








On the Offensive: the Role of Outer Membrane Vesicles in the Successful Dissemination of New Delhi Metallo- β -lactamase (NDM-1)

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ABSTRACT The emergence and worldwide dissemination of carbapenemase-producing Gram-negative bacteria are a major public health threat. Metallo- β -lactamases (MBLs) represent the largest family of carbapenemases. Regrettably, these resistance determinants are spreading worldwide. Among them, the New Delhi metallo- β -lactamase (NDM-1) is experiencing the fastest and largest geographical spread. NDM-1 β -lactamase is anchored to the bacterial outer membrane, while most MBLs are soluble, periplasmic enzymes. This unique cellular localization favors the selective secretion of active NDM-1 into outer membrane vesicles (OMVs). Here, we advance the idea that NDM-containing vesicles serve as vehicles for the local dissemination of NDM-1. We show that OMVs with NDM-1 can protect a carbapenem-susceptible strain of *Escherichia coli* upon treatment with meropenem in a *Galleria mellonella* infection model. Survival curves of *G. mellonella* revealed that vesicle encapsulation enhances the action of NDM-1, prolonging and favoring bacterial protection against meropenem inside the larva hemolymph. We also demonstrate that *E. coli* cells expressing NDM-1 protect a susceptible *Pseudomonas aeruginosa* strain within the larvae in the presence of meropenem. By using *E. coli* variants engineered to secrete variable amounts of NDM-1, we demonstrate that the protective effect correlates with the amount of NDM-1 secreted into vesicles. We conclude that secretion of NDM-1 into OMVs contributes to the survival of otherwise susceptible nearby bacteria at infection sites. These results disclose that OMVs play a role in the establishment of bacterial communities, in addition to traditional horizontal gene transfer mechanisms.

IMPORTANCE Resistance to carbapenems, last-resort antibiotics, is spreading worldwide, raising great concern. NDM-1 is one of the most potent and widely disseminated carbapenem-hydrolyzing enzymes spread among many bacteria and is secreted to the extracellular medium within outer membrane vesicles. We show that vesicles carrying NDM-1 can protect carbapenem-susceptible strains of *E. coli* and *P. aeruginosa* upon

Citation Martínez MMB, Bonomo RA, Vila AJ, Maffia PC, González LJ. 2021. On the offensive: the role of outer membrane vesicles in the successful dissemination of New Delhi metallo- β -lactamase (NDM-1). *mBio* 12:e01836-21. <https://doi.org/10.1128/mBio.01836-21>.

Editor Martin J. Blaser, Rutgers University
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This article is a direct contribution from Robert A. Bonomo, a Fellow of the American Academy of Microbiology, who arranged for and secured reviews by Gary Dunny, University of Minnesota Medical School, and Shahriar Mobashery, University of Notre Dame.

Received 31 July 2021

Accepted 26 August 2021

Published 28 September 2021

treatment with meropenem in a live infection model. These vesicles act as nanoparticles that encapsulate and transport NDM-1, prolonging and favoring its action against meropenem inside a living organism. Secretion of NDM-1 into vesicles contributes to the survival of otherwise susceptible nearby bacteria at infection sites. We propose that vesicles play a role in the establishment of bacterial communities and the dissemination of antibiotic resistance, in addition to traditional horizontal gene transfer mechanisms.

KEYWORDS outer membrane vesicles, NDM-1 carbapenemase, cross-species protection, *Galleria mellonella*, *E. coli*, *P. aeruginosa*, metallo- β -lactamase, NDM

The abuse and/or misuse of antibiotics in veterinary and clinical practices is driving an increase of multidrug-resistant (MDR) bacterial strains, among which Gram-negative bacteria producing β -lactamases are becoming the most prevalent (1, 2). Carbapenems are a family of β -lactam antibiotics with the broadest spectrum of activity and greatest potency against Gram-negative bacteria. As a result, carbapenems are often used as last-resort drugs to combat MDR bacteria (3). However, their therapeutic efficacy is challenged by the dissemination of genes encoding carbapenemases, enzymes able to inactivate carbapenems (4). Carbapenemases are currently found in *Enterobacterales* and Gram-negative nonfermenters (5, 6). As a result, the World Health Organization has classified these resistant organisms as Priority 1 strains to steer research efforts to combat them. This scenario has been further aggravated in the context of COVID-19 since MDR strains are fueled by the administration of antibiotics to hospitalized patients (7).

Metallo- β -lactamases (MBLs) stand as one of the major groups of carbapenemases (8, 9). MBLs are Zn(II)-dependent enzymes able to hydrolyze and inactivate virtually all classes of β -lactam antibiotics. Despite active research in this area, commercially available MBL inhibitors are not presently available (10, 11). Among MBLs, the New Delhi metallo- β -lactamase (NDM-1) is continuing to show the fastest and widest geographical spread since its first identification in 2008 (12, 13). The dissemination of the NDM family thus raises a major public health concern.

