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Anti-*Listeria* Activities of *Galleria mellonella* Hemolymph Proteins▽†

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We report the use of antimicrobial hemolymph proteins from the model host *Galleria mellonella* as an inhibitor for various *Listeria* strains, providing a novel source for antilisterial therapeutics. We also have shown that specific virulence-associated genes known to mediate antimicrobial resistance of *Listeria* in mammalian models indicated a similar function in *Galleria*.

The Gram-positive bacterium *Listeria monocytogenes* is able to cause food-borne infections in humans, such as listeriosis, which develops into fatal sepsis, meningitis, and meningoencephalitis (7, 12, 15). Listeriae are ubiquitously distributed in the environment and are resistant to extreme food-manufacturing processes. Recently, *L. monocytogenes* contamination in a food plant in Canada resulted in 23 casualties, along with 57 confirmed cases (Public Health Agency of Canada; www.phac-aspc.gc.ca). The pathogenic potential of *Listeria* is further attributed to a growing number of strains resistant to antimicrobial compounds and particularly to antibiotics (5, 19). Thus, it has become extremely important to identify novel sources for antilisterial therapeutics of both clinical and industrial interest.

The mechanisms of bacterial resistance against cationic antimicrobial peptides (CAMPs) are not clearly understood, and there are only few reports relating antimicrobial sensitivity of *Listeria* (5, 19). Identification of the two-component system *virR/virS* in *Listeria* revealed its dual role in virulence and resistance against CAMPs (13, 20). It is known that the transcriptional regulator VirR independently regulates expression of *mprF* and the genes comprising the *dlt* operon, which are well known for providing resistance against CAMPs of both animal and bacterial origin (2, 3, 20, 22). MprF synthesizes the lysylphosphatidyl glycerol membrane phospholipids (22), and the *dlt* operon (comprising of the genes *dltA*, *dltB*, *dltC*, and *dltD*)-encoded proteins are mainly responsible for adding D-alanine residues to the cell wall-associated lipotechoic acids (LTAs) (1). Both of these candidate genes maintain the positive charge balance of the bacterial cell wall, facilitating CAMP resistance.

Recently, we reported that following innate immune induction, the hemolymph of the lepidopteran greater wax moth *Galleria mellonella* produces antimicrobials which inhibited *L. monocytogenes* growth (16). Using the *Galleria* model, we have represented the comparative virulence attributes of different *Listeria* species and *L. monocytogenes* serotypes, similar to the findings with other mammalian models. However, *Galleria* can resist septic *L. monocytogenes* infection, and a very high 50% lethal dose (LD_{50}) value (10^6 CFU/larva) is required for larval mortality. Here we report that the different *Listeria* species and *L. monocytogenes* serotypes are sensitive to the induced antimicrobial hemolymph proteins of *Galleria*. Accordingly, our findings demonstrate a similar role of virulence-associated genes like *virR*, *dltB*, and *mprF* in antimicrobial resistance against *Galleria* hemolymph, comparable to the earlier findings using mammalian models (1, 13, 22).

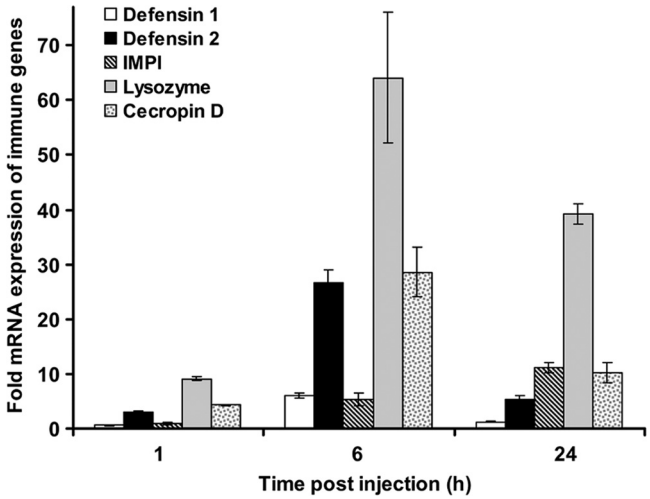

FIG. 1. Semiquantitative induction of immune-responsive genes in *Galleria* after challenge with heat-killed bacteria. The transcription levels of defensin 1, defensin 2, IMPI, lysozyme, and cecropin D following injection of heat-killed *L. monocytogenes* were determined by quantitative real time RT-PCR analysis and are shown relative to the expression levels of mock-injected animals. Values were normalized on expression levels of the housekeeping gene 18S and represent the results of three independent determinations ± SD.

