC* mutant might be defective in the ability to produce peptidoglycan upon vancomycin stress.

To further corroborate this hypothesis, we performed $^{14}$C-*N*-acetylglucosamine incorporation measurements to assess the rate of PGN synthesis during growth with and without vancomycin treatment. Bacteria were grown to the mid-log phase before vancomycin and $^{14}$C-*N*-acetylglucosamine were added to the cultures. In this experiment, an even lower concentration of vanco-

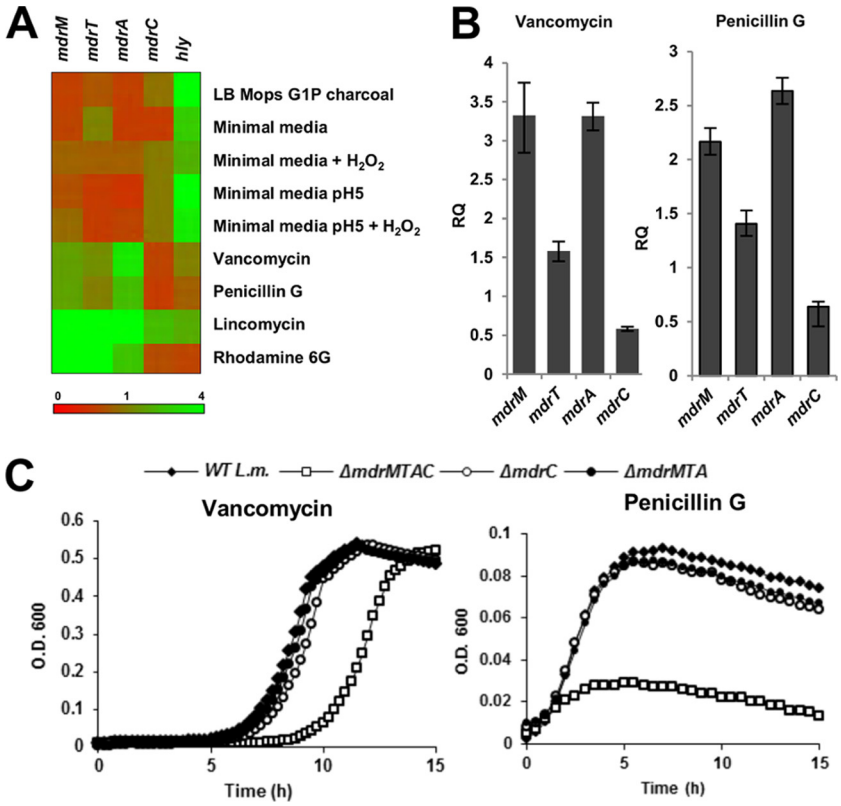


**FIG 4** MdrM-like transporters are required for cell wall stress responses. (A) RT-qPCR analysis, presented as a heat map, of transcriptional levels of the *mdrM*, *mdrT*, *mdrA*, *mdrC*, and *hly* genes in WT *L. monocytogenes* grown under different *in vitro* conditions: BHI supplemented with vancomycin (1 μg ml$^{-1}$), penicillin G (0.08 μg ml$^{-1}$), lincomycin (3 μg ml-$^{1}$), or rhodamine 6G (50 μM); LB with MOPS (morpholinepropanesulfonic acid) and G1P charcoal; or minimal medium at pH 7, minimal medium at pH 5, or minimal medium with $H_2O_2$ at pH 5. Transcription levels are represented as relative quantity (RQ) compared to the levels in BHI or in minimal medium at pH 7, respectively. Data represent 3 biological repeats. In all samples, statistical deviation did not exceed 15% with a 95% confidence level. (B) RT-qPCR analysis of transcriptional levels of the *mdrM*, *mdrT*, *mdrA*, and *mdrC* genes in WT *L. monocytogenes* grown in BHI supplemented with vancomycin (1 μg ml$^{-1}$) or penicillin G (0.08 μg ml$^{-1}$) for 2 h. Transcription levels (RQ) are relative to the levels in BHI without drugs. The data represent 3 biological repeats. Error bars represent 95% confidence intervals. (C) Growth curve of WT *L. monocytogenes* and MDR mutants in BHI medium supplemented with vancomycin (1 μg ml$^{-1}$) or penicillin G (0.08 μg ml$^{-1}$). The data represent 3 biological repeats. The experiment was performed in a 96-well format in a Synergy HT Biotek plate reader. Representative growth curves are shown. Error bars representing the standard deviation of the triplicate sample are hidden by the symbols. Growth curve experiments were performed in at least 3 independent biological repeats.