Recent studies indicate that the wide dissemination of NDM-1 into different hosts can be accounted for by unique protein determinants of this enzyme (14). On one hand, unlike other MBLs, NDM-1 harbors an optimized signal peptide that is efficiently processed in a broad range of hosts without generating any fitness cost (15). On the other hand, NDM-1 is unusual among MBLs (and also compared to other β -lactamases) in that it is a lipoprotein anchored to the outer membrane (OM) of Gram-negative bacteria (16–18). This cellular localization endows the enzyme with two major advantages: (i) it protects NDM-1 from the action of periplasmic proteases under conditions of Zn (II) scarcity promoted by the host immune system during an infection, and (ii) it enables NDM-1 secretion into outer membrane vesicles (OMVs) (16). Indeed, NDM-1 is optimized to be selectively incorporated into OMVs as a folded and active enzyme in a broad range of clinically relevant pathogens (14).

OMVs are membrane-enclosed “bacterial packs” secreted by all Gram-negative bacteria (18). These vesicles are proposed to act as “care packages” within bacterial communities. OMVs have been associated with diverse functions including promotion of pathogenesis, bacterial survival under stress conditions, or regulation of microbial interactions, among others (18). OMVs can carry a wide range of molecules, including nucleic acids, virulence factors, resistance determinants, and multiple antigenic proteins (19). Purified OMVs containing β -lactamases exhibit potent hydrolytic activity against β -lactam antibiotics (16, 20). Moreover, OMVs from *Escherichia coli* carrying NDM-1 not only display carbapenemase activity but also can protect nearby populations of carbapenem-susceptible bacteria *in vitro* (16). The finding of vesicle-mediated transfer of plasmids (21) including the *bla*_{NDM-1} gene (22, 23) is of great concern since the synergy resulting from simultaneous gene and protein transport could lead to further dissemination of resistance. These results trigger

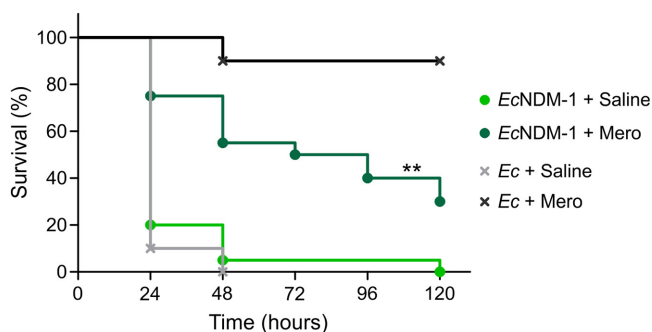


FIG 1 Survival curves of *G. mellonella* larvae infected with *E. coli* expressing NDM-1 (*EcNDM-1*) or *E. coli* transformed with empty pMBLe (*E. coli*). Each group was subsequently injected with meropenem at 10 mg/kg (Mero) or saline solution (Saline). The data were plotted using the Kaplan-Meier method, and comparisons between groups were made using the log rank test. **, $P < 0.01$, compared to the control (*E. coli* + Mero).

new questions. Can MBL-loaded vesicles protect susceptible bacteria *in vivo*? What is the extent of this mechanism of protection during an antibiotic treatment within a host? Is the amount of vesicles secreted at the infection sites enough to protect nearby populations of sensitive bacteria?

In order to unravel these questions, we investigated the effect of these vesicles *in vivo*, using the *Galleria mellonella* larva infection model. *G. mellonella* caterpillars are the larva stage of the greater wax moth. They have been extensively used to study fungal and bacterial infections (24–26). The larval immunity is remarkably like the human innate immune system (24, 27). Furthermore, this model allows working at 37°C, an important advantage compared to the widely used *Caenorhabditis elegans* model when studying human pathogens (28, 29). Additionally, *G. mellonella* larvae can be accurately injected with defined doses of bacteria, resulting in consistent survival/mortality rates (27).

Using this *in vivo* infection model, we show that OMVs loaded with NDM-1 enhance the survival of susceptible bacteria against treatment with meropenem and that NDM-1-harboring vesicles are more efficient than soluble NDM-1 in protecting bacteria. We also demonstrate that during a coinfection with a resistant *E. coli* strain expressing NDM-1 and a meropenem-susceptible *Pseudomonas aeruginosa* strain, the vesicles released by *E. coli* are able to protect *P. aeruginosa* from a meropenem treatment. We conclude that secretion of NDM-1 into vesicles contributes to the survival of otherwise susceptible nearby bacteria at infection sites. Based on these results, we posit that OMVs play a role in the establishment and development of bacterial communities, in addition to horizontal gene transfer mechanisms.

RESULTS

Expression of NDM-1 protects *E. coli* from a meropenem treatment in *Galleria mellonella*. Larvae infected with 2×10^6 CFU/larva of carbapenem-susceptible *E. coli* ATCC 25922 transformed with empty pMBLe (*E. coli*) were unable to survive after 48 h postinfection, while showing a survival rate of 90% when treated with meropenem (Fig. 1). Instead, *E. coli* ATCC 25922 cells transformed with the pMBLe-NDM-1 plasmid expressing NDM-1 (*EcNDM-1*) were able to infect the larvae under meropenem treatment, with less than 40% larva survival compared to the control group. This experiment shows that *E. coli* cells expressing NDM-1 are able to escape or resist the action of a carbapenem treatment in a *G. mellonella* infection model.

