

Cyclic di-AMP Is Critical for *Listeria monocytogenes* Growth, Cell Wall Homeostasis, and Establishment of Infection

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ABSTRACT *Listeria monocytogenes* infection leads to robust induction of an innate immune signaling pathway referred to as the cytosolic surveillance pathway (CSP), characterized by expression of beta interferon (IFN- β) and coregulated genes. We previously identified the IFN- β stimulatory ligand as secreted cyclic di-AMP. Synthesis of c-di-AMP in *L. monocytogenes* is catalyzed by the diadenylate cyclase DacA, and multidrug resistance transporters are necessary for secretion. To identify additional bacterial factors involved in *L. monocytogenes* detection by the CSP, we performed a forward genetic screen for mutants that induced altered levels of IFN- β . One mutant that stimulated elevated levels of IFN- β harbored a transposon insertion in the gene *lmo0052*. *Lmo0052*, renamed here PdeA, has homology to a cyclic di-AMP phosphodiesterase, GdpP (formerly YybT), of *Bacillus subtilis* and is able to degrade c-di-AMP to the linear dinucleotide pApA. Reduction of c-di-AMP levels by conditional depletion of the diadenylate cyclase DacA or overexpression of PdeA led to marked decreases in growth rates, both *in vitro* and in macrophages. Additionally, mutants with altered levels of c-di-AMP had different susceptibilities to peptidoglycan-targeting antibiotics, suggesting that the molecule may be involved in regulating cell wall homeostasis. During intracellular infection, increases in c-di-AMP production led to hyperactivation of the CSP. Conditional depletion of *dacA* also led to increased IFN- β expression and a concomitant increase in host cell pyroptosis, a result of increased bacteriolysis and subsequent bacterial DNA release. These data suggest that c-di-AMP coordinates bacterial growth, cell wall stability, and responses to stress and plays a crucial role in the establishment of bacterial infection.

IMPORTANCE *Listeria monocytogenes* is a Gram-positive intracellular pathogen and the causative agent of the food-borne illness listeriosis. Upon infection, *L. monocytogenes* stimulates expression of IFN- β and coregulated genes dependent upon host detection of a secreted bacterial signaling nucleotide, c-di-AMP. Using a forward genetic screen for mutants that induced high levels of host IFN- β expression, we identified a c-di-AMP phosphodiesterase, PdeA, that degrades c-di-AMP. Here we characterize *L. monocytogenes* mutants that express enhanced or diminished levels of c-di-AMP. Decreased c-di-AMP levels by conditional depletion of the diadenylate cyclase (DacA) or overexpression of PdeA attenuated bacterial growth and led to bacteriolysis, suggesting that its production is essential for viability and may regulate cell wall metabolism. Mutants lacking PdeA had a distinct transcriptional profile, which may provide insight into additional roles for the molecule. This work demonstrates that c-di-AMP is a critical signaling molecule required for bacterial replication, cell wall stability, and pathogenicity.

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Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen that is the causative agent of the food-borne illness listeriosis. Both the infection cycle and a murine model of infection have been well characterized, making *L. monocytogenes* an attractive model organism for studying basic aspects of infection and immunity (1). During infection, *L. monocytogenes* rapidly escapes from a phagocytic vacuole into the host cytosol, where bacterial replication and spread to neighboring cells occur.

Several innate immune pathways are stimulated upon cytosolic entry of *L. monocytogenes* (2). One pathway is mediated by inflammasome activation, resulting in pyroptosis, an inflamma-

tory host cell death. Levels of pyroptosis induced by *L. monocytogenes* are low compared to those induced during infection with other intracellular pathogens and are a result of detection of bacterial DNA released during infrequent bacteriolysis (3). Cytosolic *L. monocytogenes* also triggers the expression of interferon-beta (IFN- β) and coregulated genes (4) via a pathway termed the cytosolic surveillance pathway (CSP), which is dependent on the recently described cytosolic pattern recognition receptors STING and DDX41 (5–7). However, the role of c-di-AMP and CSP activation in *L. monocytogenes* pathogenesis is unclear, as STING-deficient mice are as susceptible to infection as wild-type mice (5).

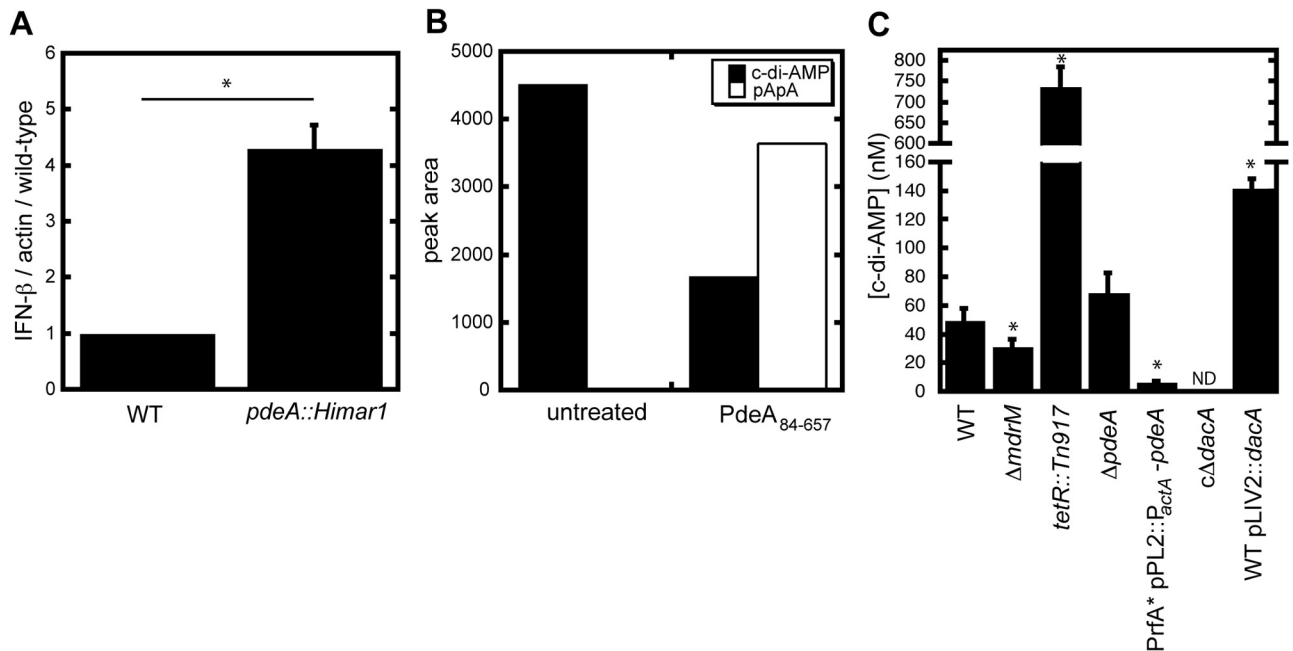


FIG 1 PdeA is a c-di-AMP phosphodiesterase. (A) qRT-PCR analysis of IFN- β expression in bone marrow-derived macrophages infected with wild-type *L. monocytogenes* (WT) or *pdeA::Himar1* mutants. (B) HPLC detection of c-di-AMP (black bars) and pApA (white bars) following incubation of c-di-AMP in the absence or presence of recombinant PdeA₈₄₋₆₅₇ protein. HPLC data are representative of more than three independent experiments. (C) Detection of secreted c-di-AMP from *L. monocytogenes* mutants. Samples from the indicated strains were taken during stationary phase, and c-di-AMP secretion was evaluated by HPLC following chloroacetaldehyde derivatization of the samples. Values are averages of three independent measurements of samples from the indicated strains. Error bars represent standard deviations from the mean. *, $P < 0.05$ compared to the wild type, using Student's *t* test. ND, none detected.