mycin was used (0.8 μg ml$^{-1}$) to reduce the growth inhibition of the Δ*mdrMTAC* mutant (see Fig. S4 in the supplemental material). Every 30 min, samples of bacteria were filtrated, washed, and counted for $^{14}$C labeling. In line with our hypothesis, this analysis demonstrated that, upon vancomycin treatment, the rate of *N*-acetylglucosamine incorporation was significantly slower in the Δ*mdrMTAC* mutant than in the WT bacteria (Fig. 5D). These differences in PGN synthesis were detectable even before inhibition of Δ*mdrMTAC* cell growth by vancomycin was observed (see Fig. S4). Taken together, these results suggest that the MTAC transporters play a role in enhancing PGN synthesis upon vancomycin stress.

**MdrM-like transporters are not involved in PGN′s assembly, structure, or immunostimulatory activity.** To examine if the MTAC transporters are involved in PGN polymer assembly, we compared the PGN structures of the Δ*mdrMTAC* mutant and WT bacteria with and without vancomycin treatment. Cell wall was extracted from bacteria and digested with mutanolysin to generate a soluble mixture of PGN muropeptides. Muropeptides were separated by reversed-phase high-pressure liquid chromatography (RP-HPLC) and analyzed. Notably, no difference was observed in the muropeptide profiles or their cross-linking levels between WT and Δ*mdrMTAC* bacteria (as evident from the detected peaks in the HPLC profile in Fig. 6A and B). Of note, a moderate difference of ~30% in the peptidoglycan N-acetylation level was observed, with the Δ*mdrMTAC* mutant displaying more N-acetylated muropeptides than WT bacteria (Fig. 6A and C). Next, the immunostimulatory properties of cell walls derived from each strain were compared. Cell wall extracts from Δ*mdrMTAC* and WT bacteria grown with and without vancomycin were added to BMD macrophages, and interleukin-6 (IL-6) induction was measured using RT-qPCR analysis. IL-6 was chosen as it is induced by both type I interferon and proinflammatory responses. As shown in Fig. 6D, all extracts activated the same level of IL-6, indicating that the immunostimulatory potency of the Δ*mdrMTAC* cell wall is unchanged, in accordance with the overall similar structure of Δ*mdrMTAC* PGN to wild-type PGN. Taken together, these results indicate that the MTAC transporters are probably not involved in PGN polymer assembly but play a role in the regulation of PGN synthesis during vancomycin stress.

**c-di-AMP and Mdr-MTAC transporters regulate the response to cell wall stress.** It was recently shown that *L. monocyto-*