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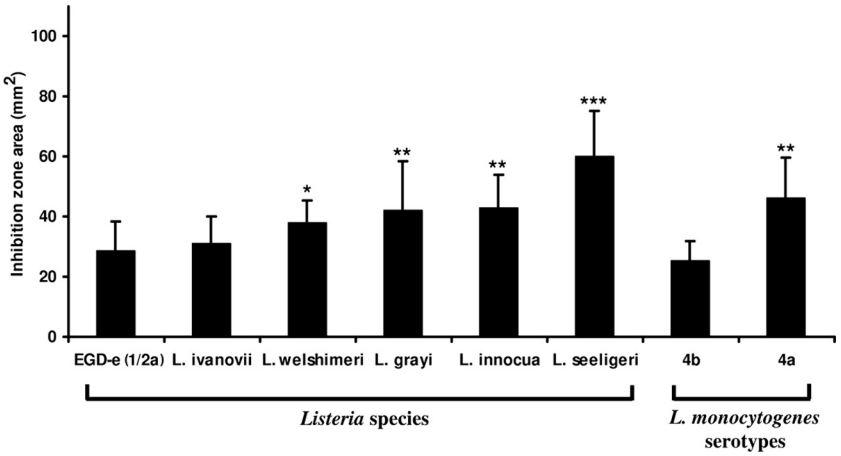

FIG. 2. The hemolymph of preimmune activated larvae produces antimicrobial effectors that inhibit the growth of various *Listeria* spp. Growth of the nonpathogenic *Listeria* species *L. seeligeri*, *L. innocua*, *L. grayi*, and *L. welshimeri* is significantly inhibited with respect to that of the pathogenic strain EGD-e. There were no significant changes in growth inhibition between *L. ivanovii* ($P < 0.2$) and the *L. monocytogenes* serotypes 4b ($P < 0.2$) and 1/2a. Only the *L. monocytogenes* serotype 4a shows high sensitivity toward antimicrobial effectors from *Galleria* hemolymph. A hemolymph sample of an individual larva was tested for all *Listeria* species, and each repetition contained hemolymph samples from at least 20 larvae. Results represent mean values for at least three independent experiments per strain using 20 larvae (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

Preparation of bacterial cultures, rearing of *Galleria*, injection methods, RNA isolation, reverse transcriptase PCR (RT-PCR), and antimicrobial assays were performed as described by Mukherjee et al. (16). For RT-PCR analysis, we used appropriate primers to amplify the housekeeping gene 18S RNA, lysozyme, insect metalloproteinase inhibitor (IMPI), defensin 1, and defensin 2, as described previously (16). In the case of cecropin D amplification, we used the primers cecropin D-forward (5′-GCCATGTTCTTCACCACGAC-3′) and -reverse (5′-TCAGTCACCGCCTTTAATGAT-3′), respectively.

It is known that innate immune activation leads to production of antimicrobial peptides in *Galleria* (6, 23). Following administration of immune elicitors such as heat-killed *Listeria*, we found induction of antimicrobial peptide-encoding genes like galiomycin (defensin 1), gallerimycin (defensin 2), IMPI, lysozyme, and cecropin D (Fig. 1). Transcriptional activation is represented as the fold change of immune-related genes in challenged *Galleria* relative to those in unchallenged larvae and normalized using the housekeeping gene 18S. The amounts of immune-related mRNAs of gallerimycin and lysozyme were

