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c-di-AMP Secreted by Intracellular *Listeria monocytogenes* Activates a Host Type I Interferon Response

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Abstract

Intracellular bacterial pathogens, such as *Listeria monocytogenes*, are detected in the cytosol of host immune cells. Induction of this host response is often dependent on microbial secretion systems, and in *L. monocytogenes*, is dependent on multi-drug efflux pumps (MDRs). Using *L. monocytogenes* mutants that over-expressed MDRs, we identified cyclic diadenosine monophosphate (c-di-AMP) as a secreted molecule able to trigger the cytosolic host response. Over-expression of the di-adenylate cyclase, *dacA* (*lmo2120*), resulted in elevated levels of the host response during infection. C-di-AMP thus represents a putative bacterial secondary signaling molecule that triggers a cytosolic pathway of innate immunity and is predicted to be present in a wide variety of bacteria and archaea.

The mammalian innate immune system is comprised of receptors that collectively serve as a pathogen sensor to monitor the extracellular, vacuolar and cytosolic cellular compartments (1). Recognition of microbes within these distinct compartments leads to cellular responses that are commensurate with the microbial threat. While both pathogenic and non-pathogenic microbes interact with extracellular and vacuolar compartments, infectious disease agents often mediate their pathogenesis by directly entering the cytosol or through delivery of virulence factors into the host cell cytosolic compartment. Thus the innate immune system may distinguish between pathogenic and non-pathogenic microbes by monitoring the cytosol (2, 3).

Several distinct pathways of innate immunity are present in the host cell cytosol. One, termed the cytosolic surveillance pathway (CSP), detects bacterial, viral and protozoan pathogens, leading to the activation of interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), resulting in the induction of interferon-β (IFN-β) and co-regulated genes (4). Some ligands that activate this pathway are known, for example, viral and bacterial nucleic acids (5). However, the ligands and host receptors that lead to IFN-β production after exposure to non-viral microbes, including *L. monocytogenes*, *M. tuberculosis*, *F. tularensis*, *L. pneumophila*, *B. abortus*, and *T. cruzi*, remain unknown (4–9).

Expression of *L. monocytogenes* multidrug efflux pumps (MDRs) of the major facilitator superfamily controls the capacity of cytosolic bacteria to induce host expression of IFN-β (10). Ectopic expression of multiple MDRs enhances IFN-β production, while one, MdrM, controls the majority of the response to wild-type bacteria (10). Given that MDRs transport small molecules (<1000 Da), we hypothesized that *L. monocytogenes* secretes a bioactive

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small molecule that is recognized within the host cell cytosol. To identify the bioactive ligand(s) secreted by *L. monocytogenes* MDRs, we performed solid phase extraction (SPE) of the culture supernatant from an MdrM over-expressing *L. monocytogenes* strain (*marR*-, DP-L5445) that exhibits an IFN- β hyper-activating phenotype (11). Delivery of the fraction to the macrophage cytosol using reversible digitonin permeabilization (12) resulted in a dose-dependent increase in type-I IFN (Fig. 1A). Addition of this fraction in the absence of digitonin resulted in no IFN production, consistent with cytosolic detection of the active ligand.

In *L. monocytogenes* strains that exhibit variable levels of MDR expression, IFN- β production correlates with increases in transporter levels (10). Supernatants from four *L. monocytogenes* strains, *mdrM*-, WT, *marR*-, and *tetR::Tn917*, each with increasing levels of MDR expression were tested for activity. Comparable to infection assays, MDR expression correlated with IFN inducing activity of the culture supernatants (Fig. 1B). The *tetR::Tn917* strain exhibited significantly higher activity than any other strain; while the *mdrM*- strain lacked detectable activity above background.

Fractionation of the active samples obtained from each MDR strain was performed using reversed-phase high performance liquid chromatography (RP-HPLC). The active component of each supernatant eluted as a single peak from the column with similar retention time (Fig. 1C), consistent with each containing the same active ligand. Furthermore, the magnitude of the active peak correlated with MDR expression. The sample with the highest activity exhibited a significant absorbance at 260 nm (Fig. S1A). Incubation with anion but not cation exchange resin removed the active molecule from solution (Fig. S1B) and treatment of the active sample was resistant to DNase (Fig. S1C). These observations were consistent with a non-DNA, nucleic acid as the active component.

To identify the IFN- β -inducing metabolite contained in the fractions, samples were analyzed by high-resolution mass spectrometry. A single ion ($m/z = 659.11$, $z = 1$) was identified as exclusively present in the active fractions and absent in the inactive samples (Fig. S2A). The parent ion mass was consistent with cyclic di-adenosine monophosphate (c-di-AMP, Fig. S2B). Collision induced dissociation was performed to further characterize the identified ion (Fig. 2A). Quantification of c-di-AMP in each sample showed that *mdrM*-, WT, and *marR*- strains had 23%, 34%, and 35% as much c-di-AMP in the culture supernatants relative to *tetR::Tn917* (53 nM). Thus IFN- β -inducing activity of *L. monocytogenes* supernatants correlates linearly with c-di-AMP concentration (Fig. S3).