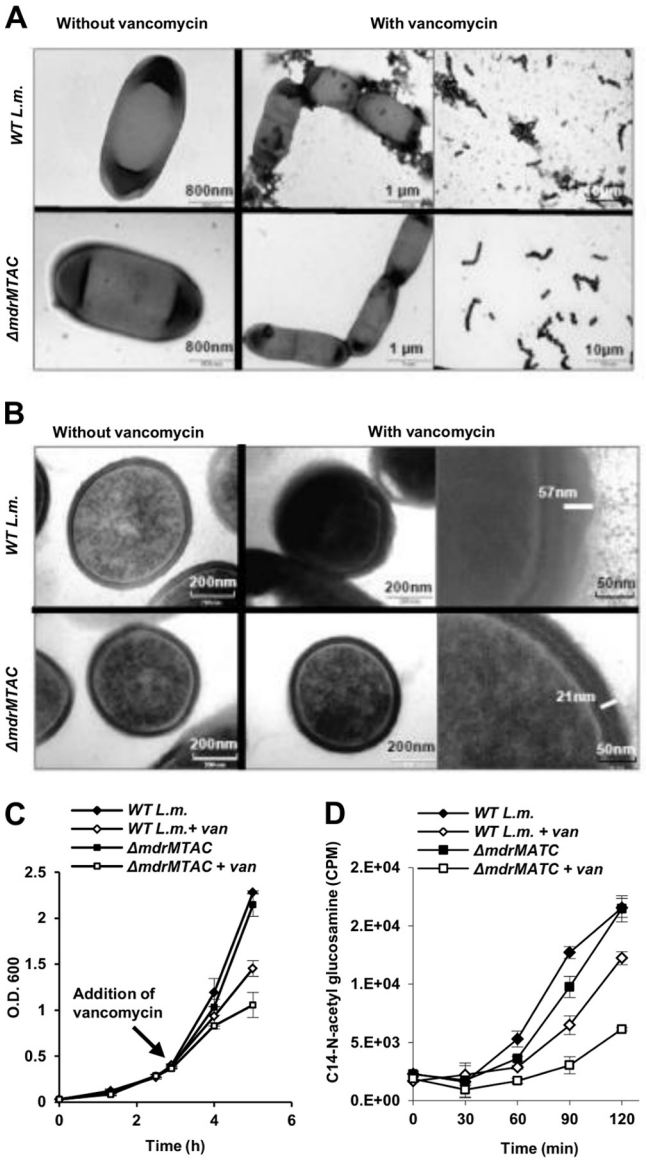


**FIG 5** The Δ*mdrMTAC* mutant does not overproduce peptidoglycan in response to vancomycin stress. (A) Negative-staining TEM images of WT *L. monocytogenes* and the Δ*mdrMTAC* mutant grown with and without vancomycin treatment. Growth curves are presented in panel C. (B) TEM section images of WT *L. monocytogenes* and the Δ*mdrMTAC* mutant grown with and without vancomycin treatment. Growth curves are presented in panel C. Images in panels A and B represent 3 independent biological repeats; a total of 35 frames were taken for each strain and condition. (C) Growth curves of bacteria taken for TEM analysis (A and B). Vancomycin (1 μg ml$^{-1}$) was added at an $OD_{600}$ of 0.4, and bacteria were harvested 2 h later for fixation and staining. The data are means of 3 independent biological experiments. Error bars represent standard deviations. (D) Analysis of the peptidoglycan synthesis rate in WT *L. monocytogenes* and the Δ*mdrMTAC* mutant, grown with and without vancomycin (0.8 μg ml$^{-1}$) treatment, as measured by incorporation of $^{14}$C-*N*-acetylglucosamine. Vancomycin and *N*-acetylglucosamine were added during bacterial growth at an $OD_{600}$ of 0.4, and incorporation of $^{14}$C-*N*-acetylglucosamine was analyzed at 30-min intervals upon addition (see the growth curves presented in Fig. S4 in the supplemental material). Error bars represent standard deviations of triplicate samples. The data represent 2 biological repeats.

