

## A critical role for peptidoglycan *N*-deacetylation in *Listeria* evasion from the host innate immune system

Ivo G. Boneca<sup>1,\*</sup>, Olivier Dussurget<sup>2,3,4</sup>, Didier Cabanes<sup>2,3,4,§</sup>, Marie-Anne Nahori<sup>2,3,4</sup>, Sandra Sousa<sup>2,3,4,§</sup>, Marc Lecuit<sup>2,3,4</sup>, Emmanuel Psyllinakis<sup>12,13</sup>, Vassilis Bouriotis<sup>12,13</sup>, Jean-Pierre Hugot<sup>14,15</sup>, Marco Giovannini<sup>16,17</sup>, Anthony Coyle<sup>18,€</sup>, John Bertin<sup>18,£</sup>, Abdelkader Namane<sup>5</sup>, Jean-Claude Rousselle<sup>5</sup>, Nadège Cayet<sup>6</sup>, Marie-Christine Prévost<sup>6</sup>, Viviane Balloy<sup>7,8</sup>, Michel Chignard<sup>7,8</sup>, Dana J. Philpott<sup>9,#</sup>, Pascale Cossart<sup>2,3,4,\*</sup>, Stephen E. Girardin<sup>10,11,§</sup>

<sup>1</sup> Institut Pasteur, Unité de Pathogénie Bactérienne des Muqueuses, Paris, France

<sup>2</sup> Institut Pasteur, Unité des Interactions Bactéries-Cellules, Paris, France

<sup>3</sup> INSERM U604 Paris, France,

<sup>4</sup> INRA USC2020, Paris, France

<sup>5</sup> Institut Pasteur, Plateforme de Protéomique, Paris, France

<sup>6</sup> Institut Pasteur, Plateforme de Microscopie électronique, Paris, France

<sup>7</sup> Institut Pasteur, Unité Défense Innée et Inflammation, Paris, France

<sup>8</sup> INSERM E336, Paris, France

<sup>9</sup> Institut Pasteur, Groupe Immunité Innée et Signalisation, Paris, France

<sup>10</sup> Institut Pasteur, Unité de Pathogénie Microbienne Moléculaire, Paris, France

<sup>11</sup> INSERM U389 and Groupe INSERM Avenir “Peptidoglycan and Innate Immunity”, Paris, France

<sup>12</sup> University of Crete, Department of Biology, Enzyme Biotechnology group, Heraklion, Greece

<sup>13</sup> Institute of Molecular Biology and Biotechnology, Heraklion, Greece

<sup>14</sup> Hôpital Robert Debré, Department of Paediatric Gastroenterology, Paris, France

<sup>15</sup> INSERM U458, Paris, France.

<sup>16</sup> Fondation Jean Dausset – CEPH, Génomique Fonctionnelles des Tumeurs Solides, Paris, France

<sup>17</sup> INSERM U674, Paris, France

<sup>18</sup> Millennium Pharmaceuticals, Cambridge, Massachusetts, USA

§ Present address: Group of Molecular Microbiology, Instituto de Biologia Celular e Molecular, Porto, Portugal.

# Present address: Department of Immunology, University of Toronto, Toronto, Ontario, Canada.

§ Present address: Department of Laboratory Medicine & Pathobiology, University of Toronto, Toronto, Ontario, Canada.

£ Present Address: Synta Pharmaceuticals, Corp., Lexington, MA 02421.

€ Present Address: Medimmune, Gaithersburg, MD 20878.

\* To whom correspondence should be addressed: [bonecai@pasteur.fr](mailto:bonecai@pasteur.fr); tel.: 33-(0)1-44389516; Fax.: 33-(0)1-40613640, [pcossart@pasteur.fr](mailto:pcossart@pasteur.fr); tel.: 33-(0)1-45688841; Fax.: 33-(0)1-45688706.

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## ABSTRACT

*Listeria monocytogenes* is a human intracellular pathogen, which is able to survive in the gastrointestinal environment and replicate in macrophages thus bypassing the early innate immune defenses. Peptidoglycan (PG) is an essential component of the bacterial cell wall readily exposed to the host and, thus, an important target for the innate immune system. Characterization of the PG from *L. monocytogenes* demonstrated deacetylation of *N*-acetylglucosamine residues. We identified a PG *N*-deacetylase gene, *pgdA*, in *L. monocytogenes* genome sequence. Inactivation of *pgdA* revealed the key role of this PG modification in bacterial virulence as the mutant was extremely sensitive to the bacteriolytic activity of lysozyme, and, growth was severely impaired following oral and intravenous inoculations. Within macrophage vacuoles, the mutant was rapidly destroyed and induced a massive IFN- $\beta$  response in a TLR2 and Nod1-dependent manner. Together, these results reveal that PG *N*-deacetylation is a highly efficient mechanism used by *Listeria* to evade innate host defenses. Presences of deacetylase genes in other pathogenic bacteria indicate that PG *N*-deacetylation could be a general mechanism used by bacteria to evade the host innate immune system.