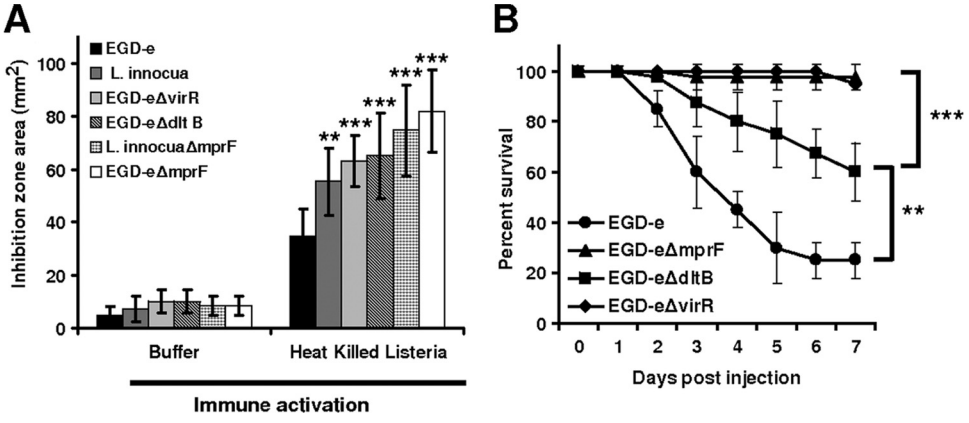

FIG. 3. Growth inhibition and survival of listerial isogenic mutants compared to their wild types in the *Galleria mellonella* infection model. (A) In response to the hemolymph samples obtained from larvae injected with heat-killed *Listeria*, pathogenic EGD-e and its isogenic mutants EGD-eΔ*mprF*, EGD-eΔ*virR*, and EGD-eΔ*dltB*, along with *L. innocua* and *L. innocua*Δ*mprF*, showed significant high growth inhibitions in comparison to the hemolymph samples obtained from larvae injected with buffer ($P < 0.0005$). Significantly high growth inhibition was observed for EGD-eΔ*mprF*, followed by EGD-eΔ*virR* and EGD-eΔ*dltB*, with respect to the pathogenic EGD-e strain. Also, the *mprF* deletion strain of *L. innocua* showed high sensitivity to the activated hemolymph proteins of *Galleria* in comparison to that of its wild-type strain ($P < 0.0005$). A hemolymph sample of an individual larva was tested for all *Listeria* mutants, and each repetition contained hemolymph samples from at least 20 larvae. Results represent mean values of at least three independent determinations ± SD (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). (B) Inoculation with 10^6 CFU/larvae EGD-eΔ*virR* (◆) and EGD-eΔ*mprF* (▲) resulted in significantly lower mortality rates of *Galleria* larvae than infection with the wild type EGD-e strain (●). The EGD-eΔ*dltB* (■) strain caused only a partial attenuation in *Galleria*, as shown by intermediate mortality rates in respect to infection with EGD-e. Results represent mean values of at least three independent determinations ± SD from each of 20 animals per treatment (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