*genes* MdrM and MdrT facilitate c-di-AMP secretion (11, 12). We speculated that the MDR transporters might regulate the enhancement of PGN synthesis in response to vancomycin by controlling c-di-AMP secretion. To provide evidence for a physiological association between c-di-AMP and the MTAC transporters, we first asked whether c-di-AMP is produced under vancomycin stress. Since c-di-AMP production was shown to correlate with *dacA* (diadenylate cyclase) gene expression (18), *dacA* transcription levels were measured upon various vancomycin treatments using RT-qPCR analysis. WT bacteria and Δ*mdrMTAC* mutant bacteria grown in the presence of a sublethal concentration of vancomycin (1 μg ml$^{-1}$) or exposed briefly to high concentrations of vancomycin (20 μg ml$^{-1}$ for 10 min) were both observed to induce *dacA* gene transcription in comparison to nontreated bacteria. WT bacteria induced 1.5- and 4.5-fold higher transcription levels of *dacA* under the respective conditions, while the Δ*mdrMTAC* mutant induced 3- and 9-fold higher levels, respectively (Fig. 7A). To evaluate the influence of c-di-AMP production on *L. monocytogenes* growth under vancomycin stress, the *dacA* gene and *pdeA* (phosphodiesterase) gene were overexpressed in Δ*mdrMTAC* and WT bacteria from an IPTG-inducible promoter (using the integrative plasmid pLIV2) (Table 2). The overexpression of *dacA* and *pdeA* genes had only a moderate effect on the growth of WT bacteria in the presence of a sublethal concentration of vancomycin (1 μg ml$^{-1}$) (Fig. 7B); however, the growth of the Δ*mdrMTAC* mutant under the same condition was seemingly altered upon overexpression of these genes (Fig. 7C). Overexpression of the *dacA* gene suppressed the growth inhibition of the Δ*mdrMTAC* mutant by vancomycin, whereas overexpression of *pdeA* rendered the mutant more susceptible to this drug (Fig. 7C). In accordance with these observations, we further detected an increase in cell wall thickness (by 17%) in the Δ*mdrMTAC* mutant overexpressing *dacA* using TEM section analysis ($P < 0.001$, based on 30 measurements). Notably, the effect of *dacA* and *pdeA* overexpression on the growth of WT bacteria was still moderate, even when the concentration of vancomycin was increased to further inhibit growth (see Fig. S5A and B in the supplemental material). Furthermore, since overexpression of the *dacA* and *pdeA* genes had no effect on the growth of WT and Δ*mdrMTAC* bacteria in the absence of vancomycin stress (see Fig. S5C and D), we surmise that c-di-AMP and the MTAC transporters are both involved in the response to the vancomycin stress.

Finally, we studied whether exogenous addition of purified c-di-AMP to bacterial cultures could recapitulate the phenotype observed with the *dacA*-overexpressing bacteria. To this end, WT and Δ*mdrMTAC* bacteria were grown in the presence of vancomycin (1 μg ml$^{-1}$), and purified c-di-AMP or c-di-GMP was added to the bacterial cultures. Notably, both Δ*mdrMTAC* and WT bacteria exhibited a shorter lag phase when c-di-AMP was added, whereas c-di-GMP addition had no effect (Fig. 7D and E). Similarly to the *dacA* and *pdeA* overexpression experiments, c-di-AMP or c-di-GMP addition had no effect on the growth of the bacteria in the absence of vancomycin stress (see Fig. S5E and F in the supplemental material). Together, these results indicate a possible role for c-di-AMP in the response to vancomycin and, furthermore, hint at a physiological association between DacA, PdeA, and the MDR transporters in mediating a response to this stress.