L. monocytogenes multidrug resistance transporters (MDRs) of the major facilitator superfamily (MFS) secrete c-di-AMP. (c-di-AMP) during infection, resulting in the induction of IFN- β (8, 9). Given the structural similarity to c-di-GMP, a well-characterized bacterial-specific second messenger nucleotide, we hypothesize that c-di-AMP serves an analogous but distinct signaling role in *L. monocytogenes*.

In this study, we identified an *L. monocytogenes* phosphodiesterase, PdeA, that mediates c-di-AMP degradation. By genetically modifying the expression of PdeA and the diadenylate cyclase DacA, we investigated the role of c-di-AMP in *L. monocytogenes* physiology and pathogenesis. These data support a role for c-di-AMP as a critical signaling molecule affecting a number of fundamental processes in *L. monocytogenes*, with significant consequences on bacterial growth and the ability to establish infection.

RESULTS

c-di-AMP metabolism in *L. monocytogenes*. Previous studies from our laboratory identified *L. monocytogenes* DacA and MDRs as regulators of the CSP-stimulatory molecule c-di-AMP (8, 9). To identify novel determinants of CSP activation and hence c-di-AMP signaling, a forward genetic screen for mutants that affected host cell death and IFN- β production was conducted (3). Infection with a mutant that had a disruption of *lmo0052* resulted in elevated levels of IFN- β compared to infection with wild-type *L. monocytogenes* (Fig. 1A), with no changes in the induction of host cell death (data not shown). Sequence analysis identified *lmo0052* as a homolog of *Bacillus subtilis* GdpP (previously YyBT), which was shown to have c-di-AMP phosphodiesterase activity (10). *lmo0052*, renamed here PdeA, shared 50% identity

and 74% similarity with *B. subtilis* GdpP and the same multidomain structure of GdpP, with two N-terminal transmembrane spanning helices, a PAS domain, a GGDEF domain, and C-terminal DHH and DHH-associated domains. Recombinant protein lacking the transmembrane domains (PdeA₈₄₋₆₅₇) catalyzed conversion of c-di-AMP to the linear dinucleotide pApA (Fig. 1B), as assessed by high-performance liquid chromatography (HPLC). Truncated constructs lacking the PAS and GGDEF domains localized this activity to the DHH/DHHA1 domains (data not shown), consistent with GdpP activity (10).

A series of *L. monocytogenes* strains were constructed to alter c-di-AMP levels. We constructed an in-frame deletion of *pdeA* and a strain that overexpressed the protein. To achieve overexpression, *pdeA* was placed downstream of the *actA* promoter (P_{actA}) in the PrfA* G145S strain (11). Multiple attempts to delete *dacA* were unsuccessful. Therefore, we generated a conditional depletion (*c* Δ *dacA*) strain with a single IPTG-inducible copy of the gene (12, 13).

To define the effects of altered expression of DacA, PdeA, and MDRs on c-di-AMP metabolism, secreted c-di-AMP was measured after bacterial growth in chemically defined minimal medium. Ectopic overexpression of the bacterial MDR, MdrT, by transposon disruption of the negative regulator *tetR* (*tetR::Tn917*) and overexpression of DacA both resulted in increased levels of c-di-AMP in the culture supernatant as previously reported (8, 9). In contrast, *c* Δ *dacA* and overexpression of *pdeA* resulted in decreased levels of c-di-AMP in culture supernatants, indicating that both DacA and PdeA regulate c-di-AMP levels (Fig. 1C). Deletion of *pdeA* was predicted to result in increased levels of intracellular c-di-AMP, as previously reported for *B. subtilis* and *Staphylococcus*

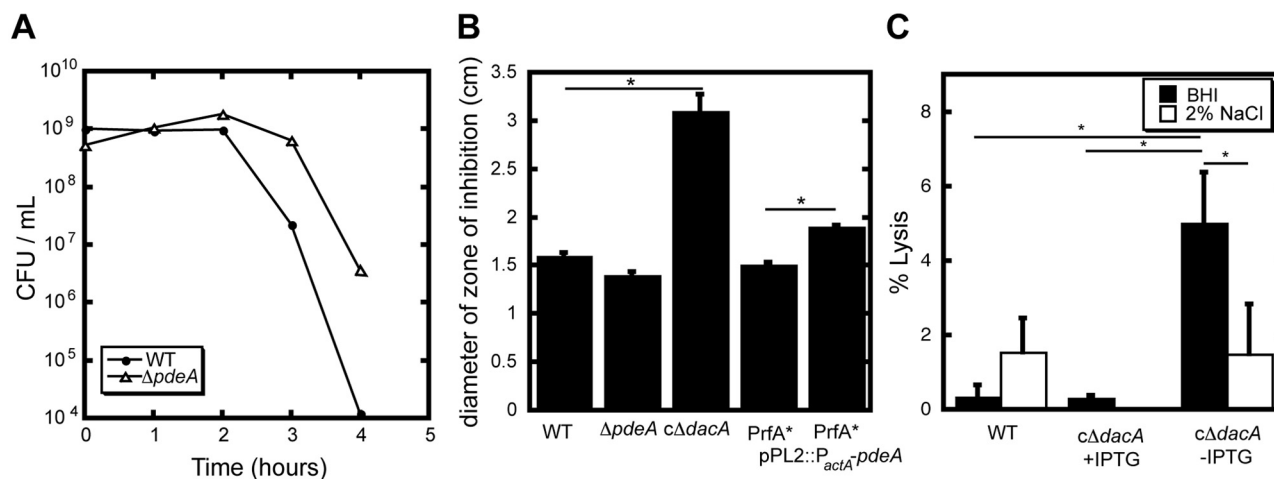


FIG 2 c-di-AMP affects acid resistance and cell wall stability. (A) Acid resistance of wild-type *L. monocytogenes* and $\Delta pdeA$ mutants was assessed by quantifying survival in BHI medium adjusted to pH 2.5. The plot is representative of three independent experiments. (B) Antibiotic susceptibility of indicated mutants as measured as zone of bacterial growth inhibition surrounding filter disks impregnated with cefuroxime (5 μ g). Values are averages from more than three independent experiments, and error bars show the standard deviation from those means. (C) *In vitro* bacteriolysis was measured as the amount of β -galactosidase activity in broth supernatant during logarithmic growth in BHI medium or BHI supplemented with 2% NaCl from the indicated strains. *, $P < 0.05$, using Student's *t* test. NM, not measured.

aureus mutants lacking GdpP (14, 15). However, PdeA deficiency did not significantly affect secreted levels under these culture conditions (Fig. 1C). This discrepancy suggests that although $\Delta pdeA$ mutants likely have high levels of intracellular c-di-AMP, there may be another factor that affects secretion in broth culture.