## INTRODUCTION

The innate immune system is central for the early recognition and clearance of pathogens. Hence, a number of microbial pathogens have developed sophisticated strategies to evade or modulate the host response to their advantage. *Listeria monocytogenes* is an invasive pathogen that survives in the harsh gastrointestinal lumen and reaches the sub-mucosa without inducing a vigorous inflammatory response. However, the mechanisms used by *L. monocytogenes* to survive during the very early steps of the infection are poorly understood. This bacterium then disseminates to different organs by spreading from cell-to-cell and by surviving in phagocytic cells such as macrophages. The ability of *L. monocytogenes* to internalize and escape into the cytosol requires key virulence factors such as the secreted toxin listeriolysin O (1), the surface protein ActA (2) and Internalin (3) which is anchored to its peptidoglycan (PG)(4) or InlB (5) which is attached to its lipoteichoic acids (LTA) (6, for reviews see references 7, 8). Hence, the cell wall of *L. monocytogenes* plays a central role in its virulence. Recently, the role of PG hydrolases in *Listeria* virulence has also been described and suggested to be important during infection (9, 10). PG is the pathogen-associated molecular pattern (PAMP) recognized by the innate immune system by some of the recently discovered intracellular pattern-recognition receptors (PRRs) such as the nucleotide-binding oligomerization domain (Nod) proteins (11). Recent work has reported a role for Nod1, Nod2 and toll-like receptor (TLR) -2 in innate immune recognition of *Listeria* (12-15). However, little is known regarding the composition and structure of *L. monocytogenes* PG except that it is a *meso*-diaminopimelic containing PG and that *N*-acetylglucosamine residues are

partially *N*-deacetylated into glucosamine (16). Whether these specific features play a role in infection has never been investigated.

Here, we report that *N*-deacetylation is a major modification of *Listeria* PG conferring to this human pathogen the ability to evade the innate immune system. The inactivation of the single PG *N*-deacetylase of *Listeria* highlights the central role of this modification in *Listeria* virulence, through survival in the gastrointestinal track, in professional phagocytes, evasion to the action of host lysozyme and modulation of inflammatory response.

## RESULTS AND DISCUSSION

To address the role of *Listeria* PG in virulence, we first prepared highly purified PG from *L. monocytogenes* strain EGDe and analyzed its muropeptide composition by reverse phase high-pressure liquid chromatography (HPLC, Figure 1A). Mass spectrometry analysis of each peak revealed that *L. monocytogenes* has several unusual modifications of its peptidoglycan (Figure 1A). Around 50 % of its muropeptides featured a glucosamine residue instead of the canonical *N*-acetylglucosamine, definitively establishing that *Listeria* *N*-deacetylates partially its PG. Interestingly, PG *N*-deacetylation has been described to confer resistance to host lysozyme (17), a major innate defense mechanism against bacterial infections.

Analysis of *L. monocytogenes* genome allowed identification of a gene encoding a putative PG deacetylase (*lmo0415*). This was the only putative PG *N*-deacetylase that could be identified. A deletion mutant of *lmo0415* was generated. Analysis of its PG confirmed that *lmo0415*, renamed *pgdA*, encodes the unique PG *N*-deacetylase of *L. monocytogenes* as all peaks corresponding to *N*-deacetylated muropeptides disappeared from the HPLC pattern (Figure 1B). As expected, the *pgdA* mutant showed sensitivity to lysozyme, resulting in five log decrease in viability compared to the parental strain EGDe in the presence of lysozyme (Figure 2). Interestingly, the sensitivity of the *pgdA* mutant was specific for lysozyme since the human serum amidase, another PG hydrolase produced by the host, had no bacteriolytic effect on the *pgdA* mutant (Figure 2A). Also, the mutant was perfectly capable to grow in BHI broth and serum, indicating that *pgdA* inactivation had no effect on its growth (Figure 2A and supplementary Figure 1) or

morphology (supplementary Figure 2). Sensitivity to lysozyme was only observed in stationary phase and resulted in cell rounding (supplementary Figure 2).