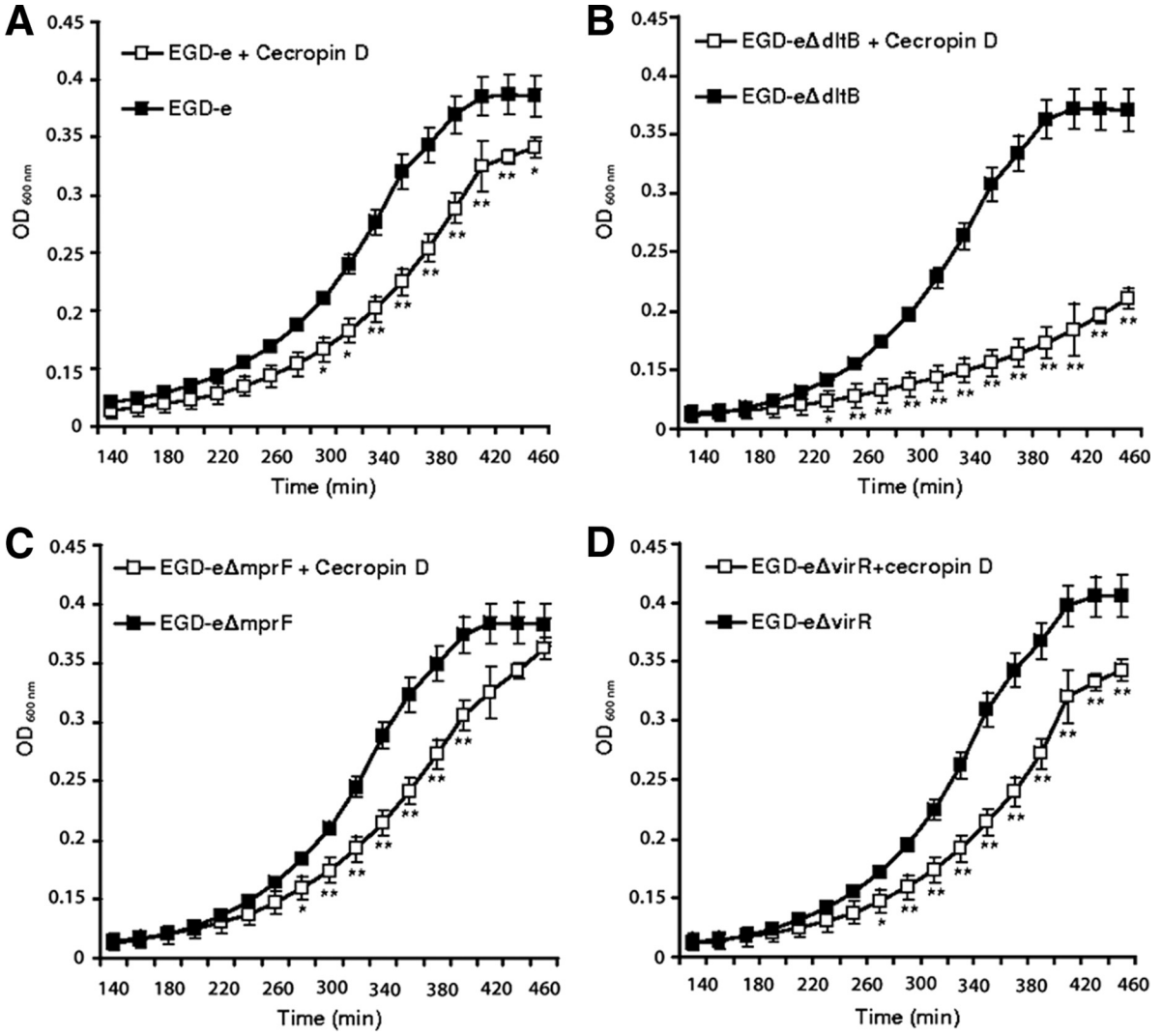

FIG. 4. Growth of the *L. monocytogenes* EGD-e wild type (A) or of the deletion mutant EGD-eΔ*dltB* (B), EGD-eΔ*mprF* (C), or EGD-eΔ*virR* (D) in the presence of the *Galleria* cationic peptide cecropin D. Bacteria were cultured in BHI broth supplemented with 160 μM cecropin D, and the optical density at 600 nm (OD_{600}) was measured hourly. Results represent mean values of at least three independent determinations ± SD (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

found to be induced about 3.0-fold and 9.1-fold at 1 h postinjection, respectively. At 6 h postinjection, we determined increased mRNA levels of IMPI (~5.2-fold), galiomycin (~6.0-fold), gallerimycin (~26-fold), cecropin D (~28-fold), and lysozyme (~64-fold). The amounts of induced mRNA for IMPI, cecropin D, and lysozyme were found to be 11-fold, 10-fold, and 39-fold after 24 h postinjection. After this time point, the hemolymph samples were collected and tested for their antimicrobial activities against human-pathogenic *L. monocytogenes*.

