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Listeria monocytogenes PdeE, a phosphodiesterase that contributes to virulence and has hydrolytic activity against cyclic mononucleotides and cyclic dinucleotides

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ABSTRACT

We have identified and partially characterized a putative HD domain hydrolase, LMOf2365_2464, which is highly expressed during listerial intracellular replication. LMOf2365_2464 is annotated as a putative HD domain-containing hydrolase. The ability of an isogenic mutant strain, F2365Δ*2464*, to adhere, invade and replicate in intestinal epithelial cells (Caco-2) was significantly lower than parent strain F2365. Colonization of mouse liver and spleen by *L. monocytogenes* F2365 was significantly higher than it was for the mutant. The recombinant protein showed phosphodiesterase activity in the presence of divalent metal ions, indicating its role in nucleotide metabolism. It has activity against several cyclic nucleotides and cyclic dinucleotides, but its strongest activity is against cyclic di-AMP and cyclic AMP. Based on this enzymatic activity, we designated LMOf2365_2464 phosphodiesterase E (PdeE).

1. Introduction

Survival and replication of *Listeria monocytogenes* in the intracellular environment is an essential step during listeriosis, which is the second most common cause of death from foodborne infections in the U.S. [1]. Coordinated regulation of virulence factor expression is critical to listerial intracellular survival. PrfA is a well-characterized regulatory protein that controls expression of listerial genes for intracellular survival. Expression of PrfA is regulated by sigmaB, which also regulates expression of listerial stress response genes [2,3].

Nucleotide signaling molecules have important regulatory functions for bacterial gene expression. For example, guanosine tetraphosphate (ppGpp) regulates sigma factor binding to core RNA polymerase and controls the bacterial stringent response [4]. Cyclic-di-nucleotides are unique to bacteria and archaea, and they often regulate bacterial gene expression related to virulence [5]. Cyclic-di-GMP is important in regulating virulence, motility, and biofilm formation in several bacterial species [6–10]. In particular, it tends to activate biofilm formation and inhibit motility [5]. In *Vibrio cholerae*, c-di-GMP inhibits virulence gene expression and is involved in transition from adaptation to an aquatic environment to survival in the human host [11]. C-di-GMP also inhibits virulence gene expression in *Salmonella*, particularly expression of virulence factors needed for resistance to oxygen radicals [12], and it inhibits virulence in *Pseudomonas aeruginosa* as well [7]. Intrabacterial levels of c-di-GMP are controlled by the action of diguanylate cyclases with GGDEF domains and by two types of c-di-GMP-specific phosphodiesterases: one type containing an EAL domain and one type containing a HD-GYP domain [13].

Similar to other bacteria, *L. monocytogenes* produces c-di-GMP, but it also produces a second signaling cyclic dinucleotide, cyclic-di-AMP [14–17]. C-di-GMP regulates expression of listerial exopolysaccharide, which protects the pathogen from disinfectants and inhibits liver colonization, and it inhibits invasion of enterocytes [16,17]. In *L. monocytogenes*, intrabacterial c-di-GMP is regulated by diguanylate cyclases containing GGDEF domains (DgcA, DgcB, and DgcC) and phosphodiesterases with EAL domains (PdeB, PdeC, and PdeD); its genome does not encode phosphodiesterases with HD-

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GYP domains [16]. Through the activity of di-adenylate cyclase DacA and phosphodiesterase PdeA, c-di-AMP regulates establishment of listerial infection by controlling growth rate, intracellular growth, stress response, and cell wall stability [15]. PdeA represents a different type of c-di-AMP-specific phosphodiesterases that contains a GGDEF-like domain and a DHH domain. Another phosphodiesterase, PgpH, also has high affinity and specificity for c-di-AMP and is the primary hydrolase that degrades c-di-AMP during growth in broth [18].

In the current study, we show that a putative HD domain hydrolase, LMOf2365_2464, which is highly expressed during listerial intracellular replication [19], contributes to virulence in mice. LMOf2365_2464 is a 215 amino acid protein with a conserved HD domain, but it is in a poorly characterized family of proteins that are distinct from the previously described listerial phosphodiesterases PdeA, PdeB-D, and PgpH. HD domain is associated with phosphohydrolases and has highly conserved histidine and aspartate residues. HD domain proteins require coordination of divalent cations for activity, and they hydrolyze a variety of substrates containing a phosphodiester bond including nucleotide triphosphates, nucleotide diphosphates, cyclic dinucleotides, and cyclic mononucleotides [20]. We observed that LMOf2365_2464 plays a role in listerial intracellular survival and replication in both macrophage (J774A.1) and intestinal epithelial cells (Caco-2). Recombinant LMOf2365_2464 protein demonstrated phosphodiesterase activity in the presence of divalent cations, and the protein was designated phosphodiesterase E (PdeE).

2. Materials and methods

2.1. Ethics statement

All mice experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University.