As shown above, the *pgdA* mutant is extremely sensitive to host lysozyme. Since lysozyme is particularly abundant in macrophages, to evaluate the possible impact of *N*-deacetylation on infection, we compared the ability of the *pgdA* mutant and that of strain EGDe to infect and survive in macrophages (Figure 2C and D). The *pgdA* mutant was severely impaired in its ability to survive and multiply in RAW264.7 macrophages (Figure 2C), in peptone elicited peritoneal macrophages (PEM from C57/BL6J mice; Figure 2D) and in bone marrow derived macrophages (BMDM from C57/BL6J mice; data not shown). For example, after 8 hours of infection of RAW264.7 macrophages, the difference in viable bacteria between the *pgdA* mutant and strain EGDe was 1,5 Log. Next, infected macrophages were observed by electron microscopy (EM). Figures 3A and B illustrate EM images of macrophages infected with strain EGDe and the *pgdA* mutant, respectively, after 8 h of infection (see also Figure 3E and F). While strain EGDe was present exclusively in the macrophage cytosol, the *pgdA* mutant accumulated in vacuoles preventing its cytosolic multiplication. EM observations of infected macrophages at different time points after infection showed that the *pgdA* mutant was mainly detected in vacuoles compared to the parental strain EGDe (Figures 3C and D) suggesting an early intracellular killing. Strikingly, the *pgdA* mutant was found frequently in large vacuoles with multiple bacteria or bacterial debris (Figure 3A, B, E and F). We also infected non-phagocytic Caco 2 epithelial cells with strain EGDe and its *pgdA* mutant. The *pgdA* mutant was not impaired in its ability to be internalized (data not shown) indicating that the *pgdA* mutant was not generally affected in bacterial stability. Moreover, the *pgdA*

mutant produced normal amounts in listeriolysin O showing that impaired escape from phagosomes in macrophages is not due to a defect of listeriolysin O production (data not shown).

Virulence and dissemination of *Listeria* in its host strongly relies on survival in macrophages as illustrated by the very strong attenuation of the listeriolysin O mutant (1). We thus performed *in vivo* challenge of BALB/c and C57/BL6J mice by the intra venous (IV) route with strain EGDe and the *pgdA* mutant. In both mice backgrounds, the *pgdA* mutant was severely attenuated with LD50s of  $1,7 \times 10^6$  and  $1,1 \times 10^8$  bacteria in C57/BL6J and BALB/c mice, respectively, compared to  $2,3 \times 10^4$  and  $1,7 \times 10^4$  bacteria, respectively, for the parental strain EGDe. Accordingly, bacterial counts for the *pgdA* mutant were lower than the wild type in both liver and spleen (Figure 4A and B). In addition, the *pgdA* mutant, although sensitive to lysozyme *in vitro*, was as resistant as the wild type strain to the action of serum in the presence or absence of complement (supplementary Figure 1). Hence, the impaired virulence of the *pgdA* mutant is not related to an impaired survival in the blood stream.

The oral route is the natural route of *Listeria* infection. We thus also infected orally human E-cadherin (hEcad) transgenic mice, which are permissive to *Listeria* oral infection (18), and monitored bacterial counts in different organs at 3 h, 24 h, 48 h and 72 h post-infection (Figure 4C to G). The *pgdA* mutant was strongly impaired in surviving in the intestinal lumen and 48 h post-infection, no bacteria could be detected in this compartment (Figure 4C). Furthermore, bacterial counts of the *pgdA* mutant were compared to the wild type lower in the intestine, in the mesenteric lymph nodes (Figure 4D and E, respectively) and, as observed after IV inoculation, in the liver and the spleen

(Figure 4F and G, respectively). Hence, the *pgdA* mutant is attenuated in virulence both at very early stages of the infection and at later stages after dissemination from the intestine to the liver and the spleen. These results reveal that the *pgdA* mutant is highly sensitive to both the very first host innate immune responses and at later stages of the infection. This is in agreement with the fact that Paneth cells in the small intestine abundantly produce lysozyme and other antimicrobial agents.

We then investigated the impact that lack of PG *N*-deacetylation might have on the inflammatory response. Since the Nod proteins detect PG and PG fragments such as muropeptides (19, 20), we first analyzed the ability of highly purified PG from *L. monocytogenes* EGDe to activate the Nod pathways by measuring NF- $\kappa$ B activation with the classical luciferase reporter assay in HEK293T cells (21). The native PG of *L. monocytogenes* activated poorly NF- $\kappa$ B in both Nod1 and Nod2-dependent manners compared to *E. coli* PG (supplementary Figure 3) as previously reported (22). However, a complete pre-digestion with a muramidase increased induction of NF- $\kappa$ B in a Nod2-dependent (20 fold) and Nod1-dependent (6 fold) manner (Supplementary Figure 3A). The fully acetylated native PG from the *pgdA* mutant was better detected by the Nods (Supplementary Figure 3B), compared to the native PG of parental strain EGDe. Taken together, our results suggested that *L. monocytogenes* contains in its native PG Nod agonists but that these are not readily available to the host. Hence, our hypothesis that *Listeria* also *N*-deacetylates its PG as a strategy to avoid generation and presentation of cell wall components to the PRRs such as the Nods or TLR2. As a proof of principal, we were able to show that an *in vitro* fully *N*-deacetylated PG from *Helicobacter pylori* lost



completely its ability to be sensed by both Nod1 and Nod2 (Supplementary Figure 3C) compared to native *H. pylori* PG.