Protection of susceptible *E. coli* by OMVs-NDM-1. We then evaluated the possible toxicity of OMVs in *G. mellonella* larvae. We purified OMVs carrying NDM-1 (OMVs-NDM-1 here) from cultures of *EcNDM-1*, and as a control, we used enzyme-free OMVs (OMVs-EF) purified from *E. coli* cells transformed with the empty pMBLe plasmid. After injection of OMVs (14.64 mg of vesicles per kg of body weight of larva), the larvae

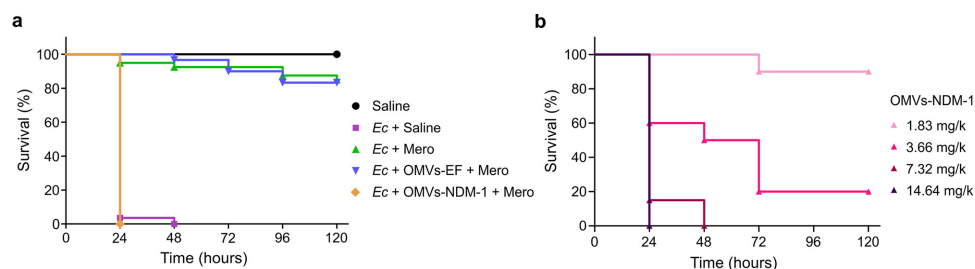


FIG 2 (a) Survival curves of *G. mellonella* larvae infected with *E. coli* + OMVs-EF or OMVs-NDM-1 and subsequently treated with meropenem (Mero) at 10 mg/kg. Saline, saline solution. (b) Survival curves of *G. mellonella* infected with *E. coli* plus different amounts of OMVs-NDM-1 and treated with 10 mg/kg meropenem 1 h postinfection.

showed the typical pigmentation due to the process of melanization of the insect immune response. Neither OMVs-NDM-1 nor OMVs-EF affected the viability of the larvae even 120 h after injection (see Table S1 in the supplemental material), confirming the viability of this infection model.

Larvae infected with *E. coli* and treated with meropenem showed a survival rate of 80%, while all the larvae that did not receive the antibiotic died after 48 h (Fig. 2a). Similar experiments were performed by adding OMVs-NDM-1 (14.64 mg/kg) and OMVs-EF. Injection of empty OMVs resulted in similar survival levels as those for infected larvae without the addition of vesicles. In stark contrast, injection of OMVs-NDM-1 led to 100% larval deaths at 24 h postinfection, revealing that the carbapenem-susceptible strain was able to thrive in the presence of meropenem (Fig. 2a). We conclude that OMVs loaded with active NDM-1 can protect susceptible bacteria *in vivo*.

The minimum amount of OMVs needed to provide carbapenem resistance was next determined by testing larval survival after administering different amounts of OMVs-NDM-1. As shown in Fig. 2b, 7.32 mg/kg of vesicles was able to provide resistance to meropenem administered in a single dose. Based on these results, we performed all experiments with this amount of OMVs-NDM-1.

OMVs-NDM-1 are stable in the larva hemolymph for at least 6 h. We next assessed whether NDM-1 in vesicles was able to maintain its activity after residing for long periods in larval hemolymph. For this purpose, we injected vesicles at different times before inoculation with *E. coli* (22, 6, and 0 h before inoculation) and evaluated the protective effect of OMVs-NDM-1 toward treatment with meropenem.

Injection of OMVs-NDM-1 22 h before *E. coli* inoculation resulted in 80% larva survival at the end of the assay (Fig. 3), i.e., a reduced protection against the antibiotic. In contrast, OMVs-NDM-1 administered 6 h before infection led to only 15% of larvae surviving at the end of the assay, a significant protective activity against meropenem. In this case, injection of OMVs-NDM-1 simultaneously with inoculation (0 h) resulted in no larva survival after 48 h. These results show that OMVs-NDM-1 are stable in the larva hemolymph, retaining significant bacterial protection against meropenem for at least 6 h. This allows us to discard the idea that the *G. mellonella* immune system response compromises the stability of OMVs for this period.

Encapsulation within the vesicle scaffold enhances the protective activity of NDM-1. NDM-1 is located in the lumen of vesicles as a lipoprotein anchored to the vesicle membrane. To assess whether the vesicle scaffold provides an advantage to NDM-1 regarding bacterial protection in *G. mellonella* compared to the free soluble protein, we performed experiments comparing OMVs-NDM-1 with equal amounts of soluble enzyme, normalized by immunoblotting (Fig. S1). For these experiments, OMVs-NDM-1 or soluble enzyme was administered 6 h before inoculation with *E. coli*.