Deletion of *pdeA* affects bacterial response to acid stress. The effects of c-di-GMP on gene expression are well documented (16–18). Therefore, we hypothesized that altered levels of c-di-AMP would have effects on RNA levels as well. To test this, we performed whole-genome microarray analysis comparing $\Delta pdeA$ mutants to wild-type bacteria (see Table S1 in the supplemental material). Two of the most highly (3- to 8-fold) upregulated genes in PdeA-deficient mutants were those encoding the glutamate decarboxylase, *gadD2* (*lmo2362*), and a glutamate transporter, *gadT2* (*lmo2363*). Together, GadD2 and GadT2 comprise one of two glutamate decarboxylase systems in *L. monocytogenes* and are associated with bacterial survival under severe acid stress (19, 20). We tested the effects of PdeA deficiency in acidified culture medium (pH 2.5). After 4 h, nearly 2 logs more *L. monocytogenes* $\Delta pdeA$ mutants than wild-type bacteria were recovered (Fig. 2A), consistent with a role for PdeA in acid resistance and previous reports in *B. subtilis* and *L. lactis* (10, 21).

Deletion of *pdeA* leads to increased expression of resuscitation-promoting factors. Microarray transcriptional analysis of $\Delta pdeA$ mutants also identified increased expression of *lmo2522* (see Table S1 in the supplemental material). Although the increase was not significant by statistical analysis of microarrays (SAM), we also observed upregulation of *lmo0186* that was confirmed by quantitative reverse transcription-PCR (qRT-PCR). *Lmo2522* and *Lmo0186* are homologous to the *B. subtilis* resuscitation-promoting factors/stationary-phase survival (Rpf/Sps) family members YocH and YabE (22, 23). Rpf, the first Rpf/Sps family member, was initially identified in *Micrococcus luteus* as an autolysin required for stimulating growth of dormant bacteria (24). We hypothesized that the absence of these two proteins may affect *L. monocytogenes* replication in broth culture or an *in vivo*

mouse model of infection. However, deletion of *lmo2522* and *lmo0186*, either independently or in combination, did not affect the *L. monocytogenes* growth rate or the time necessary to exit stationary phase (see Fig. S1A in the supplemental material). Similarly, no effect on the ability of *L. monocytogenes* to replicate *in vivo* was observed in the spleens and livers of mice infected with $\Delta lmo2522$, $\Delta lmo0186$, or $\Delta lmo2522 \Delta lmo0186$ mutants (see Fig. S1B in the supplemental material). Nevertheless, the effects of c-di-AMP levels on Rpf/Sps transcription established a potential link between PdeA, c-di-AMP levels, and bacterial cell wall metabolism and growth control.

Intracellular c-di-AMP levels affect bacterial growth rate. Growth rates of *L. monocytogenes* mutants with altered *DacA* expression were measured. *DacA* overexpression did not affect *in vitro* bacterial replication, while conditional *dacA* depletion resulted in a doubling time of 84 min, compared to 44 min for wild-type *L. monocytogenes* (Table 1; also, see Fig. S2A in the supplemental material). In the presence of IPTG, the $c\Delta dacA$ strain grew similarly to wild-type *L. monocytogenes*. To confirm that synthesis of c-di-AMP was critical for growth and not another feature of the *DacA* protein, two experiments were performed. First, we characterized growth of strains with altered PdeA expression. De-

TABLE 1 c-di-AMP levels affect bacterial *in vitro* doubling time

<i>L. monocytogenes</i> genotype or description	Doubling time (min) ^a
WT	44
$\Delta pdeA$	44
$c\Delta dacA$ +IPTG	44
$c\Delta dacA$ +IPTG	84
$c\Delta dacA$ pLIV::disA +IPTG	48
$c\Delta dacA$ pLIV::disA -IPTG	113
PrfA* G145S	41
PrfA* G145SpPL2::PactA-pdeA	56

^a Bacterial doubling times were calculated during logarithmic growth of each strain. Values are representative of at least two independent experiments.

letion of *pdeA* did not affect *in vitro* bacterial replication (Table 1; also, see Fig. S2B). Overexpression of PdeA led to a doubling time of 56 min, compared to 41 min for the parent strain (Table 1; also, see Fig. S2C). Second, growth was characterized in a Δ *dacA* mutant containing an inducible copy of *B. subtilis* *disA* (*c* Δ *dacA* pLIV2:*disA*) (see Fig. S1C), of which *L. monocytogenes* does not have a homologue. In addition to the DAC domain, *B. subtilis* DisA contains a DNA-binding domain that leads to chromosomal localization, in contrast to the membrane-anchored DacA. This orthogonal approach to generating c-di-AMP in *L. monocytogenes* rescued the growth defect of the *dacA* conditional depletion (Table 1; also, see Fig. S2D). To address the possibility that extracellular c-di-AMP regulates the growth of *L. monocytogenes*, we supplemented *c* Δ *dacA* mutant cultures with 0 to 10 μ M c-di-AMP, far exceeding the secreted levels observed in broth culture. No change in growth rate was observed, suggesting that intracellular c-di-AMP levels synthesized by DacA regulate bacterial replication (data not shown).

c-di-AMP affects bacterial cell wall stability. To characterize cell wall stability in *L. monocytogenes*, susceptibility to peptidoglycan-targeting antibiotics was measured by antibiotic disk diffusion assays. *L. monocytogenes* Δ *pdeA* mutants were slightly more resistant to cefuroxime, whereas the DacA conditional depletion and the *pdeA*-overexpressing strains had increased susceptibility to cefuroxime (Fig. 2B) as well as penicillin and ampicillin (see Fig. S3A in the supplemental material). A similar trend was observed by measuring MICs of cefuroxime for each of the *L. monocytogenes* strains (see Fig. S3B in the supplemental material). These data indicated a role for c-di-AMP in regulating cell wall structure, consistent with similar findings reported for *S. aureus* and *B. subtilis* (15, 25).