The cytokine response of macrophages to the *pgdA* mutant was then compared it to the wild type. Despite the reduced number of viable bacteria mutant bacteria (see Figure 3A and B), the *pgdA* mutant induced a strikingly more vigorous cytokine response compared to the wild type (for example, higher amounts of interleukin (IL) 6 and interferon (IFN) –  $\beta$ ) than strain EGDe in RAW264.7 macrophages (Figure 5A, B and supplementary Figure 4). This enhanced cytokine response of the *pgdA* mutant compared to wild type EGDe was also observed in PEM (Figure 5C and D, respectively). To our knowledge, the *pgdA* mutant is the first *Listeria* mutant that stimulates IFN- $\beta$  production by PEM at such a high level.

The role of different PRRs in the enhanced cytokine response to *pgdA* mutant infection was then addressed using macrophages from Nod1<sup>-/-</sup>, Nod2<sup>-/-</sup>, MyD88<sup>-/-</sup> and TLR2<sup>-/-</sup> mice. IL-6 and IFN- $\beta$  production depended strongly on TLR2. The TLR2 response was partially dependent on MyD88, the master adaptor molecule myeloid differentiation factor 88. IL-6 production was also partially dependent on Nod1, which, surprisingly, negatively regulated IFN- $\beta$  production by PEM (Figure 5D). This negative regulatory impact on TLR2-mediated IFN- $\beta$  production of the Nod1-pathway is reminiscent of the role of Nod2 as negative regulator of TLR2-mediated T helper type 1 responses (23). Together, our results suggested that the inflammatory response induced by the *pgdA* mutant is due to an enhanced accessibility of cell wall components to TLR2 and Nod1. These results also suggested as shown in PEM that wild type *Listeria* exploit PG N-deacetylation to evade Nod1 sensing.

In conclusion, the *pgdA* mutant is a highly attenuated mutant of *Listeria*. Attenuation is of the same order as that of listeriolysin O and ActA mutants, the most attenuated *Listeria* mutants (7, 8). Thus, *N*-deacetylation of *Listeria*'s PG is a major new virulence determinant. *Listeria* escapes recognition and killing by the host by preventing processing and optimal sensing of its PG by the host. Our observations *in vitro* and *in vivo* indicate that a major consequence of PG *N*-deacetylation is an increased survival at early and also later stages of the infectious process. We propose the following model (supplementary Figure 5). *Listeria* PG *N*-deacetylation enhances the pathogen's ability to survive to first defenses of the host in the intestinal lumen, by avoiding the bacteriolytic activity of lysozyme that is massively produced by Paneth cells in the intestine (24). *Listeria* PG *N*-deacetylation might also prevent induction of enhanced production of antimicrobial peptides synthesis by escaping Nod2 detection in the intestine as previously proposed thus potentially coupling resistance to lysozyme to the Nod2 evasion (12). From the primary infection site, *Listeria* disseminates to target organs, owing to escape from lysozyme, survival in phagocytic cells and downregulation of the inflammatory response. The *pgdA* mutant sensitivity to lysozyme results in its enhanced destruction by phagocytes and release of its cell wall components such as muropeptides and LTA. The *pgdA* mutant's LTA is readily available to membrane bound TLR2 while muropeptides can be delivered to the cytosol by endogenous transporters such as hPepT1 (25) or after pore formation by listeriolysin O. Cytokine production would then ensue. PG *N*-deacetylation would thus function as double protection mechanism for *Listeria* against the innate immune defenses by escaping the action of lysozyme and providing a mechanism to evade the Nod proteins. The predominant TLR2-dependent phenotype is

most probably due to the extreme sensitivity of the *pgdA* mutant to the phagosome environment before any major contribution of the cytosolic sensing mechanism by the Nods can occur. In perfect agreement with this report, *N*-deacetylation of pneumococcal PG had been associated with pneumococcal virulence but the underlying mechanisms had not been studied in detail (26). PG *N*-deacetylation also occurs in other major gram-positive pathogens such as *Bacillus anthracis*. Genome analysis of *B. anthracis* indicates the presence of 10 open reading frames of putative deacetylases (27) suggesting that these might contribute to extreme virulence of this bioterrorism agent. Enterobacteria such as *Escherichia coli*, *Shigella flexneri* and *Yersinia pestis* have a unique lysozyme inhibitor, the small periplasmic polypeptide Ivy (28). Alternatively, *Staphylococcus aureus*, *Neisseria meningitidis* and *Mycobacterium tuberculosis* have developed mechanisms such as PG *O*-acetylation and PG *N*-glycolylation of their muramic acid residues to counteract the activity of host lysozyme (29-31), suggesting these major pathogens also might modulate the host innate immune response by modifications of their PGs. Together, our study reveals a new mechanism by which pathogens modify their PG to escape from host defenses.