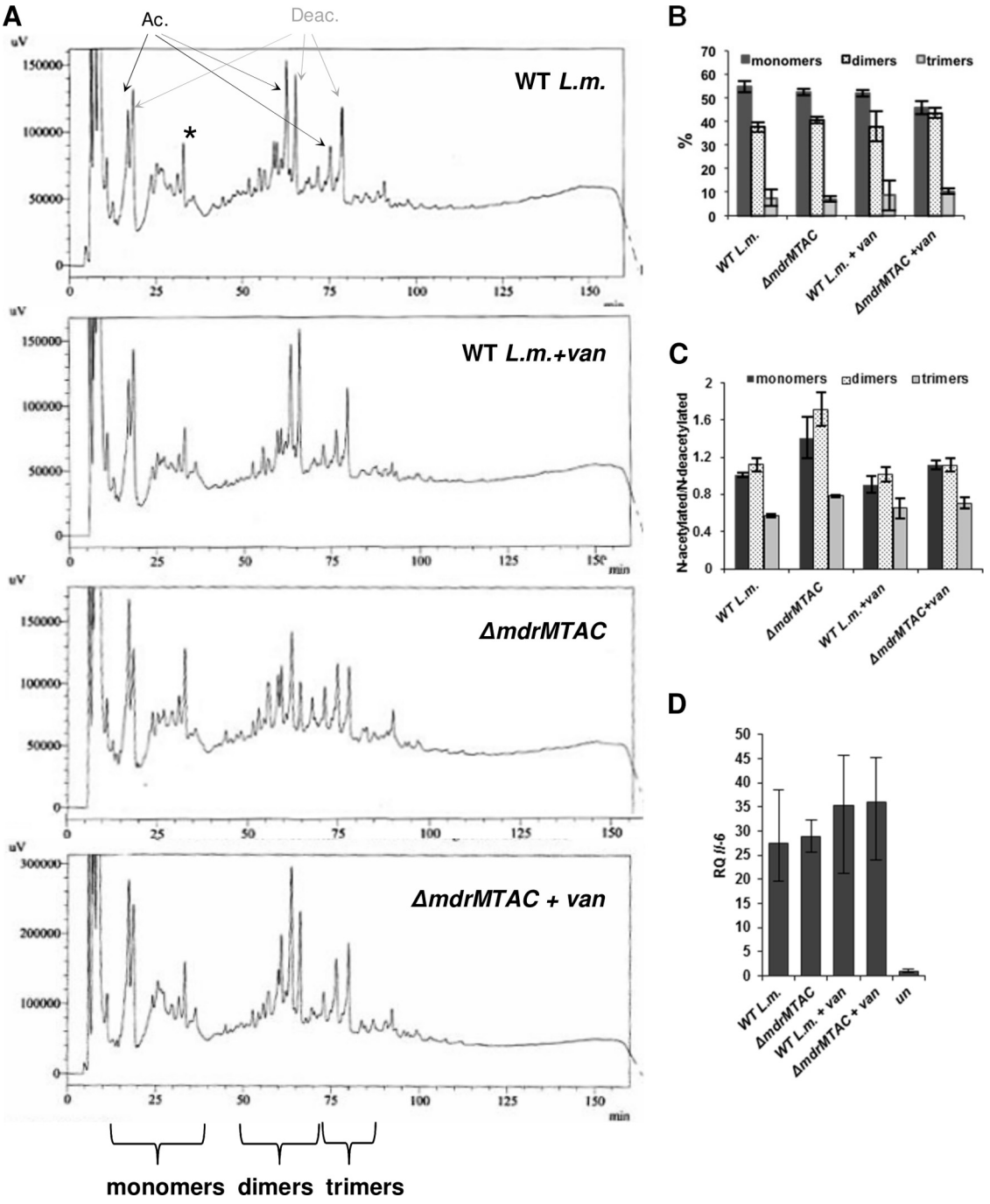


**FIG 6** MdrM-like transporters do not impact peptidoglycan composition. (A) HPLC analysis of cell-wall-derived muropeptides of WT *L. monocytogenes* and Δ*mdrMTAC* bacteria, grown with and without vancomycin (van) treatment. HPLC peaks associated with N-acetylated muropeptides are marked as "Ac," and peaks associated with N-deacetylated muropeptides are marked as "Deac." The peak highlighted with an asterisk corresponds to O-acetylated monomer. The data represent 5 biological repeats. (B) Degree of muropeptide cross-linking, presented as the percentage of monomer, dimer, and trimer muropeptides (based on the integrated area of the corresponding peaks in the HPLC analysis) of WT *L. monocytogenes* or the Δ*mdrMTAC* mutant grown with and without vancomycin treatment. The data are means of 5 biological repeats. Error bars represents standard deviations of the independent samples. (C) The degree of peptidoglycan N-acetylation in muropeptides derived from WT *L. monocytogenes* or the Δ*mdrMTAC* mutant grown with and without vancomycin treatment, presented as the ratio of the integrated area of peaks corresponding to N-acetylated/N-deacetylated muropeptides for monomeric, dimeric, and trimeric units in the HPLC analysis. The data are means of 5 biological repeats. Error bars represent standard deviations of the independent samples. (D) RT-qPCR analysis of IL-6 transcriptional levels in BMD macrophages treated for 6 h with cell wall extracts derived from WT *L. monocytogenes* and Δ*mdrMTAC* bacteria, grown with and without vancomycin (van; 1 $\mu$g ml$^{-1}$). Transcription levels are represented as relative quantity (RQ) compared to levels in untreated cells (un). The data represent 3 biological repeats. Error bars represent 95% confidence intervals (as described in Materials and Methods).