Next, we tested the ability of commercially available c-di-AMP to induce IFN- β in macrophages. C-di-AMP exhibited a dose-dependent response when delivered to the cytosol of murine BMMs (Fig. 2D). Treatment of the purified active fraction and the commercial standard with snake venom phosphodiesterase (SVPD) abolished the activity of each sample. The host pathway responsible for cytosolic detection of *L. monocytogenes* is dependent on IRF3 and independent of MyD88/Trif and mitochondrial antiviral signaling protein (MAVS) (10). Detection of c-di-AMP is MyD88/Trif and MAVS independent but requires IRF3 (Fig. S4). Thus c-di-AMP requires a parallel host-signaling pathway to activate the CSP, consistent with c-di-AMP as the relevant ligand of *L. monocytogenes*.

Here we report that the intracellular pathogen *L. monocytogenes* generates c-di-AMP, which induces the host cytosolic surveillance pathway. Di-adenylate cyclase (DAC) activity has been assigned to a domain of unknown function (previously DUF147) within the protein DisA of *B. subtilis* (13), and c-di-AMP is thought to act as a secondary signaling molecule that regulates bacterial sporulation (14). Bio-informatic analysis identified the widespread

presence of the DAC domain in bacteria and archaea, including pathogenic *Staphylococci*, *Streptococci*, *Mycobacteria*, *Chlamydia*, and *Mycoplasma* spp (15).

In *L. monocytogenes* a single gene, *lmo2120*, contains a predicted DAC domain. This gene is present in an operon with the downstream gene *lmo2119*, a gene of unknown function (Fig. 3A). Attempts to delete the gene *lmo2120* using standard techniques were unsuccessful. Genetic screens identified genes containing DAC domains in *Streptococci* and two species of *Mycoplasma* as essential (16–18), supporting a similar indispensable role in *L. monocytogenes*. However, over-expression of *lmo2120* did not affect bacterial growth but led to increased CSP activation during macrophage infection (Fig. 3B and C), consistent with DAC activity encoded by the *lmo2120* gene, which we have named here *dacA*.

MDRs are generally recognized to function in conferring resistance to small toxic molecules such as antibiotics by active efflux, preventing accumulation of lethal concentrations within the cell. A number of instances have described transport of small molecules that are not toxic (19–21), leading to the hypothesis that these transporters have evolved to transport specific natural substrates as well (22). Our observations suggested that these proteins play a broader biological role beyond general drug resistance, perhaps involved in bacterial signaling. Moreover, bacterial signaling nucleotides are generally considered to act within the cell. Here, we provided evidence that c-di-AMP is exported from the cell and thus may be involved in extracellular signaling by *L. monocytogenes*.

The ability of the host to discriminate between pathogen and non-pathogen is often mediated by the compartmentalized detection of microbial ligands. For instance, *L. monocytogenes* mutants that cannot escape the primary vacuole are avirulent and do not activate IRF3 dependent inflammation, while those that are virulent enter the cytosol, leading to type I interferon production. The results of this study showed that host cells detect and respond to cytosolic c-di-AMP, recapitulating the effects of cytosolic infection. These observations are consistent with reports in which host responses to bacterial peptidoglycan, c-di-GMP, and flagellin are dependent on cytosolic delivery (23–27). The conserved and critical role of bacterial cyclic di-nucleotides fulfills the criteria of ligands that alert the immune system to the presence of live pathogenic bacteria that engage the host cytosol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and Notes

1. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Cell Host Microbe. 2008; 3:352. [PubMed: 18541212]
2. Vance RE, Isberg RR, Portnoy DA. Cell Host Microbe. 2009; 6:10. [PubMed: 19616762]
3. Lamkanfi M, Dixit VM. Immunol. Rev. 2009; 227:95. [PubMed: 19120479]
4. O'Riordan M, Yi CH, Gonzales R, Lee KD, Portnoy DA. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:13861. [PubMed: 12359878]
5. Stetson DB, Medzhitov R. Immunity. 2006; 24:93. [PubMed: 16413926]