## MATERIAL AND METHODS

### Bacterial and cell growth conditions

*E. coli* DH5 $\alpha$  was used as hosts for the construction and preparation of plasmids. *E. coli* was cultivated in Luria Bertani solid or liquid media supplemented as appropriate with ampicillin (100  $\mu\text{g}\cdot\text{ml}^{-1}$ ). *E. coli* MC1061 (ATCC 53338) (32) and *H. pylori* strain 26695 (ATCC 700392) (33) were used to extract PG. *H. pylori* was grown microaerobically at 37°C on blood agar plates or in liquid medium consisting of brain-heart infusion (BHI; Oxoid) with 0.2%  $\beta$ -cyclodextrin (Sigma) supplemented with antibiotic-antifungal mix (34). *L. monocytogenes* EGDe (BUG1600; ATCC number: BAA-679) and its *pgdA* mutant were grown in BHI solid or liquid media. HEK293T (ATCC number: CRL-1573) cells and RAW264.7 (ATCC number: TIB-71) macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal calf serum (Gibco). Prior to transfection, HEK293T cells were seeded into 24-well plates at a density of  $10^5$  cells/ml as described previously (35). RAW264.7 macrophages were either seeded into 6-well plates or into 25 mm Petri dishes at  $10^5$  cells/ml and  $10^6$  cells/ml, respectively.

### Construction of *Listeria pgdA* mutant

A DNA fragment containing 663 bp of the sequence upstream of *lmo0415* was generated by PCR using oligonucleotides *lmo0415-1* (5'-AACAGGATCCATAACTGGAGACACGGAGAC-3') and *lmo0415-2* (5'-AACAGAATTCATTATGCACCTCACCTCAG-3'). The fragment was cloned into *Bam*HI-*Eco*RI digested pMAD (36). A DNA fragment containing 702 bp of the sequence downstream of *lmo0415* was generated by PCR using oligonucleotides *lmo0415-3* (5'-

AACAGAATTCAAATCAGTAGCTAAGATGAGTTAA-3') and *lmo0415-4* (5'-AACAAAGATCTGATTGTCAAACCTTGAAATGG-3'). The fragment was cloned into *EcoRI-BglIII* digested pMAD containing the *lmo0415* upstream fragment, constructing the pDC27. The construct was verified by sequencing. To achieve allelic exchange, pDC27 was electroporated into *L. monocytogenes* EGDe at 2500 V, 200 Ω and 25 μF. Transformants were selected at 30°C on BHI-agar medium containing erythromycin (5 μg/ml) and X-Gal (50 μg/ml). One blue colony was grown in BHI-Ery broth at 43,5 °C for 48 h, and the culture was plated onto BHI-Ery agar containing X-Gal at 43,5 °C. One blue colony was selected and grown in BHI broth at 30 °C. BHI broth was inoculated with 1 μl of a 1:10 dilution of the previous culture and incubated at 43,5 °C. Tenfold serial dilutions of this culture were plated onto BHI-X-Gal agar and incubated at 43,5 °C. White colonies were analyzed for erythromycin sensitivity, and PCR amplifications with oligonucleotides *lmo0415-1* and *lmo0415-4*, and oligonucleotides *lmo0415-5* (5'-GATGGACAGACTAATGAAAGACC-3') and *lmo0415-6* (5'-AAAGCACCTGTTTCTGCGTC-3') were performed to confirm the gene deletion.

#### *Lysozyme, PGRP-L and serum experiments*

*Listeria* strains were grown in BHI media to which was added either hen egg lysozyme (Sigma-Aldrich, 10 μg/ml final concentration), human serum amidase (or PGRP-L, kindly provided by Waldemar Vollmer, University of Tübingen, Germany; 1 μg/ml final concentration) or fetal calf serum (Gibco, final concentration at 25%). When needed, decomplexation was done by heating the fetal calf serum at 65°C for 30 minutes. Growth was monitored by following the optical density at 600 nm and by determining the number of viable bacteria per milliliter.

### Hemolytic activity

*L. monocytogenes* strains were grown on 5% horse blood agar plates. Plates were incubated at 37°C for 48h and at 4°C for 24h. The size of the clear zone around the colonies was measured, which indicated beta-hemolytic activity.