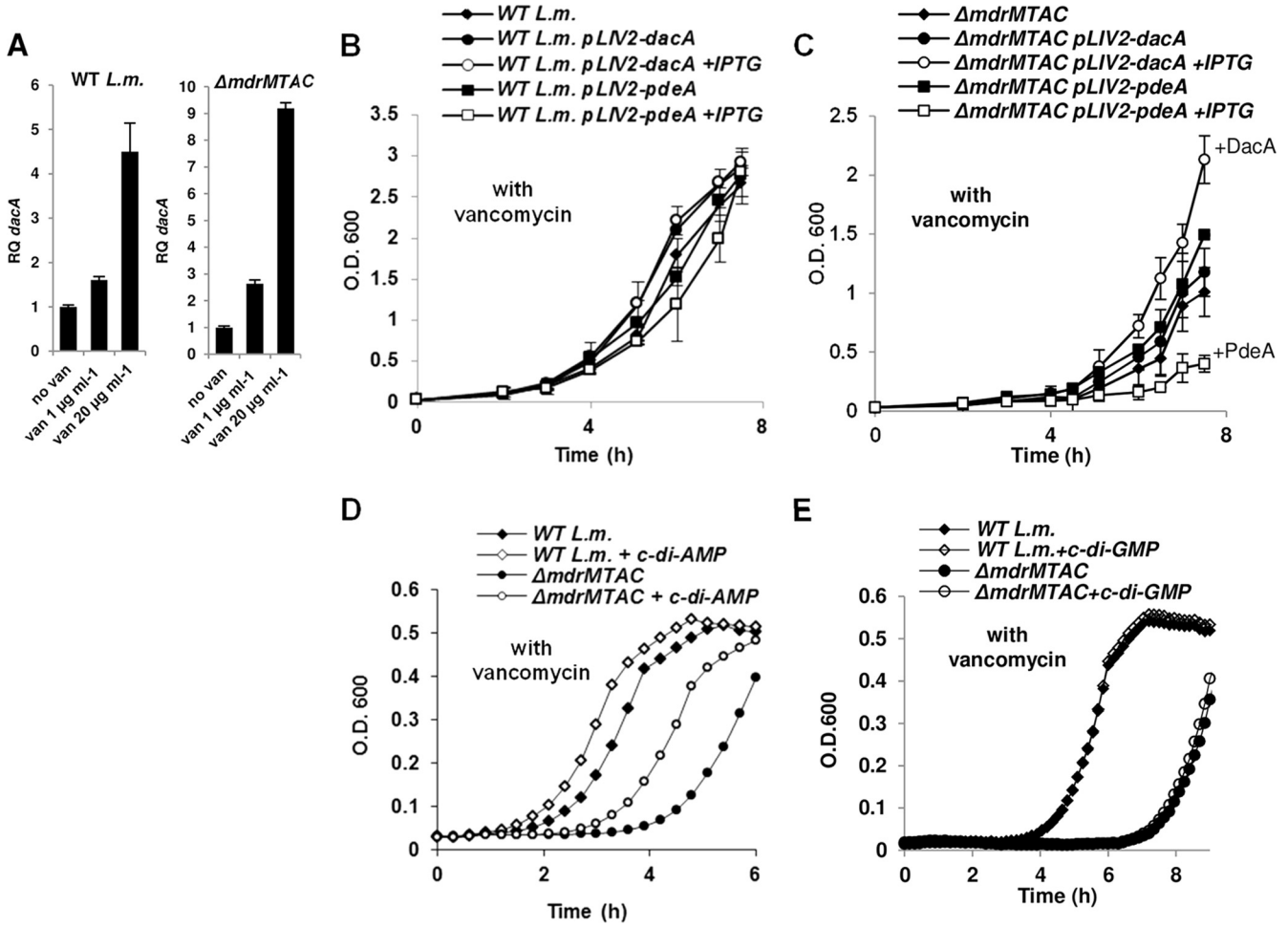


**FIG 7** Mdr-MTAC transporters and c-di-AMP are functionally associated in the response to cell wall stress. (A) RT-qPCR analysis of transcriptional levels of the *dacA* gene in WT *L. monocytogenes* and Δ*mdrMTAC* bacteria grown in BHI or supplemented with vancomycin (van; 1 μg ml$^{-1}$ for 2 h or 20 μg ml$^{-1}$ for 10 min). Transcription levels are represented as relative quantity (RQ) compared to the levels in BHI alone. The data represent 3 biological independent repeats. Error bars represent 95% confidence intervals (as described in Materials and Methods). (B) Growth curves of WT *L. monocytogenes* strains harboring the pLIV2 plasmid with an IPTG-inducible promoter, expressing *dacA* or *pdeA* genes in BHI supplemented with vancomycin (1 μg ml$^{-1}$) with or without IPTG. The experiment was performed in flasks. The data are means of 3 independent biological experiments. Error bars represent standard deviations. (C) Growth curves of Δ*mdrMTAC* bacteria harboring the pLIV2 plasmid with an IPTG-inducible promoter expressing the *dacA* or *pdeA* genes in BHI supplemented with vancomycin with or without IPTG. The experiment was performed in flasks. The data are means of 3 independent biological experiments. Error bars represent standard deviations. (D) Growth curve of WT *L. monocytogenes* or the Δ*mdrMTAC* mutant in BHI supplemented with vancomycin with and without addition of 3 μg ml$^{-1}$ c-di-AMP. This experiment was performed in 3 biological repeats in a 96-well format in a Synergy HT Biotek plate reader. Growth curves from one representative experiment are shown. Error bars representing standard deviations of a triplicate sample are hidden by the symbols. (E) Growth curve of WT *L. monocytogenes* or the Δ*mdrMTAC* mutant in BHI supplemented with vancomycin with and without addition of 3 μg