2.2. Bacterial strains and growth conditions

Listeria monocytogenes serotype 4b strain F2365 was grown in Brain Heart Infusion (BHI) broth or agar plates at 30 °C, 37 °C, and 42 °C, depending on the experiment. *Escherichia coli* was grown in Luria-Bertani (LB) broth and agar at 37 °C. Ampicillin was used at 100 μg/ml for *E. coli*, and erythromycin was used at 300 μg/ml for *E. coli* and 5 μg/ml for *L. monocytogenes*. Chloramphenicol was used at 10 μg/ml for *L. monocytogenes*.

2.3. Mutagenesis

Mutagenesis was achieved by allelic exchange of *lmof2365_2464* with a mutated allele. Overlap extension PCR was used to construct a *lmof2365_2464* deletion [21]. Two independent PCR reactions were conducted with primers A (5′-AAACCTTTTAGGAATGATGGG-3′) and B (5′-ATTATCCCTCCATCCTTCGTTTTA-3′) in one reaction and primer C (5′-TAAAACGAAGGATCGAGGGATAA-TAAAAGGGGGAGGAAAGTAAGATGG-3′) and D (5′-TTCTTTATCGGTCCGATTCTGGTA-3′) in another reaction. The two PCR products were diluted and mixed, and a gene deletion fragment was amplified using primers A and D. The amplified gene deletion was cloned into pAUL-A [2], a temperature sensitive shuttle vector. The recombinant pAUL-A plasmid was transferred into F2365 by electroporation (100 Ω, 25 μF and 2.5 kV) and selected with erythromycin at 30 °C for 48 h. Colonies were passed three times on BHI plates with erythromycin at 42 °C for 48 h to allow integration. The integrants were then grown in BHI for 8 h at 30 °C to generate gene deletion excisants.

For complementation, primers LM2464_Comp-F01 (5′-AAA-GAGCTCAACGAAGGATGGAGGGATAA-3′) and LM2464_Comp-R01 (5′-AAAGTCGACTTTTTACGCGTCTCGCAATG -3′) were used to amplify the *lmof2365_2464* gene (672 bp) from *L. monocytogenes* F2365 genomic DNA. Amplified product was digested with *Sac*I and *Sal*I and cloned into pPL2 integration vector [22]. Electrocompetent F2365Δ*2464* mutant strain was transformed with pPL2-*2464*, and positive transformants were selected by spreading on BHI agar with chloramphenicol. Chromosomal integration was confirmed using PCR and DNA sequencing.

2.4. Growth kinetics

Growth of F2365, F2365Δ*2464*, and F2365Δ*2464*::pPL2-2464 in BHI broth and HTM minimal media supplemented with tryptone [23] at 37 °C was compared. HTM media was prepared as described [24] and supplemented with 1.0 mg/ml Bacto-Tryptone (Difco Laboratories, Detroit, MI). Overnight cultures of each strain in BHI were adjusted to an optical density at 600 nm (OD_{600}) of 0.1 and diluted 1:100 into 20 ml BHI or 1:20 into HTM media containing tryptone. OD_{600} was measured at 30 min intervals until onset of stationary phase in BHI; OD_{600} was measured at 0, 4, 8, 12, 16, 20, 24 and 48 h in HTM media plus tryptone. All growth experiments were performed in duplicate in two independent experiments (four replicates total).

2.5. Mouse virulence assay

Animal studies were conducted under the approval of the Mississippi State University Institutional Animal Care and Use Committee (protocol #10–055). Mice were monitored at least three times daily to evaluate clinical expression of disease. Mice that were moribund were humanely euthanized by inhalation of CO2. Moribund mice were defined as those that were not responsive to handling or were immobile/unable to eat or drink. There were no unexpected mortalities during this study. Mice were restrained manually in one hand, and animals were restrained only as long as necessary to accomplish an intraperitoneal injection of 0.1 ml via 25 g needle.

Virulence of mutant strain F2365Δ*2464* was compared to F2365 [25]. F2365 and F2365Δ*2464* were grown to OD_{540} 1.35 and diluted in 0.9% saline. Four groups of 6–8 week old female A/J mice (Jackson Laboratory) [26], five mice per group, were inoculated intraperitoneally with 100 μl of appropriate dilutions of bacteria (1.674×10^2, 1.674×10^3, 1.674×10^4, and 1.674×10^5) for each strain. One group of five mice was inoculated with 100 μl sterile saline, and another group was not injected. Colony forming units (CFUs) per gram of tissue were determined for liver and spleen at 7 days post-infection.

2.6. Cell culture

Human colon carcinoma enterocyte-like epithelial cells (Caco-2) were grown in Eagle's Minimum Essential Medium (MEM) supplemented with 20% heat inactivated fetal bovine serum (FBS). J774A.1 murine macrophages were grown in DMEM complete medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Cells were passaged when they reached 70–80% confluency. All the cell lines used in this study were grown at 37 °C and 5% CO_2.

2.7. Adhesion assay

Two days prior to the assay, Caco-2 cells were seeded in 12 well plates with approximately 10^5 cells per plate. On the day of assay,