6. Henry T, Brotcke A, Weiss DS, Thompson LJ, Monack DM. *J. Exp. Med.* 2007; 204:987. [PubMed: 17452523]
7. Roux CM, et al. *Cell. Microbiol.* 2007; 9:1851. [PubMed: 17441987]
8. Stanley SA, Johndrow JE, Manzanillo P, Cox JS. *J. Immunol.* 2007; 178:3143. [PubMed: 17312162]
9. Vaena de Avalos S, Blader IJ, Fisher M, Boothroyd JC, Burleigh BA. *J Biol. Chem.* 2002; 277:639. [PubMed: 11668183]
10. Crimmins GT, et al. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105:10191. [PubMed: 18632558]
11. Detailed materials and methods are available as supporting online material.
12. Girardin SE, et al. *Science.* 2003; 300:1584. [PubMed: 12791997]
13. Witte G, Hartung S, Buttner K, Hopfner KP. *Mol. Cell.* 2008; 30:167. [PubMed: 18439896]
14. Bejerano-Sagie M, et al. *Cell.* 2006; 125:679. [PubMed: 16713562]
15. Romling U. *Sci. Signal.* 2008; 1:pe39. [PubMed: 18714086]
16. French CT, et al. *Mol. Microbiol.* 2008; 69:67. [PubMed: 18452587]
17. Song JH, et al. *Mol. Cells.* 2005; 19:365. [PubMed: 15995353]
18. Glass IJ, et al. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103:425. [PubMed: 16407165]
19. Woolridge DP, et al. *J. Biol. Chem.* 1997; 272:8864. [PubMed: 9083003]
20. van Helvoort A, et al. *Cell.* 1996; 87:507. [PubMed: 8898203]
21. Yang S, Lopez CR, Zechiedrich EL. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103:2386. [PubMed: 16467145]
22. Neyfakh AA. *Trends Microbiol.* 1997; 309(5)
23. Viala J, et al. *Nat. Immunol.* 2004; 5:1166. [PubMed: 15489856]
24. Y. H., Sun; H. G., Rolan; R. M., Tsolis. *J. Biol. Chem.* 2007; 282:33897. [PubMed: 17911114]
25. Molofsky AB, et al. *J. Exp. Med.* 2006; 203:1093. [PubMed: 16606669]
26. Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE. *PLoS Pathog.* 2006; 2:e18. [PubMed: 16552444]
27. McWhirter SM, et al. *J. Exp. Med.* 2009; 206:1899. [PubMed: 19652017]

Figure 1. Characterization and isolation of *L. monocytogenes* secreted IFN- β stimulatory ligand (A) IFN- β production by BMMs in response to solid phase extracts (SPE) of *marR*- *L. monocytogenes* supernatants in the presence (black bars) and absence (grey bars) of digitonin. IFN- β activity was measured using ISRE L929 cells that generate luciferase in response to type-I IFN stimulation. Data are mean of biological replicates ($N=2$). (B) IFN- β activity by BMMs in response to solid phase extracts of sterile filtered culture supernatants from *mdrM*-, wild-type (WT), *marR*-, and *tetR::Tn917* strains of *L. monocytogenes*. Negative control consists of digitonin permeabilizing solution alone (dig). Data are mean $\pm$ SD ($N=2$). Data representative of two independent experiments. (C) IFN- β stimulatory activity of culture supernatants fractionated using reversed-phase HPLC. Activity measured as in (A). Data are the mean activity of biological replicates ($N=2$).

Figure 2. Cyclic di-AMP is an IFN- β activating ligand

(A) Tandem mass spectrum resulting from collisionally activated dissociation of the singly charged positive ion at $m/z = 659.11$ formed from an active fraction of *Listeria monocytogenes*. (B) Tandem mass spectrum of commercially obtained sample of c-di-AMP (BioLog Life Sciences Institute, Denmark). Fragmentation pathways of c-di-AMP are shown in (C). The fragment ions at $m/z = 641.10$ and 312.05 correspond to neutral losses of 18 Da from the precursor ion ($m/z = 659.11$) and from the fragment ion at $m/z = 330.06$, respectively, and are consistent with neutral loss of water molecules from these respective ions. (D) Commercial c-di-AMP standard was added to BMMs in increasing amount. IFN- β production by BMMs was detected using the type I IFN reporter cell line (ISRE L929). Commercial c-di-AMP standard and the active *L. monocytogenes* fraction were treated with snake venom phosphodiesterase (SVPD). Data are mean of biological replicates $\pm$ SD ($N=2$).

Figure 3. The di-adenylate cyclase gene *dacA* (*lmo2120*) alters CSP activation during infection
(A) Predicted operon of genes *lmo2120*, renamed here *dacA*, and *lmo2119*. The gene product of *lmo2119* contains three *ybbR* domains of unknown function. The gene product of *lmo2120* contains a single di-adenylate cyclase (DAC) domain. Trans-membrane spanning segments (TM) predicted using Topcons (<http://topcons.cbr.su.se/index.php>). **(B)** Intracellular growth curves of WT *L. monocytogenes* (closed circles) and *L. monocytogenes* with an integration vector (pLIV2) containing IPTG inducible *dacA* in the absence (open circles) and presence (open squares) of IPTG (1 mM) in BMMs. Data are mean $\pm$ SD ($N=3$). **(C)** qRT-PCR analysis of IFN- β induction by each strain in BMMs. Data are mean $\pm$ SD ($N=2$). Data representative of two independent experiments.