ml$^{-1}$ c-di-GMP. The experiment was performed in 3 biological repeats in a 96-well format in a Synergy HT Biotek plate reader. Growth curves from one representative experiment are shown. Error bars representing standard deviations of a triplicate sample are hidden by the symbols.

## DISCUSSION

In this study, we investigated the physiological functions of *L. monocytogenes* MDR transporters and c-di-AMP with the goal of understanding better the mechanism that leads infected macrophages to induce IFN-β. We discovered that not only MdrM but also a set of related putative MDR transporters together mediate IFN-β induction in infected macrophages. A screen for physiological conditions that demand the activity of these transporters revealed that they are involved in regulation of peptidoglycan synthesis in response to cell wall stress. The phenotypes observed upon disturbance of peptidoglycan synthesis pointed out a possible link between the function of MDR transporters and c-di-AMP production. Although we demonstrate a physiological association between MDRs and c-di-AMP in this study, it remains unclear if the MDRs play a role in regulation of c-di-AMP intracellular concentration (via c-di-AMP efflux) or translocate c-di-AMP outside the bacterial cell to bind an extracellular target or targets, or alternatively, if the MDRs simply bind c-di-AMP to regulate various cellular processes, as was recently shown with potassium ion transporters (34). Further studies are required to discriminate between these various possibilities.

Very little is known about the physiological functions of MDR transporters in bacteria, aside from drug efflux (35, 36). Bacterial genomes contain a large arsenal of MDR transporter genes, many of which are highly similar and possess overlapping substrate specificity (9, 37, 38). The generally accepted hypothesis is that