As shown in Fig. 4, soluble NDM-1 elicited lower bacterial protection (greater larva survival and no significant difference from the control group at the end of the assay) than the vesicle-associated enzyme, which displayed a significant difference from the control group (*E. coli* + Mero). This suggests that NDM-1 inside OMVs is more effective

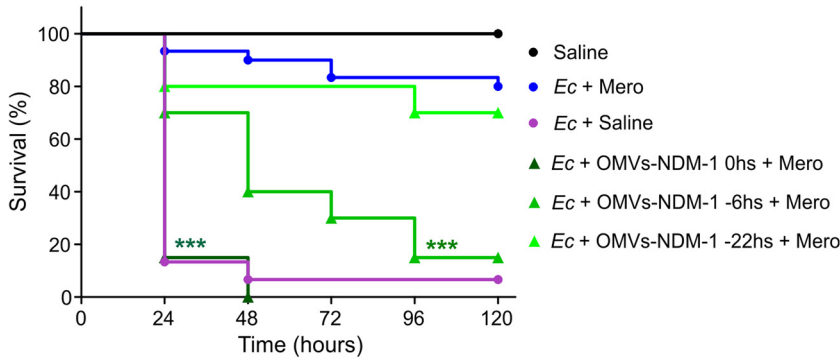


FIG 3 Effect of early injection of OMVs-NDM-1 in the hemolymph of larvae at different times before *E. coli* infection and subsequent meropenem treatment. The data were plotted using the Kaplan-Meier method, and comparisons between groups were made using the log rank test. The general statistical significance was established at $P < 0.05$, and then the Bonferroni correction was used to make comparisons of the groups against the control (*E. coli* + Mero), from which *** was defined as $P < 0.0001$. Mero, meropenem (10 mg/kg); Saline, saline solution.

in protecting a meropenem-susceptible strain than soluble free NDM-1 in the hemolymph.

OMVs-NDM-1 provide cross-species protection during a bacterial coinfection in *G. mellonella*. Next, we sought to evaluate the possible cross-species protection role of OMVs secreted by an NDM-1-producing strain on another susceptible bacterial species during coinfection upon antibiotic treatment. We chose *P. aeruginosa* PAO1 as the coinfecting susceptible strain, which is incapable of thriving on its own in the hemolymph of *G. mellonella* in the presence of meropenem (Fig. S2).

There are some caveats regarding this experiment, since a cross-protection phenomenon *in vivo* might result from one (or a combination) of four possible mechanisms: (a) hydrolysis of meropenem by *E. coli* cells expressing NDM-1 in the periplasm, (b) hydrolysis of meropenem by secreted OMVs-NDM-1, (c) an impaired *G. mellonella* innate immune system due to *E. coli* overgrowth that indirectly allows *P. aeruginosa* to grow, or (d) horizontal transfer of the *bla*_{NDM-1} gene from *E. coli* to *P. aeruginosa*. Regarding this last point, despite pMBLE being neither conjugative nor mobilizable, we transformed *P. aeruginosa* with a plasmid (pBBR1MCS-2) belonging to the same incompatibility group (16), reducing the possibilities of a *bla*_{NDM-1} transfer event.

In order to assess the mechanism of action of OMVs in cross-protection of *P. aerugi-*

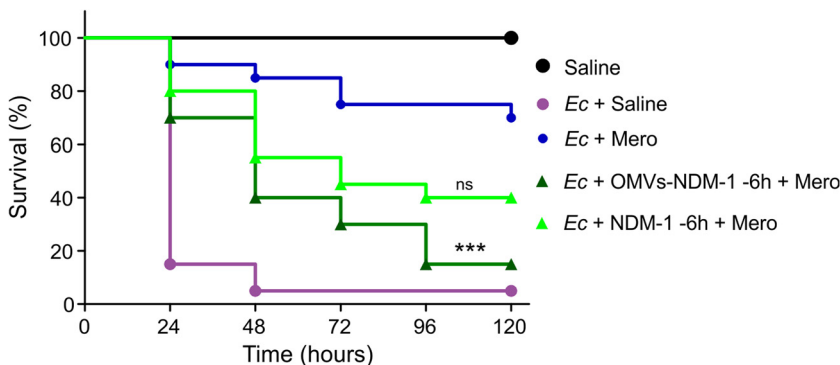


FIG 4 *In vivo* bacterial protection against meropenem of soluble free NDM-1 compared to OMVs-NDM-1. Vesicles containing NDM-1 or soluble protein were injected 6 h prior to infection with *E. coli*. The data were plotted using the Kaplan-Meier method, and comparisons between groups were made using the log rank test. The general statistical significance was established at $P < 0.05$, and then the Bonferroni correction was used to make comparisons of the groups against the control (*E. coli* + Mero), redefining the statistical significance as $P < 0.025$. Mero, meropenem (10 mg/kg); Saline, saline solution.

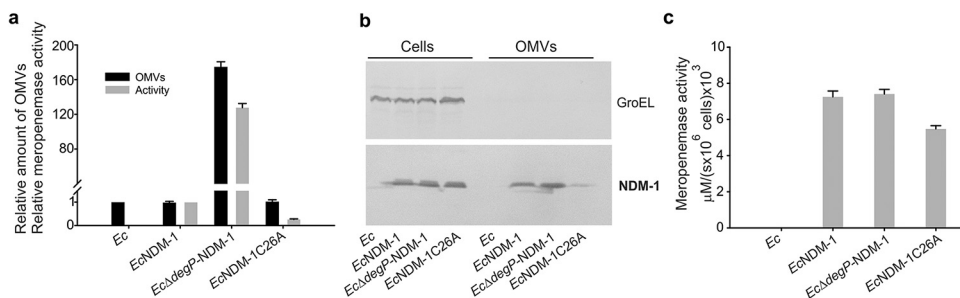


FIG 5 (a) Relative production of OMVs (black bars) and OMV meropenemase activity (gray bars). (b) Immunodetection of NDM-1 in bacterial cells and normalized amounts of OMVs purified from culture supernatants. GroEL detection was used as loading control for cells and as a cytoplasmic contamination marker for OMVs. (c) Meropenemase activity of *E. coli*, *EcNDM-1*, *EcΔdegP-NDM-1*, and *EcNDM-1C26A* cells washed from vesicles. The activity was normalized by the number of cells.