Induction of CAMPs and their secretion into the hemolymph resulted in growth inhibition of *Listeria* in brain heart infusion medium (BHI). All *Listeria* species tested in this study were sensitive to the antimicrobial hemolymph proteins of *Galleria* (Fig. 2). Nonpathogenic *Listeria*, such as *Listeria seeligeri* (21), showed highest sensitivity, followed by *L. innocua* (8), *L. grayi* (10), and *L. welshimeri* (9) with respect to pathogenic *L. monocytogenes* (Fig. 2). No significant difference in growth inhibition was recorded between pathogenic *L. monocytogenes* and *Listeria ivanovii*. Both were least sensitive to the induced antimicrobials of *Galleria*. Among the different *L. monocytogenes* serotypes, the 4b strain, which is mainly responsible for *Listeria* epidemics, causes high mortality rates in *Galleria* and shows only low sensitivity to the hemolymph, along with the 1/2a strain. In contrast, the 4a serotype was significantly more sensitive to hemolymph antimicrobials than the other *L. monocytogenes* serotypes (Fig. 2). We also investigated the role of specific virulence-associated genes in *L. monocytogenes*, *virR*, *mprF*, and *dltB*, against the antimicrobial hemolymph proteins using isogenic deletion mutants (see the supplemental material). We found that *virR*, *mprF*, and *dltB* in *L. monocytogenes* are important factors to counteract the induced antimicrobial activities of *Galleria* hemolymph. The Δ*virR*, Δ*mprF*, and Δ*dltB* mutants showed significantly higher antimicrobial sensitivities than their isogenic wild-

type strain, EGD-e (Fig. 3A). However, deletion of *mprF* from nonpathogenic *L. innocua* (18) (see the supplemental material) also resulted in a more sensitive strain than its wild type (Fig. 3A).

Our infection studies with *Galleria* gave similar results, as was previously demonstrated in the mouse model. The strains EGD-eΔ*virR* and EGD-eΔ*mprF* (at a 10^6 CFU/larva concentration) were strongly attenuated, whereas EGD-eΔ*dltB* showed only a partial reduction in pathogenicity compared to the wild type (Fig. 3B). It has been shown that that deletion of the *dlt* operon from *Bacillus cereus* resulted in reduced survival of *Galleria* larvae upon oral infection (2). The varied *in vitro* and *in vivo* survivability of EGD-eΔ*dltB* in *Galleria* hemolymph and a whole-animal model possibly indicates the specialized role of other virulence-associated proteins, like MprF, in providing resistance against antimicrobial host defense, such as phagocytosis and cationic antimicrobial peptides.

Confirming the antimicrobial effects of *Galleria* hemolymph, we have tested the effect of cecropin D, a CAMP of the greater wax moth (4, 11), against pathogenic EGD-e and its isogenic deletion mutants, EGD-eΔ*mprF*, EGD-eΔ*dltB*, and EGD-eΔ*virR*. Cecropin is known for exhibiting strong antimicrobial activities, especially against Gram-positive bacteria (4, 11). Cecropin D was chemically synthesized by standard Merrifield solid-phase peptide synthesis (14, 17), purified by reverse-phase high-performance liquid chromatography (RP-HPLC), and controlled by electrospray ionization mass spectrometry (ESI-MS) (see Fig. S1A and B in the supplemental material). Purity was >98%, and the molecular weight (MW) was found to be 4,256.2 (theoretical MW, 4,255.9). Antimicrobial assays were performed as described by Cytryńska et al. and Kim et al. (4, 11). Growth inhibition in the presence of cecropin D was observed for all tested strains in BHI (Fig. 4A, B, C and D). However, the direct lethality associated with the growth inhibitions of *Listeria* following cecropin D treatment is yet to be reported. Similarly, the human cationic peptide LL37 and bacterial polymyxin B showed strong growth arrest of pathogenic EGD-e and its isogenic mutants, EGD-eΔ*mprF*, EGD-eΔ*dltB*, and EGD-eΔ*virR*, in BHI (see Fig. S2A and B in the supplemental material). The antilisterial effects of cationic peptides such as cecropin D indicate their great potential as new therapeutics and justify more efforts for the identification and production of other novel antimicrobial candidates for treating bacterial infections.

Conclusion. We report the contributions of the *mprF*, *virR*, and *dltB* genes to CAMP resistance in *Galleria mellonella*, indicating the involvement of the members comprising the VirR regulon for antimicrobial resistance of *Listeria*. The varied sensitivities of different *Listeria* species and serotypes to the hemolymph proteins advocate for the development of *Galleria*-derived antimicrobial peptides for therapeutic use to counteract the development of multidrug-resistant pathogens.

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