### PG purification, HPLC analysis, in vitro N-deacetylation and MALDI-PSD analysis.

*E. coli*, *L. monocytogenes* and *H. pylori* PGs were prepared from exponentially growing bacteria and purified as previous described (22). From native PG, muropeptides were generated using the muramidase mutanolysin M1 (Sigma-Aldrich), separated by HPLC, purified and analyzed by MALDI-PSD as previously described (31). *N*-deacetylation of *H. pylori* PG was done as previously described using the recombinant BC1960 deacetylase from *B. cereus* (27).

### Expression plasmids, transient transfections, and NF- $\kappa$ B activation assays.

The expression plasmid for FLAG-tagged hNod1 was from Gabriel Nuñez (University of Michigan Medical School, Ann Arbor, Michigan, United States) and has been described previously (37). The expression plasmid for hNod2 was from Gilles Thomas (Fondation Jean Dausset/CEPH, Paris, France). HEK293T cells were used for transfections as described previously (35). Synergistic activation of NF- $\kappa$ B by PGs, muramyl peptides, and related compounds in cells overexpressing Nod1 or Nod2 was studied as described by Inohara et al. (21). Briefly, HEK293T cells were transfected overnight with 1 ng of hNod1 or 1 ng of hNod2 plus 75 ng of Ig luciferase reporter plasmid. PG samples (0.1  $\mu$ g/ml) were digested with 0.25  $\mu$ g/ $\mu$ l mutanolysin or human serum amidase. At the same time, 0.3  $\mu$ g of PG preparations or 10 pmol of muramyl-peptides were added to the cell culture medium, and synergistic NF- $\kappa$ B-dependent luciferase activation was measured

after 24 h of co-incubation. NF- $\kappa$ B-dependent luciferase assays were performed in duplicate, and data reported represent at least three independent experiments. Data were standardized with positive controls: *N*-acetylmuramic acid-dipeptide for hNod2 and *N*-acetylmuramic acid-tripeptide for hNod1. hNod1 and hNod2 were activated with PG (0.3  $\mu$ g/ml) as previously described (38).

#### Infection of murine macrophages.

PEM and BMDM were isolated from BALB/c, C57/BL6J (Charles River), TLR2<sup>-/-</sup>, MyD88<sup>-/-</sup>, Nod1<sup>-/-</sup>, Nod2<sup>-/-</sup> mice. PEM were established as described (39). For primary cultures of BMDM, the content of murine femurs and tibias were flushed and cells grown for a week in RPMI supplemented with 2mM glutamine, 10% FCS and 6ng/ml M-CSF. Nonadherent cells were recovered and incubated in RPMI supplemented with 2mM glutamine, 10% FCS and 6ng/ml M-CSF for 24h to obtain a homogenous monolayer of macrophages. PEM or BMDM (10<sup>6</sup> cells per well) were infected with *L. monocytogenes* strains (MOI=10) grown to an OD<sub>600</sub>=0.8 and incubated at 37°C for 1h to allow bacterial phagocytosis. Extracellular bacteria were eliminated by 3 washes in RPMI supplemented with 10%FCS and 10  $\mu$ g/ml gentamicin was added for the time of infection. Macrophages were lysed with 0.2% triton X-100 at different time points (1h30, 3h and 7h) for 10 min and the number of CFU was assessed by plating serial dilutions on BHI agar plates which were incubated at 37°C.

#### Epithelial Caco 2 cells and RAW264.7 macrophage infections

The *Listeria* strains were grown to OD<sub>600nm</sub>=0.6-0.8, washed (x3) and diluted in DMEM such that the MOI was about 100 and 25 bacteria per Caco-2 cell and RAW264.7 macrophage, respectively (OD<sub>600nm</sub> =1 correspond to 10<sup>9</sup> bacteria approximately).

Bacteria suspensions were added to mammalian cells for 1h, cells were then washed and non-invasive bacteria were killed by adding 20 µg/ml gentamicin for 1h30. After washing (x3), cells were lysed in 0.2 % Triton X-100 at different desired time points (0h, 1h30, 3h, 5h, 7h and 8h30), and the number of viable bacteria released from the cells was assessed after serial dilutions of the lysates on BHI agar plates. Supernatants were collected at the different time points to determine cytokine production. For electron microscopy observations, infected cells were recovered and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C.

#### Electron microscopy

Fixed samples were rinsed three times with 0.1M cacodylate buffer, postfixed with 2% osmium tetroxide, 1% potassium ferricyanide in 0.1M cacodylate buffer for 1h at room temperature, and washed again with 0.1 M cacodylate buffer and water. The samples were dehydrated through a graded series of ethanol, one time with propylene oxide and overnight in a mixture of Epon 812 / propylene oxide at room temperature. After embedded in Epon 812, samples polymerized for 48h at 60°C. Ultrathin sections (70 to 80 nm) were cut with a diamond knife, stained with uranyl acetate and Reynolds lead citrate and viewed in a Jeol JEM 1010 (Jeol, Tokyo, Japan) transmission electron microscope at 80 kV using an Eloïse Mega View III camera and AnalySIS Pro Software version 3.1 (Eloïse SARL, Roissy, France).