nosa, we constructed a series of *E. coli* variants with similar capacities to hydrolyze meropenem within the cell but able to secrete various amounts of NDM-1 in vesicles. We used two strains: (i) *EcΔdegP-NDM-1*, exhibiting a hypervesiculation phenotype due to deletion of the *degP* gene (30, 31), and (ii) *EcNDM-1C26A*, expressing a soluble variant of NDM-1 generated by a mutation that eliminates the lipidation site of NDM-1, resulting in smaller amounts of NDM-1 being secreted into vesicles (16).

Next, we measured the amount of vesicles secreted by these strains, the carbapenemase activity of these vesicles and inside the cells, and the levels of NDM-1 in vesicles and cells (measured by immunodetection). *EcΔdegP-NDM-1* showed a 180-fold-increased secretion of OMVs, as expected (Fig. 5a and b). These vesicles contained similar amounts of NDM-1 as did vesicles produced by strain *EcNDM-1*. *EcNDM-1C26A* secreted equal amounts of vesicles as *EcNDM-1* but with 5-fold less NDM-1. The variable amounts of secreted NDM-1 were also evident by measuring the meropenemase activity of the OMVs, indicating that NDM-1 is secreted as a fully active enzyme. On the other hand, cells of variants *EcNDM-1*, *EcΔdegP-NDM-1*, and *EcNDM-1C26A* displayed similar rates of meropenem hydrolysis after washing the OMVs (Fig. 5c), in good agreement with the levels of NDM-1 present in the cells (Fig. 5b) and MIC values of meropenem (Table S2). These results are summarized in Table S3. It is noteworthy that variations in the amount of secreted NDM-1 have no significant impact on the levels of intracellular enzyme as the amount of NDM-1 present in *EcNDM-1* cells is ~1,000 times higher than that secreted in vesicles (see Materials and Methods).

Then, we performed a series of coinfection experiments on meropenem-susceptible *P. aeruginosa* with the four *E. coli* strains in larvae. In these experiments, we measured the bacterial load within the larvae after 24 h of coinfection and treatment with meropenem (Fig. 5). In order to independently monitor the growth of each bacterium, we used a kanamycin-resistant *P. aeruginosa* strain (pBBR1MCS-2; Km^r), while all the *E. coli* strains used were gentamicin resistant (pMBLe plasmids; Gm^r). CFU of *E. coli* or *P. aeruginosa* was determined by plating each larva homogenate on LB agar supplemented with gentamicin or kanamycin, respectively. This allowed individual counting without interference from *G. mellonella* normal flora or the coinfecting bacterium.

We infected *G. mellonella* larvae with a mixture of *E. coli* cells expressing NDM-1 and susceptible *P. aeruginosa* cells and treated the larvae with meropenem. *P. aeruginosa* cells were able to survive despite the presence of meropenem (Fig. 6) with the different *E. coli* variants expressing NDM-1 (groups 1, 2, and 3), in contrast to cells coinfecting with carbapenem-susceptible *E. coli* (group 4). Larvae coinfecting with susceptible *E. coli* and without antibiotic treatment (5) presented an average count of 4×10^9 CFU/larva for *E. coli* and 5×10^8 CFU/larva for *P. aeruginosa* (group 5). Thus, in the absence of meropenem, both strains can cause infection within the larvae. Upon treatment with meropenem, a considerable reduction in the number of CFU was observed in