We next assessed bacterial lysis during growth in broth by constitutively expressing β -galactosidase in wild-type *L. monocytogenes* and *c* Δ *dacA* mutants and measuring β -galactosidase release into the culture medium by hydrolysis of *ortho*-nitrophenyl- β -galactoside (ONPG) (26). The *L. monocytogenes* *c* Δ *dacA* mutant released nearly 10-fold more β -galactosidase than wild-type bacteria (Fig. 2C). A weakened cell wall may lead to osmotic stress, resulting in bacteriolysis. Therefore, *c* Δ *dacA* and wild-type strains were grown in medium containing betaine (100 μ M) or carnitine (100 μ M), neither of which affected lysis or growth rate (data not shown). However, increased broth hypertonicity (2% NaCl) significantly reduced lysis of *c* Δ *dacA* mutants (Fig. 2C), whereas wild-type bacteria exhibited a small but significant increase in bacteriolysis. Together these observations support a direct correlation between c-di-AMP production and cell wall stability in *L. monocytogenes*.

c-di-AMP is required for *L. monocytogenes* virulence. To investigate the role of c-di-AMP during *L. monocytogenes* infection, we examined bacterial intracellular replication in primary bone marrow-derived macrophages. The intracellular growth kinetics of the *pdeA*-deficient mutants were indistinguishable from those of wild-type bacteria, whereas strains that overexpressed *pdeA* upon cytosolic entry were unable to replicate to the same level (Fig. 3A). Conditional depletion of DacA resulted in attenuated bacterial replication (Fig. 3B) with comparable bacterial growth rates (70 min between 2 and 5 h postinfection) to those observed in broth culture (Table 1). While *c* Δ *dacA* bacteria were able to reach the same final density as wild-type *L. monocytogenes* in broth culture, significantly lower levels of viable bacteria were recovered

from infected macrophages at 5 and 8 h postinfection. To further characterize the role of c-di-AMP levels on *L. monocytogenes* virulence in a mouse model of infection, mice were infected intravenously with each of the mutants. The bacterial loads recovered from both livers and spleens were indistinguishable between Δ *pdeA* and wild-type-*L. monocytogenes*-infected mice (Fig. 3C). In contrast, nearly 4 logs fewer *c* Δ *dacA* bacteria were recovered 48 h postinfection compared to recovery from mice infected with wild-type bacteria or to mice maintained on water containing IPTG throughout infection with *c* Δ *dacA* mutants (Fig. 3D). Together, these results support the idea that c-di-AMP is required for *in vitro* replication in broth culture, intracellular growth in macrophages, and establishment of infection *in vivo*.

Altered c-di-AMP metabolism affects host innate immune activation. To characterize the host response to bacterial mutants, we infected murine bone marrow-derived macrophages and quantified transcription of IFN- β as a measure of CSP activation. PdeA-deficient bacteria stimulated 5-fold more IFN- β than wild-type *L. monocytogenes* (Fig. 4A). Given that Δ *pdeA* mutants do not exhibit increased host cell death or DNA delivery, which would be indicative of increased lysis during infection, these observations suggest increased c-di-AMP secretion in the host cell cytosol. Surprisingly, infection with *c* Δ *dacA* mutants also resulted in a significant increase in IFN- β (Fig. 4A), despite lower levels of bacterial growth in macrophages and decreased c-di-AMP secretion *in vitro*. Given that *c* Δ *dacA* mutants lyse in broth culture, we hypothesized that released bacterial DNA may result in CSP stimulation. Cytosolic DNA also activates the AIM2 inflammasome, leading to caspase-1 cleavage and pyroptosis, an inflammatory host cell death (27–29). Infection with *c* Δ *dacA* mutants led to a 2-fold increase in macrophage cell death relative to levels observed with wild-type *L. monocytogenes* or *c* Δ *dacA* mutants in the presence of IPTG (Fig. 4B). Infection of macrophages deficient for the DNA inflammasome receptor, AIM2, resulted in reduced cell death following infection with *c* Δ *dacA* mutants, indicating that bacterial DNA released by lysing bacteria stimulated pyroptosis (see Fig. S4 in the supplemental material). To quantify the extent of bacteriolysis during intracellular infection, we utilized a bacterial reporter plasmid encoding firefly luciferase under control of a cytomegalovirus promoter (3). A 4.5-fold increase and 0.5-fold decrease in luciferase expression were observed in macrophages infected with *c* Δ *dacA* and Δ *pdeA* mutants, respectively (Fig. 4C), indicating that the *c* Δ *dacA* mutants are more susceptible to bacteriolysis while Δ *pdeA* mutants may be more resistant. Together, these findings establish a correlation between c-di-AMP levels and cell wall stability during infection (Fig. 4C).

DISCUSSION

The results of this study extend the repertoire of proteins involved in c-di-AMP metabolism in *L. monocytogenes* by identifying a phosphodiesterase that regulates levels of the signaling nucleotide secreted during infection (Fig. 5). In addition, we provide evidence that c-di-AMP is required for optimal *L. monocytogenes* growth and contributes to bacterial cell wall stability. Our data show that c-di-AMP production is necessary for *L. monocytogenes* to establish and maintain growth within the host. Finally, we demonstrate that perturbation of c-di-AMP levels, both enhanced and diminished, directly affects detection by host innate immune pattern recognition receptors.