#### Mice experiments

Male mice six to ten week old were used for this study. C57/BL6J were purchased from Janvier (Le Genest, France) or Charles River (L'Arbresle, France). TLR2<sup>-/-</sup> and MyD88<sup>-/-</sup> mice initially provided by S. Akira (Osaka, Japan) were further backcrossed in C57/BL6J



to reach the 8<sup>th</sup> backcross by Michel Chignard (Institut Pasteur). *Nod1*<sup>-/-</sup> were generated under specific pathogen-free conditions in the animal facilities of the Institut Pasteur (Paris), from breeding pairs that had been kindly supplied by J. Bertin (Millenium Inc, MA). *Nod2*<sup>-/-</sup> were initially provided by J.-P. Hugot (Hôpital Robert Debré, Paris, France). *Nod1*<sup>-/-</sup> and *Nod2*<sup>-/-</sup> were further backcrossed in C57/BL6J to reach the 8<sup>th</sup> backcross by Stephen Girardin (Institut Pasteur). *iFABP-hEcad* transgenic mice were generated at the Institut Pasteur and were previously described (18). Animals were housed in polycarbonate cages in isolators and fed a commercial pellet diet with water *ad libitum*. Animal handling and experimentation was performed in accordance with institutional guidelines and current French legislation (Law No. 87-848) and European regulations on Animal Welfare and with Public Health Service recommendations. LD50 were performed after IV challenge of mice with increasing doses of wild type EGDe and its *pgdA* mutant. Survival was monitored during three weeks and LD50 were calculated as previously described (40).

Kinetics of infection was monitored by IV in C57/BL6J mice at 72 h after challenge with a sub-lethal dose of strain EGDe and its *pgdA* mutant ( $5 \times 10^3$  bacteria per mouse). Oral infections were performed as described previously (18). Female *iFABP-hEcad* transgenic mice (6- to 8-week-old) starved for 24 h were injected intragastrically with  $5 \times 10^9$  bacteria mixed with PBS 150 mg/ml CaCO<sub>3</sub>. At 3, 24, 48 or 72 h after infection, the organs were dissected. The small intestine was rinsed and incubated for 2 h in 100 mg/l gentamicin to kill extracellular bacteria from the intestinal lumen. Monitoring of *Listeria* in the intestinal lumen was done by determining the bacterial load of *Listeria* per gram of feces using a *Listeria* selective media (Oxford media, Oxoid).

Cytokine production.

Supernatants of macrophages (RAW264.7 and PEM) infected by strain EGDe and isogenic *pgdA* mutant were used to determine the production of IL-6, IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\beta$ . IL-6, IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\beta$  amounts were determined using the Quantikine mouse IL-6, IL-1 $\beta$ , TNF- $\alpha$  (R&D Systems) and mouse interferon Beta (PBL Biochemical Laboratories) ELISA kits, respectively, as recommended by the manufactures.

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## **Abbreviations**

Electron microscopy, EM; high-pressure liquid chromatography, HPLC; interferon- $\beta$ , IFN- $\beta$ ; interleukin-6, IL-6; intravenous, IV; lipoteichoic acid, LTA; myeloid differentiation factor 88, MyD88; nucleotide-binding oligomerization domain, Nod; pathogen associated molecular pattern, PAMP; peritoneal macrophage, PEM; peptidoglycan, PG; pattern recognition receptor, PRR; TOLL-like receptor, TLR

## Figures Legend

**Figure 1.** Characterization of *Listeria* peptidoglycan. (A) Each muropeptide peak highlighted by a number was purified by HPLC, desalted and analyzed by MALDI-TOF. Structural assignment was done by muropeptide fragmentation using MALDI-PSD. The structure of major muropeptides is indicated by full arrows. Structures or substructures in red and black indicate fully acetylated and *N*-deacetylated moieties, respectively, of the different muropeptides. Peaks 1 to 22 correspond to monomeric muropeptides, peaks 23 to 44 to dimeric muropeptides and 46 and over to trimeric muropeptides. Around 50 % of the muropeptides present a glucosamine residue instead of the canonical *N*-acetylglucosamine residue. (B) HPLC analysis of the muropeptide composition of *Listeria* wild type EGDe strain and its *pgdA* isogenic mutant. Each muropeptide peak was purified by HPLC and analyzed by matrix-assisted laser desorption ionization post-source decay (MALDI-PSD) mass spectrometry. Muropeptide peaks indicated with an asterisk correspond to *N*-deacetylated muropeptides. *N*-deacetylated muropeptides characteristic of the parental strain EGDe were completely absent from the elution pattern of the *pgdA* mutant. The abbreviations stand for: GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine; M, *N*-acetylmuramic acid, TriPDAP, L-alanyl- $\gamma$ -D-glutamyl-*meso*-diaminopimelic acid; TriPDAPNH<sub>2</sub>, L-alanyl- $\gamma$ -D-glutamyl-amidated *meso*-diaminopimelic acid; TetraPDAP, L-alanyl- $\gamma$ -D-glutamyl-*meso*-diaminopimelyl-D-alanine; TetraPDAPNH<sub>2</sub>, L-alanyl- $\gamma$ -D-glutamyl-amidated *meso*-diaminopimelyl-D-alanine.