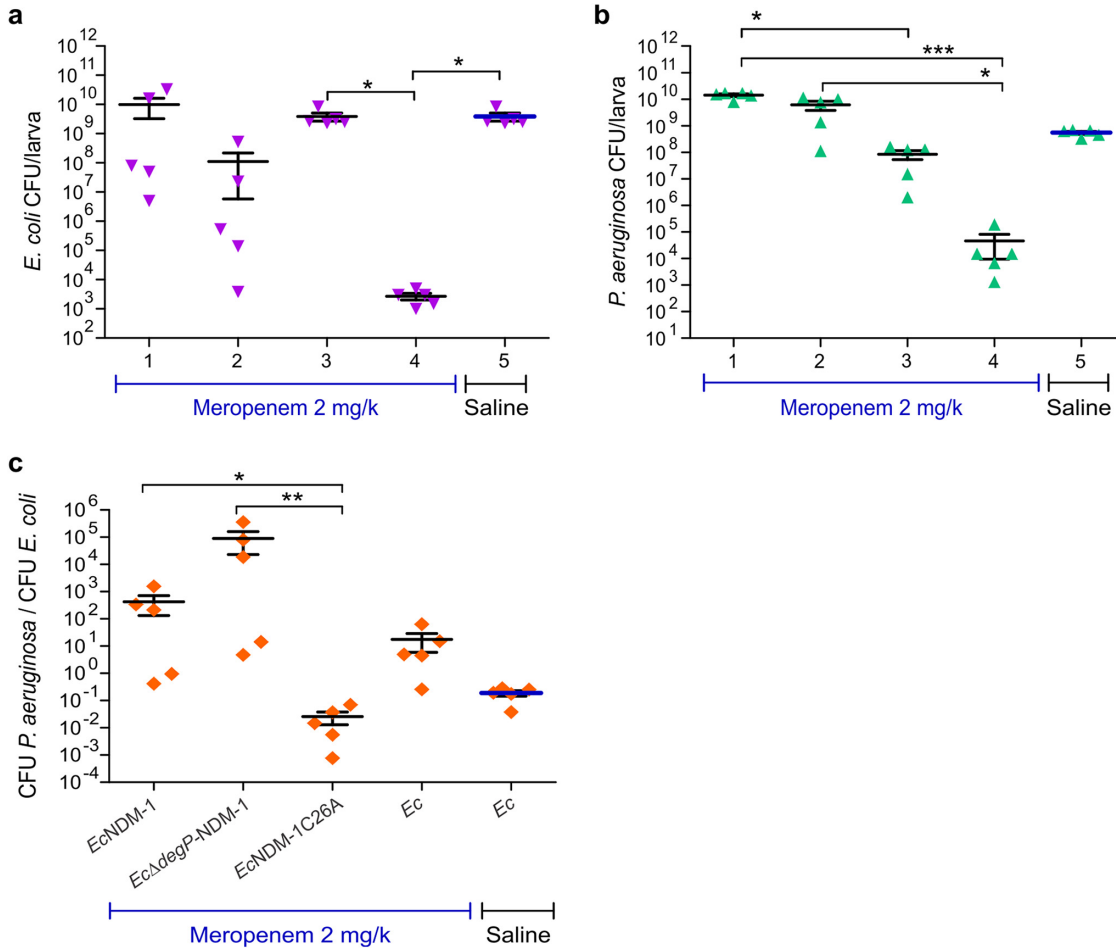


FIG 6 (a and b) *E. coli* (a) and *P. aeruginosa* (b) coinfection in *G. mellonella*. The scatter plot depicts the CFU for each individual larva at 24 h after the coinfection. (c) Protection index calculated as CFU *P. aeruginosa*/CFU *E. coli*, for each individual larva. Coinfection groups: (1) *Ec*NDM-1 + *P. aeruginosa*/meropenem at 2 mg/kg; (2) *Ec*Δ*degP*-NDM-1 + *P. aeruginosa*/meropenem at 2 mg/kg; (3) *Ec*NDM-1C26A + *P. aeruginosa*/meropenem at 2 mg/kg; (4) *E. coli* + *P. aeruginosa*/meropenem at 2 mg/kg; (5) *E. coli* + *P. aeruginosa*/saline solution. Kruskal-Wallis nonparametric statistical analysis: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (see Table S4 for further details). Mero, meropenem; Saline, saline solution.

both cases (≈ 4 log for *E. coli* and ≈ 3 log for *P. aeruginosa*) compared to the group without antibiotic, showing that meropenem is active in the coinfection model (group 4). On the other hand, the group of larvae coinfecting with *Ec*NDM-1 and treated with meropenem (group 1) showed $>10^8$ CFU/larva of *E. coli*, as a result of the resistance to meropenem caused by NDM-1. Interestingly, in this group the count of CFU/larva of *P. aeruginosa* showed an increase of more than 5 log units, compared to the *P. aeruginosa* count in the group coinfecting with *E. coli* not producing NDM-1 (*E. coli*) and treated with antibiotic (group 4). Considering that the CFU per larva for *E. coli* was $>10^8$ for the three variants expressing NDM-1, this reveals that all strains producing NDM-1 exhibited similar levels of meropenem resistance during the coinfection, in agreement with their MIC values for meropenem (Table S2).

We conclude that *E. coli* cells producing NDM-1 are able to cross-protect *P. aeruginosa* from a meropenem treatment in *G. mellonella*. *P. aeruginosa* cells protected by *E. coli* were unable to grow in gentamicin-supplemented plates, allowing us to discard any event of pMBLe-NDM-1 plasmid transfer and maintenance in *P. aeruginosa*. The possibility that *P. aeruginosa* has acquired the NDM-1 gene by recombination to the chromosome, rendering cells sensitive to gentamicin but resistant to carbapenems, is very unlikely within the time frame of the experiment. If recombination events were

significant, a substantial population of the *P. aeruginosa* cells would harbor the NDM-1 or NDM-1 C26A gene, at similar proportions since the two genes are expected to integrate in the chromosome with the same frequency. In this way, in the presence of meropenem the CFU of *P. aeruginosa* would parallel the resistance profiles of *P. aeruginosa* producing NDM-1 or NDM-1 C26A. However, while MIC values of meropenem for *P. aeruginosa* pMBLe-NDM-1 and *P. aeruginosa* pMBLe-NDM-1 C26A were equal (Table S2), EcNDM-1 and EcNDM-1C26A elicited different levels of cross-protection to *P. aeruginosa*. These results allow us to attribute the survival of *P. aeruginosa* to meropenem hydrolysis by NDM-1 produced by *E. coli* and not by horizontal gene transfer events.