Bacterial second messengers, such as cAMP, c-di-GMP, and

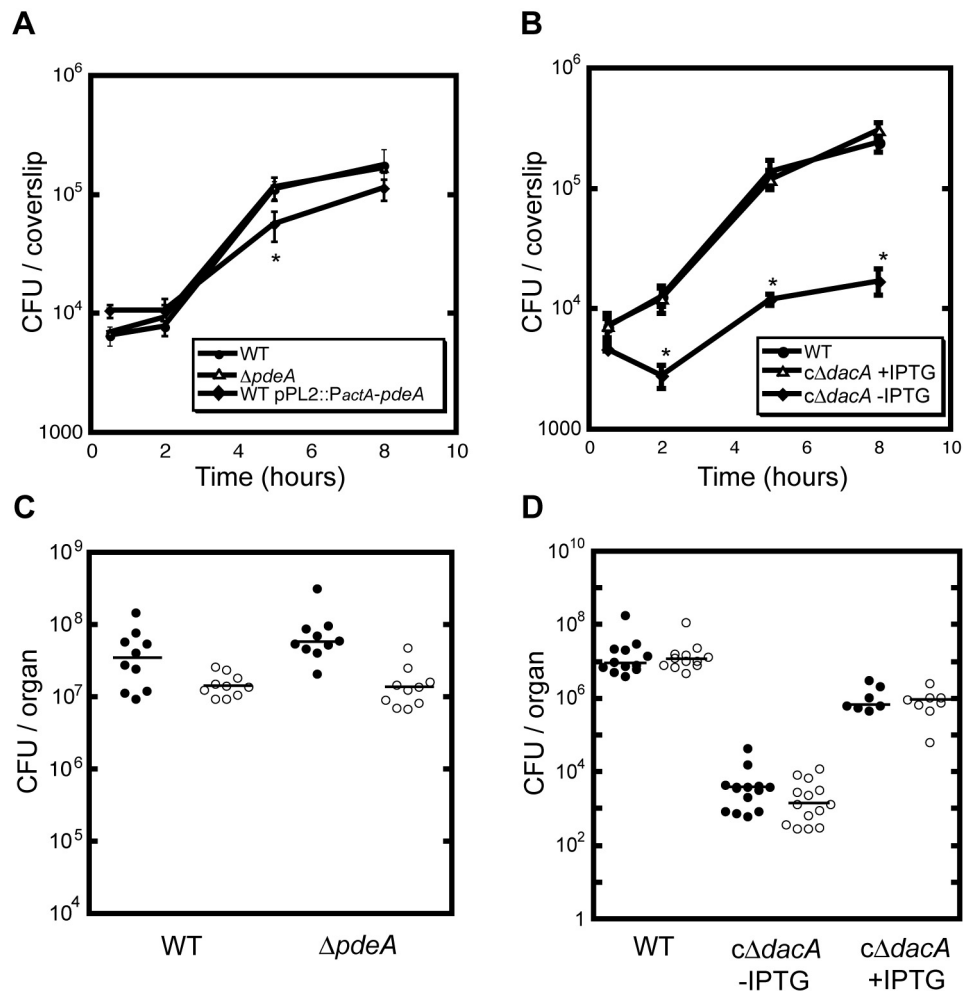


FIG 3 C-di-AMP levels affect *L. monocytogenes* ability to establish infection. (A) Representative intracellular growth of wild-type *L. monocytogenes*, the $\Delta pdeA$ mutant, or *pdeA*-overexpressing mutants in bone marrow-derived macrophages. (B) Representative intracellular growth of wild-type *L. monocytogenes* or *dacA*-conditional mutants in the presence or absence of IPTG in bone marrow-derived macrophages. Error bars represent the standard deviation from the mean of triplicates within the representative experiment. (C) C57BL/6 mice were infected with 1×10^5 CFU of the wild type or $\Delta pdeA$ mutants. Organs were harvested at 48 hpi, and bacterial burden per spleen (closed circles) or liver (open circles) was enumerated. (D) C57BL/6 mice were infected with 1×10^5 CFU of the wild type or *dacA*-conditional mutants in the absence or presence of 10 mM IPTG in the drinking water. Organs were harvested at 48 hpi, and bacterial burden per spleen (closed circles) or liver (open circles) was enumerated. Median values are presented as horizontal lines. *, $P < 0.05$.

ppGpp, function as signaling molecules whose levels are rapidly altered by generation and degradation of the signal in response to environmental cues. In *L. monocytogenes*, DacA mediates production and PdeA regulates degradation of the second messenger c-di-AMP. Although only a single report regarding regulation of a diadenylate cyclase exists (30), *in vitro* biochemical characterization showed that GdpP/PdeA proteins have multiple functions and regulatory inputs, including heme and nitric oxide binding (31), ATPase activity (10), and ppGpp binding (10). We speculate that GdpP/PdeA homologues function as a hub to control levels of c-di-AMP by integrating environmental and bacterium-derived signals.

Regulation of PdeA activity within the context of infection of macrophages or mouse models remains largely unexplored. Our data show that *L. monocytogenes* $\Delta pdeA$ mutants did not exhibit increased c-di-AMP secretion during growth in broth despite increased IFN- β production by infected host cells. These observations are consistent with *in vivo* regulation of c-di-AMP levels by

PdeA during intracellular infection, perhaps in response to host-derived signals. Given the increased resistance of $\Delta pdeA$ mutants to acid stress, phagosomal acidification may be a signal for the bacteria to alter c-di-AMP levels during infection. Indeed, pretreatment of BMDM with bafilomycin A, which prevents vacuolar acidification, led to lower levels of IFN- β induced by both $\Delta pdeA$ mutants and wild-type bacteria (C. E. Witte and D. A. Portnoy, unpublished data). These observations do not address why c-di-AMP levels remain unchanged during broth growth. We hypothesize that feedback inhibition or the presence of another phosphodiesterase regulates nucleotide levels during growth in broth. Nonsporulating members of the *Actinobacteria*, including *Mycobacteria*, *Gordonia*, and *Rhodococcus*, have predicted DAC domain-containing proteins but do not have GdpP/PdeA homologs, suggesting the existence of alternative mechanisms to regulate c-di-AMP levels.

A variety of stresses and cellular cues likely modulate DacA and PdeA activity, controlling c-di-AMP levels and altering pheno-

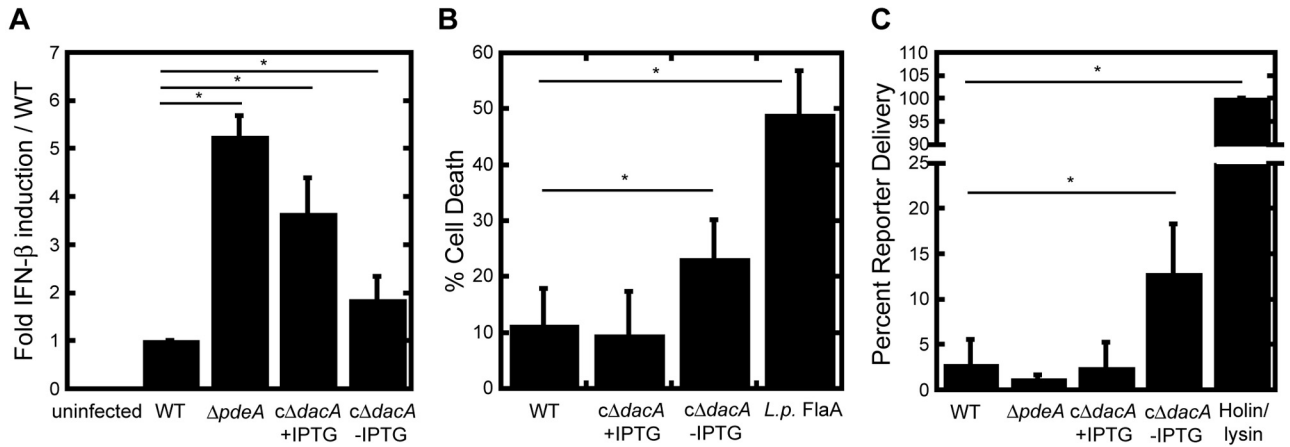


FIG 4 *L. monocytogenes* mutants with altered c-di-AMP have increased detection by host innate immune pathways. (A) IFN- β induction was measured by qRT-PCR following a 4-h infection of bone marrow-derived macrophages with the indicated strains. (B) Cell death was measured by lactate dehydrogenase release following a 6-h infection of bone marrow-derived macrophages with the indicated strains. “*L. p. FlaA*” indicates *L. monocytogenes* expressing the Nlr4-stimulatory flagellin from *Legionella pneumophila* (4). Data are relative to a 100% Triton-induced lysis control and are the averages of at least three independent experiments. (C) Bacteriolysis was measured by delivery and expression of luciferase from the reporter plasmid pBHE573 following 6-h infection of IFNAR $^{-/-}$ bone marrow-derived macrophages. Bacteriolysis data are relative to infection with the holin/lysin-expressing strain (5) and are the averages from at least three independent experiments. Error bars represent the standard deviations from the mean. *, $P < 0.05$, using Student’s *t* test.