**Figure 2.** Effect of lysozyme on growth and impaired survival in macrophages of the *pgdA* mutant. Strain EGDe and its isogenic *pgdA* mutant were grown in BHI media and incubated with lysozyme (10 µg/ml) or the human serum amidase (1 µg/ml). The *pgdA* mutant was selectively sensitive to the action of lysozyme upon entry into stationary phase (A). Decrease of the optical density of the *pgdA* mutant correlated with a loss of viability (B) while the parental strain was insensitive to lysozyme. Lysozyme induced cell rounding of the *pgdA* mutant (see supplementary Figure 2). RAW264.7 macrophages (C) and PEM (D) were infected with wild type EGDe and its *pgdA* mutant. Sensitivity of the *pgdA* mutant to lysozyme correlated with its impaired survival in macrophages.

**Figure 3.** Impaired survival of the *pgdA* mutant in macrophages. Panels A and B illustrate RAW264.7 cells after 8 h of infection with the parental strain EGDe and the *pgdA* mutant, respectively. Impaired survival was correlated with delay in escape of the *pgdA* mutant (C) from phagosomes compared to the wild type strain EGDe (D). Panels E to F illustrate peritoneal macrophages (PEM) after 7 h of infection with the parental strain EGDe (E) and the *pgdA* mutant (F). The scale bars correspond to 1 and 2 µm for the inserted and for the main pictures, respectively. Impaired survival correlated with delay in escape from phagosomes and bacterial lysis of the *pgdA* mutant compared to the wild type strain EGDe.

**Figure 4.** Impaired virulence of the *pgdA* mutant *in vivo*. BALB/c and C57/BL6J mice were challenged by intra-venous injection with the parental strain EGDe and its *pgdA* mutant with a sub-lethal dose ( $5 \times 10^3$  CFU per mice). After 72 h, mice were sacrificed

and bacterial counts in the liver (A) and the spleen (B) were determined. Transgenic human E-cadherin were used as model for the oral route of infection and colonization of several organs was followed after 3 h, 24 h, 48 h and 72 h post-challenge. Interestingly, the *pgdA* mutant was particularly vulnerable to persistence in the intestinal lumen as assayed by bacterial counts per grams of feces (C). Survival was more robust in the intestine (D) and the mesenteric lymph nodes (E) where colonization was particularly impaired after 72 h. The mutant was impaired in the survival of the liver (F) and spleen (G) at all time points as in the IV model (A and B).

**Figure 5.** Enhanced inflammatory response of the *pgdA* mutant. Cytokine production such as IL-6 (A) and IFN- $\beta$  (B) by RAW264.7 macrophages was enhanced by the *pgdA* mutant compared to the parental strain EGDe (see also supplementary Figure 4). PEM of wild type C57/BL6J, Nod1<sup>-/-</sup>, Nod2<sup>-/-</sup>, TLR2<sup>-/-</sup> and MyD88<sup>-/-</sup> mice were infected with strains EGDe and its isogenic *pgdA* mutant. After 7 h of infection, the inflammatory response was enhanced as measured by the amount of IL-6 (C) and, particularly, IFN- $\beta$  (D) production. The cytokine response was mainly dependent on TLR2 and Myd88 although their contribution varied according to the cytokine measured. IFN- $\beta$  production depended almost entirely on TLR2 in a MyD88-dependent and -independent manner (D). IL-6 production was only partially dependent on TLR2 and Myd88 (C), and required also Nod1. Surprisingly, Nod1 seemed to function as an inhibitor of IFN- $\beta$  production, since the *pgdA* mutant induced three folds more IFN- $\beta$  in Nod1<sup>-/-</sup> compared to C57/BL6J peritoneal macrophages (D). Note that the role of Nod1 is entirely restricted to the



response to the *pgdA* mutant and does not participate in the response to the wild type EGDe strain.