Another remarkable finding is that the coinfection with the strain expressing the NDM-1 C26A variant (EcNDM-1C26A) showed an average count of *P. aeruginosa* (8×10^7 CFU/larva) 2 orders of magnitude lower than that for larvae coinfecting with EcNDM-1 (1×10^{10} CFU/larva) or Ec Δ degP-NDM-1 (6×10^9 CFU/larva). Since cells from the three variants expressing NDM-1 exhibited similar hydrolytic activities against meropenem, these differences can be accounted for by the amount of NDM-1 released in *E. coli* vesicles. Moreover, the fact that EcNDM-1C26A counts were similar to or even higher than those of EcNDM-1 allows us to discard an effect resulting from an overwhelmed innate immune system. Finally, in the case of Ec Δ degP-NDM-1, the counts of *P. aeruginosa* were similar to those in coinfection with EcNDM-1. In contrast, the counts of Ec Δ degP-NDM-1 were lower than those of EcNDM-1, indicating that hypervesiculating cells are more effective in protecting *P. aeruginosa*. The lower counts of Ec Δ degP-NDM-1 cells are in agreement with the role assigned to DegP as a protease/chaperone involved in the relief of envelope stress, which is expected to be more pronounced in the *G. mellonella* hemolymph.

To quantify the protective capacity of *E. coli* variants toward *P. aeruginosa*, we defined a protective index independent of the number of bacteria inside each larva. This index is calculated as the ratio between the CFU/larva of *P. aeruginosa* and the CFU/larva of *E. coli* for each individual larva. Thus, this ratio represents the number of *P. aeruginosa* cells protected by each *E. coli* cell. As shown in Fig. 6c, the protective index correlates with the amount of NDM-1 secreted in vesicles. The index value for EcNDM-1 is approximately 4×10^2 (meaning that each *E. coli* cell protected approximately 400 *P. aeruginosa* cells), but for Ec Δ degP-NDM-1, which is able to produce 180-fold more vesicles, that index is approximately 8×10^4 (indicating that each *E. coli* cell protected more than 80,000 *P. aeruginosa* cells). Interestingly, this increase in bacterial protection matches the increase in NDM-1 secretion in the Δ degP background. On the other hand, the index for EcNDM-1C26A (which produces a periplasmic and soluble version of NDM-1) is approximately 0.04, meaning that 25 *E. coli* cells are necessary to protect one *P. aeruginosa* cell.

Overall, we conclude that secretion of NDM-1 into OMVs is a relevant mechanism of cross-protection against carbapenems between coexisting populations of different bacterial species during an infection.

DISCUSSION

Microbial pathogenesis research has historically focused on the study of infections as single-species events. The advent of next-generation sequencing tools has revealed that many infections involve more than one bacterial species (32, 33). The growing body of evidence revealed by these new techniques has led researchers to propose that the classical single-species analysis for a pathogen response to antibiotics is, at least, incomplete. Indeed, many infections are known to involve multiple pathogens (32, 34) or interactions between pathogens and commensals (35, 36). However, the understanding of how interspecies interactions influence the impact of antibiotics on microbial communities is still limited. It is known that resistant species can protect more susceptible species by degrading antibiotics via antibiotic-degrading enzymes, causing the detoxification of the microenvironment or growth medium (37–39). Additionally, secretions from one species can induce resistance mechanisms in others,

for example, by activating stress-response pathways (40) or efflux pump expression (41).

Among antibiotic-degrading enzymes, the carbapenemase NDM-1 raises a major public health concern. We have previously shown that membrane anchoring contributes to the unusual stability of NDM-1 and favors secretion of this enzyme in OMVs in a wide range of bacterial hosts (14, 16). In this work we show the role of these vesicles in the cross-protection of bacteria otherwise susceptible to carbapenems *in vivo*.

Using *G. mellonella* as an *in vivo* model of infection, we showed that OMVs loaded with NDM-1 enable the survival of susceptible bacteria under meropenem treatment inside the larvae and that these NDM-1-harboring vesicles were more efficient than free soluble NDM-1 for bacterial protection. Moreover, OMVs-NDM-1 remain active inside the larvae for at least 6 h postinjection. This finding demonstrates *in vivo* the positive impact of this membrane protein on the fitness of bacterial communities compared to other soluble lactamases and highlights the importance of this enzyme in the global antibiotic resistance.

We also demonstrated that during a coinfection with an OMV-NDM-1-producing *E. coli* strain and a meropenem-sensitive *P. aeruginosa* strain, the vesicles released by *E. coli* were able to protect *P. aeruginosa* from the meropenem treatment. Although the possible protective role of OMVs-NDM-1 toward nearby microorganisms has been previously proposed (16), this is the first *in vivo* demonstration that this cross-species protection actually occurs during the course of a coinfection and that this protection takes place by protein transport mediated by the vesicles. It is worth notice that the *E. coli* variants produced approximately the same amount of cellular active NDM-1 enzyme (Fig. 4), supporting the hypothesis that the observed differences were exclusively due to variations in NDM-1 secretion.

Our results further suggest that horizontal genetic transfer may not always be necessary for the transfer of antibiotic resistance, at least for short-term survival during an antibiotic treatment. Susceptible bacteria that participate in a mixed infection may be protected against carbapenems, showing a resistant phenotype *in vivo* if a strain that secretes OMVs carrying NDM-1 participates in the coinfection.