typic outputs. Our results establish two fundamental processes regulated by c-di-AMP: peptidoglycan homeostasis and bacterial growth. Bacteria with a reduced capacity to generate c-di-AMP lysed spontaneously during growth in rich medium and in macrophages, while the sensitivity of *L. monocytogenes* mutants to cell wall-targeting antibiotics was inversely correlated with c-di-AMP levels. We propose that lysis under conditions of low levels of c-di-AMP is due to a weakened cell wall that cannot withstand the

high internal pressure, estimated to be between 15 and 25 atm in Gram-positive bacteria (32). Similar to $c\Delta dacA$ mutants, spheroplasts and various peptidoglycan-defective mutants are susceptible to lysis and are osmotically stabilized by the addition of sucrose or NaCl (33). A similar role for c-di-AMP in maintaining cell wall homeostasis was reported in *B. subtilis*, where inactivation of *B. subtilis* GdpP rescued bacterial resistance to cefuroxime and overexpression increased antibiotic susceptibility (25). Addition-

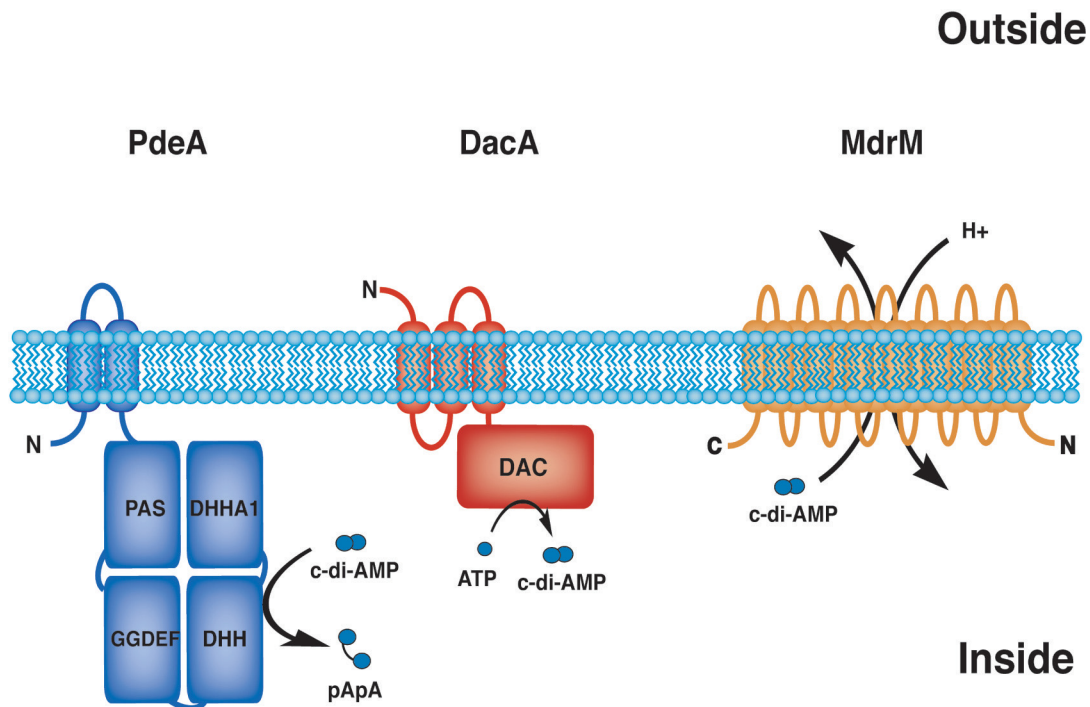


FIG 5 Model of c-di-AMP metabolism in *L. monocytogenes*, levels of which are regulated by the diadenylate cyclase DacA, which synthesizes the molecule, the phosphodiesterase PdeA, which is capable of degradation, and MDR transporters that secrete c-di-AMP from the cell.

ally, mutation of the *S. aureus* GdpP resulted in increased peptidoglycan cross-linking and rescued the severe growth defects of mutants lacking lipoteichoic acids (15). Although transcriptional analysis identified altered expression of cell wall-metabolizing proteins (see Table S1 in the supplemental material), the mechanism by which cell wall stability is affected is currently unresolved. Also of note, *glmM*, which encodes glucosamine mutase, is adjacent to *dacA* on the *L. monocytogenes* chromosome. GlmM generates the precursor for all peptidoglycan biosynthesis, glucosamine-1-phosphate. This genetic organization is highly conserved among bacteria that contain DacA homologs and is consistent with a link between c-di-AMP and amino sugar metabolism.

The second major phenotype we report is a direct correlation between c-di-AMP production and *L. monocytogenes* growth rate. Because the $c\Delta dacA$ strains may still contain low levels of DacA, we cannot definitively define DacA as essential to bacterial replication. However, a recent study with *B. subtilis* as well as previous high-throughput screens defined DacA homologs in *Mycoplasma* and *Streptococcus* spp. as equally important for bacterial growth (30, 34–36). How and why c-di-AMP is crucial for bacterial growth remains an intense area of research. PdeA is encoded within an operon containing a ribosomal protein and replicative DNA helicase, both predicted to be essential. This genomic architecture is conserved among many microbes with PdeA homologs and suggests a link between c-di-AMP metabolism and bacterial translation and replication. Furthermore, we identified altered expression of two peptidoglycan-metabolizing proteins referred to as resuscitation-promoting factors or stationary-phase survival factors (Rpf/Sps). In *Micrococcus luteus* these proteins promote growth of dormant bacteria and in *B. subtilis* stimulate emergence from stationary-phase growth (24). Therefore, the correlation between c-di-AMP and Rpf/Sps expression levels is consistent with a progrowth effect of c-di-AMP, although deletion of *lmo2522* and/or *lmo0186* did not result in a growth defect under the conditions tested here.

c-di-AMP, c-di-GMP, and the newly discovered cyclic GMP-AMP (cGAMP) (37) represent small bacterial secondary messengers that fulfill the criteria of pathogen-associated molecular patterns (PAMPs) in that they are essential and/or conserved microbial molecules recognized by host innate immune receptors. *L. monocytogenes* mutants that lack c-di-AMP still trigger the host IFN- β response likely due to DNA released during bacteriolysis. The host response to cyclic dinucleotides and DNA requires the host protein STING. Interestingly, it was recently discovered that the STING-dependent response to DNA involves a host-derived cyclic dinucleotide, cGAMP, as a secondary messenger (38). Perhaps it is more than coincidental that host innate immune recognition of *L. monocytogenes* involves both bacterial and host-derived cyclic dinucleotide secondary messengers.