MDR transporters evolved independently during evolution to cope with a wide array of physiological substrates, allowing bacteria to survive diverse ecological niches (39). Studies in the last decade have revealed diverse functions of MDR transporters that are not related to drug efflux (36, 40). For example, involvement of MDR transporters in lipid transport, pH homeostasis (41), virulence (42, 43), and quorum sensing (44) has been documented, with fatty acids, ions, bile salts, antibacterial peptides, and precursors of quorum-sensing molecules suggested as natural substrates (45–51). Despite the advance in our understanding of MDR transporters, *in silico* prediction of MDR transporter functions remains challenging. The initial discovery that the *L. monocytogenes* MdrM transporter modulates the type I interferon response raised many questions regarding the mechanism of this function. Primarily, it was not clear whether this function evolved specifically to subvert the host immune system or, alternatively, represents the inadvertent consequence of a more basic bacterial physiological function. Later the report that c-di-AMP is secreted by MdrM and leads to IFN-β induction (11) further highlighted the need to better understand the natural biological process that involves MdrM and c-di-AMP. Here we show that four homologous *L. monocytogenes* MDR transporters not only are triggering IFN-β induction during infection but also are novel players in the response to cell wall stress. Remarkably, the Δ*mdrMTAC* mutant lacking the four transporters failed to trigger enhanced production of PGN in response to vancomycin stress, a mechanism known to facilitate vancomycin resistance (32). In light of the observation that the growth defect of the Δ*mdrMTAC* mutant under vancomycin stress was not accompanied by production of aberrant PGN, we propose that the MDR transporters play a regulatory role in PGN synthesis rather than a direct role in PGN biogenesis. The findings that increased production of c-di-AMP (via *dacA* overexpression) or addition of purified c-di-AMP rescued the growth inhibition of the Δ*mdrMTAC* mutant under vancomycin stress support that c-di-AMP is involved downstream of the transporters' function. However, the possibility that c-di-AMP affects the observed phenotypes indirectly cannot be excluded (14, 34, 52, 53).

Cyclic di-AMP has been recently demonstrated to play a role in the response to cell wall stresses in both *S. aureus* and *Bacillus subtilis*. Two independent genetic screens highlighted c-di-AMP as a key regulator of lipoteichoic acid (LTA)- and PGN-related stress. In *S. aureus*, a genetic screen designed to identify suppressor mutations that restore the ability of LTA-deficient mutants to grow identified mutations in *pde* gene and revealed a global role for c-di-AMP in cell wall regulation (15). In *B. subtilis*, a search for genes that facilitate intrinsic resistance to β-lactam antibiotics also identified a *pde* gene and c-di-AMP as key players (16). Notably, increased production of c-di-AMP resulted in enhanced resistance of *B. subtilis* bacteria to β-lactams (16), a phenotype that is similar to our observation that overexpression of the *dacA* gene suppressed the growth defect of the Δ*mdrMTAC* mutant under vancomycin stress. Interestingly, a connection between c-di-AMP, MDRs, and cell wall stress was pointed out previously in a study that examined global genomic changes in a β-lactam-resistant methicillin-resistant *S. aureus* (MRSA) strain. In this study, mutations were identified in three genes encoding a penicillin binding protein, PDE, and an MDR transporter (54). While it was not clear to the authors how these genes associate, we now surmise that c-di-AMP and MDR transporters function together to regulate cell wall synthesis. Notably, this transporter's function might be part of an intrinsic mechanism of bacteria to overcome cell wall stress. Such a function is most likely required during infection of mammalian cells, where bacteria are subjected to various host antimicrobial mechanisms targeting the bacterial cell wall. In this regard, the *in vivo* virulence defect of the Δ*mdrMTAC* mutant may be linked to the transporters' function in resistance to cell wall stress; however, since the transporter's function poses additional effects on the innate immune system, the observed *in vivo* phenotype is complex to decipher.

Finally, in light of the growing notion that bacteria use diverse cyclic nucleotide (or dinucleotide) messengers (such as c-di-GMP and c-di-AMP) to regulate basic processes (e.g., adhesion, biofilm, virulence, DNA damage, and cell wall stress) (55, 56), it is not surprising that the innate immune system developed mechanisms to detect these vital molecules. The study presented here further supports the premise that c-di-AMP is a vital molecule that exhibits multiple functions during *L. monocytogenes* infection of mammalian cells.

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