MATERIALS AND METHODS

Bacterial strains, growth medium, and cell culture. *L. monocytogenes* strains generated for this study are listed in Table S2 in the supplemental material. For *in vitro* growth, acid stress susceptibility, and gene expression experiments, wild-type 10403 *S. L. monocytogenes* and isogenic strains were grown in brain heart infusion (BHI) medium at either 30°C or 37°C overnight to stationary phase, except for c-di-AMP secretion assays, in which *L. monocytogenes* strains were grown in a defined minimal medium (39). All *Escherichia coli* strains used for in-frame gene construction, complementation, and protein expression were grown on Luria-Bertani (LB)

medium. Antibiotics were used at the following final concentrations: streptomycin, 200 $\mu\text{g/ml}$; chloramphenicol, 7.5 to 20 $\mu\text{g/ml}$; kanamycin, 30 $\mu\text{g/ml}$.

Bone marrow-derived macrophages were prepared as previously described (40). IFNAR^{-/-} mice were previously described (41). Immortalized C57BL/6 macrophages were a gift from Russell Vance. AIM2 short hairpin RNA knockdown vectors were a gift from Katherine Fitzgerald. Lentivirus-mediated knockdowns were performed using the pLKO.1 system as previously described (42).

Deletions of *pdeA* (*lmo0052*), *lmo2522*, and *lmo0186* were performed by allelic exchange (43). Overexpression of *pdeA* was achieved by placing the gene downstream of the P_{*actA*}. Briefly, P_{*actA*} and *pdeA* were amplified and then combined by SOE PCR. The product was digested and ligated into a digested pPL2-derivative vector (44). The plasmid was transformed into *E. coli* SM10. Following confirmation by sequencing at the UC Berkeley, Sequencing Facility, it was conjugated into wild-type *L. monocytogenes* or PrfA* G145S mutants.

Construction of DacA conditional depletion strain. The IPTG-inducible pLIV2:*dacA* plasmid (8) was modified to contain a tetracycline resistance cassette (Tet^r). Tet^r was amplified from the Tn917 transposon, digested with NdeI, and ligated into the pLIV2:*dacA* plasmid to replace the chloramphenicol resistance cassette. The resulting plasmid, pLIV2-Tc:*dacA*, was subsequently integrated into the *L. monocytogenes* genome as described previously. Deletion of the chromosomal copy of *dacA* was accomplished by allelic exchange (43).

The $c\Delta dacA::pLIV2-disA$ strain DP-L5937 was constructed by replacing the IPTG-inducible *dacA* of the $c\Delta dacA$ strain with an IPTG-inducible *B. subtilis* *disA* gene. Briefly, *disA* was amplified from an asporogenic derivative of *B. subtilis* strain ZB307 (45) and then digested and ligated into an IPTG-inducible vector, pLIV2 (12). The resulting plasmid, pLIV2-*disA*, was integrated into the 10403s chromosome and transduced using the listeriophage U153 (46) into the $c\Delta dacA$ strain, replacing the IPTG-inducible *dacA* element (Tet^r) with an IPTG-inducible *disA* (Cm^r), and was confirmed by PCR and antibiotic resistance.

The $c\Delta dacA$ strain was maintained in solid and liquid media containing 500 μM IPTG. Prior to infection or broth growth analysis, bacteria were passaged overnight in BHI liquid culture in the absence of IPTG to deplete DacA levels. Infection and growth in nutritive medium were subsequently analyzed in the presence or absence of 500 μM IPTG.

PdeA protein expression and purification. Using *L. monocytogenes* genomic DNA as a template, *pdeA* fragments encoding the PAS-GGDEF-DHH/DHHA1 (PdeA₈₄₋₆₅₇) truncation were amplified. This fragment was ligated into the T7 expression vector pET-28(b), introducing a C-terminal 6 \times His tag. The plasmids were transformed into Rosetta *E. coli* BL21, and protein expression was analyzed as previously described (10). The bacteria were pelleted by centrifugation, resuspended in 20 ml lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5% glycerol, 0.1% β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), and lysed with a cell disruptor (Branson). Cell debris was pelleted by centrifugation for 30 min followed by filtration. The supernatant was incubated with 1 ml nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) for 1 h at 4°C and then washed with 50 ml wash 1 buffer (lysis buffer with 20 mM imidazole) followed by 20 ml wash 2 buffer (lysis buffer with 50 mM imidazole). The bound protein was eluted using a step gradient method with elution buffers containing 50 mM Tris (pH 8.0), 150 mM NaCl, 5% glycerol, and 200 mM, 300 mM, or 500 mM imidazole. The fractions were assessed for purity by SDS-PAGE, and fractions with estimated >95% purity were dialyzed overnight at 4°C in Tris (50 mM, pH 8.0) and NaCl (150 mM). For storage, 5% glycerol was added and aliquots were flash frozen.

Enzymatic synthesis of c-di-AMP. Recombinant DisA was expressed and purified as described previously (47). Purified DisA (0.6 μM final) was added to a solution of ATP (1 mM), Tris (40 mM, pH 7.5), NaCl (100 mM), β -mercaptoethanol, and MgCl₂ (20 mM). Reaction mixtures were incubated for 24 h at 30°C. Protein was precipitated by boiling the

sample for 10 min and removed by filtration. Following 5-fold dilution with deionized water, nucleotide was applied to anion exchange resin (Q-Sepharose, 10 ml; Pharmacia). The resin was washed with 5 bed volumes of deionized water. Elution of adsorbed nucleotide was accomplished with ammonium acetate (NH_4Ac , 2 M) until the A_{260} in the eluate returned to baseline levels. Dissolved c-di-AMP was speed vacuumed to dryness and then resuspended in water. This process was repeated two more times to ensure complete removal of NH_4Ac . Sample purity was assessed by HPLC analysis and confirmed to be >98% based upon peak absorbance (data not shown).

Derivatization and detection of secreted c-di-AMP. To assess secretion of c-di-AMP, bacterial mutants were grown in defined minimal medium (39) for 24 h at 37°C with shaking. Bacteria were removed from the supernatant by centrifugation. Samples (50 μl) of culture supernatants were combined with 50 μl sodium acetate (1 M, pH 4.5) and 5 μl chloroacetaldehyde (4 M) and incubated at 80°C for 20 min to derivatize all adenyl purine molecules, as previously described (48). Samples were analyzed using an HPLC system (Agilent 1100 series) fitted with a Nova-Pak C_{18} column (150 by 3.9 mm, 4 m; Waters) equipped with an Alltima C_{18} -LL guard column (5 m; Alltech). Solvent A consisted of sodium phosphate (30 mM), tetrabutylammonium bisulfate (5 mM, pH 6.0), and solvent B contained 100% acetonitrile. The samples were eluted using an isocratic flow of 14% solvent B over 14 min followed by a gradient from 14 to 40% B over 0.5 min, 40% B for 5 min, a return to 14% B over 0.5 min, and 5 min at 14% B to re-equilibrate the column. The injection volume was 100 μl , and the flow rate was 0.7 ml/min. Excitation of N^6 -etheno-derivatized c-di-AMP was done at 278 nm, and emission was monitored at 418 nm. Nucleotides were quantified by comparing the fluorescent peak area to similarly derivatized standards of purified c-di-AMP. Concentrations of standards were determined spectrophotometrically based upon the absorbance at 259 nm ($\epsilon = 30,000 \text{ M}^{-1} \text{ cm}^{-1}$).

PdeA enzyme activity. Reaction mixtures containing 40 μM c-di-AMP were incubated with 1.5 to 2.5 μM enzyme in assay buffer consisting of Tris (100 mM, pH 8.0), potassium chloride (20 mM), manganese sulfate (0.5 mM), and reactions were allowed to proceed for 30 min at 37°C prior to analysis using an HPLC system (Agilent 1100 series) Nova-Pak C_{18} column (150 by 3.9 mm, 4 m; Waters) equipped with an Alltima C_{18} -LL guard column (5 m; Alltech). Solvent A consisted of sodium phosphate (30 mM), tetrabutylammonium bisulfate (5 mM, pH 6.0), and solvent B contained 100% acetonitrile. The samples were eluted using a linear gradient from 5 to 12% solvent B over 20 min followed by a gradient from 12 to 40% over 3 min. The injection volume was 100 μl , and the flow rate was 0.7 ml/min; c-di-AMP and pApA (Biolog) standards were run in each experiment.

Bacterial transcriptional analysis. Microarray analysis of *L. monocytogenes* $\Delta pdeA$ was compared to analysis of the WT as previously described (49). Selected genes that exhibited >2-fold increases in transcription were confirmed by qRT-PCR.

Susceptibility to acid stress. Bacterial cultures were grown to stationary phase in BHI broth ($\text{OD} \approx 2.5$) at 37°C with shaking, and then the pH was adjusted to 2.5 with HCl. Viable-cell counts were performed at various intervals by plating on LB plates.

Bacteriolysis in broth and in macrophages. A U153 transducing lysate was generated with donor strain DP-L967, which contains a Tn917-LTV3 insertion that leads to constitutive expression of the contained *lacZ* gene. Transduction of the $c\Delta dacA$ and wild-type *L. monocytogenes* strains was performed as described previously (46). Broth lysis was assessed as the amount of β -galactosidase activity secreted into the broth supernatant during exponential growth. Overnight cultures of each strain were grown at 37°C with shaking. Cultures were diluted 1:100 in fresh BHI medium and grown to mid-log phase (OD at 600 nm [OD_{600}] = 0.5). Bacteria were removed by centrifugation, and culture supernatants were sterilized with syringe filters (Millex GP, 0.22 μm ; Millipore). The bacterial pellet was resuspended in fresh BHI and lysed by vortexing for 10 min at 4°C with zirconia/silica beads (~200 μl ; Biospec Products Inc.). The lysed bacteria

were then centrifuged to remove cellular debris and the beads. Cellular lysates and sterile filtered culture supernatants of each strain were diluted (1- to 16-fold) and separately mixed with Z buffer (100 μl , 0.1 M phosphate, 0.01 M KCl, 1 mM MgSO_4 , 50 mM β -mercaptoethanol, pH 7.0) and ONPG (4 mg/ml in 0.1 M phosphate, pH 7.0). Samples were placed in a 96-well plate, and A_{420} was monitored at 37°C. β -Galactosidase activity was calculated based on the rate of change in the A_{420} . Dilutions of bacterial lysates were used to generate a standard curve of lysis. Broth lysis was calculated based upon the activity observed in the culture supernatant relative to this standard curve.

To assess intracellular bacteriolysis, *L. monocytogenes* strains were engineered to carry the luciferase reporter plasmid pBHE573 and used to infect IFNAR^{-/-} macrophages, as previously described (3). *L. monocytogenes* engineered to express bacteriophage holin and lysin was used as a 100% lysis control, to which other values were normalized (3).

Antibiotic susceptibility. Approximately 2×10^7 CFU of bacteria were spread on BHI plates and allowed to dry. Sterile 7-mm-diameter paper disks (Whatman 3 MM) containing 5 μg cefuroxime, 5 μg ampicillin, or 5 μg penicillin were placed on the plates. Following 24 h of incubation at 37°C, the diameter of the zone of growth inhibition surrounding the disks was measured. Antibiotic susceptibility was also assessed by determining the MIC. Briefly, stationary-phase cultures were diluted 1:100 into fresh BHI medium containing 2-fold serial dilutions of cefuroxime. Cultures were grown for 12 h at 37°C, and OD_{600} readings were taken. The MIC was determined as the antibiotic concentration required to inhibit bacterial growth by 75%.

In vivo mouse infections. This study was performed in accordance with the guidelines in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (50). Protocols were approved by the Animal Care and Use Committee of the University of California, Berkeley.

Prior to infection, bacterial strains are grown to stationary phase ($\text{OD}_{600} = 1.2$) at 30°C, then diluted into fresh BHI, and grown at 37°C with shaking until the OD_{600} was 0.3 to 0.5. Cultures were diluted in 1× PBS and used to intravenously infect female C57BL/6 mice between 6 and 8 weeks of age with a final inoculum of 10^5 bacteria. At 48 h postinfection, mice were sacrificed and organs collected. Bacterial burdens were enumerated by plating organ homogenates on LB plates and incubating overnight.

Host response to infection. Macrophage cell death was assessed by lactate dehydrogenase release and qRT-PCR of IFN- β was performed as previously described (3). *L. monocytogenes* mutants expressing *Legionella pneumophila* flagellin were used as positive controls for robust inflammasome activation (51).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00282-13/-/DCSupplemental>.

Figure S1, TIF file, 0.1 MB.

Figure S2, TIF file, 0.2 MB.

Figure S3, TIF file, 0.1 MB.

Figure S4, TIF file, 0.1 MB.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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Daniel A. Portnoy has a consulting relationship with and a financial interest in Aduro Biotech, and both he and the company stand to benefit from the commercialization of the results of this research.

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