In summary, we posit that OMVs are efficient and protective NDM-1 transporters, being relevant participants in the dissemination of carbapenem resistance between species. The search for genes responsible for hypervesiculating phenotypes (like mutations that truncate outer membrane structures such as lipopolysaccharide and enterobacterial common antigen [42], among NDM-1-producing bacteria) may further consider them possible new pathogenic markers. Finally, since OMVs can also carry plasmids with β -lactamase genes, as reported for *bla*_{NDM-1} (22) and *bla*_{OXA-24} (21), the simultaneous secretion of protein and genetic material suggests that OMVs contribute to the dissemination of antimicrobial resistance, and this may not be limited to MBLs. This scenario opens new working hypotheses and suggests that new therapeutic options interfering with vesicle secretion should be explored.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents. *Escherichia coli* ATCC 25922 was used for construction of the Δ *degP* mutant, OMV purification, and *Galleria mellonella* infection experiments, without plasmid or transformed with pMBLe, pMBLe-NDM-1, or pMBLe-NDM-1 C26A plasmids. The pMBLe plasmids, constructed in a previous work (16), allow isopropyl- β -D-thiogalactopyranoside (IPTG)-induced expression of MBLs (with their natural leader peptides) at physiological levels from a pTac promoter. The *E. coli* ATCC 25922 Δ *degP* strain was generated by Lambda Red-mediated recombination as previously described (43). Briefly, *E. coli* ATCC 25922 containing pKD46 plasmid was transformed with a PCR product generated with primers degP-P1 (5'-ACAGCAATTTTGC GTTATCTGTTAATCGAGACTGAAATACGTGTAGGCTGGAGCTGCTTCG-3') and degP-P2 (5'-GGAGAACCCCTCCCGTTTTCAGGAAGGGGTTGAGGGAGACATATGAATATCCTCCTTA-3') and pKD3 plasmid as the template.

Once obtained, *E. coli* cells carrying the *degP* deletion were transformed with pMBLe or pMBLe-NDM-1. *Pseudomonas aeruginosa* PAO1 transformed with pBBR1-MCS2 (kanamycin resistance) was used in coinfection experiments. Unless otherwise noted, all strains were grown aerobically at 37°C in LB broth (Lennox) medium supplemented with 200 μ g/ml gentamicin (*E. coli* pMBLe strains) or 1 mg/ml kanamycin (*P. aeruginosa*). Chemical reagents were purchased from Sigma-Aldrich, molecular biology enzymes from Promega, and primers from Invitrogen.

Purification and quantification of outer membrane vesicles (OMVs). OMVs were purified from early-stationary-phase cultures of *E. coli* as already described (14, 16). Briefly, 250 ml of LB medium inoculated with *E. coli* (wild type or $\Delta degP$), harboring any of the pMBLE plasmids, was used for growth at 37°C up to an optical density at 600 nm (OD_{600}) of 0.4, induction was done with 20 μ M IPTG, and growth continued overnight with agitation. Cells were harvested, and the supernatant was filtered through a 0.45- μ m membrane (Millipore). OMVs were precipitated with 55% (wt/vol) ammonium sulfate with stirring at 4°C for 3 h, separated by centrifugation at $12,800 \times g$ for 10 min, resuspended in 10 mM HEPES, 200 mM NaCl, pH 7.4, and dialyzed overnight against >100 volumes of the same buffer. The samples were then filtered through a 0.45- μ m membrane, layered over an equal volume of 50% (wt/vol) sucrose solution, and ultracentrifuged at $150,000 \times g$ for 1 h and 4°C. Pellets, containing OMVs, were washed once with 10 mM HEPES, 200 mM NaCl, pH 7.4, and further purified by sucrose isopycnic density gradient ultracentrifugation in equal buffer. Fractions containing OMVs (those possessing an SDS-PAGE pattern corresponding to the main outer membrane proteins) were pooled, washed with 10 mM HEPES, 200 mM NaCl, pH 7.4, and stored at -80°C until use. As a control, 10 μ l of a 300- μ g/ml OMV preparation was plated on LB agar and determined to be sterile.

OMV samples were quantified and normalized according to total protein concentration, with the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). OMV normalization was further verified by quantifying *E. coli* Omps F/C and A by densitometry (Image J software) from Coomassie blue-stained SDS-PAGE of vesicle samples or by using the fluorescent lipophilic dye FM4-64 (Molecular Probes) as previously described (44). The absence of cellular contaminants due to cell lysis during culture growth or cell manipulation was verified by immunodetection of cytoplasmic GroEL in OMV preparations as previously described (16).

Expression and purification of soluble NDM-1. Recombinant NDM-1 was expressed and purified as previously described (16). Briefly, the NDM-1 gene coding for residues 39 to 270 (without the lipidation site) was expressed from a modified version of pET28 plasmid harboring a tobacco etch virus (TEV) protease cleavage site. *E. coli* BL21(DE3) pET28-NDM-1 cells were grown at 37°C in M9 minimal medium until reaching an OD_{600} of 0.6, and protein expression was induced by addition of 0.5 mM IPTG and 0.5 mM ZnSO_4 during 16 h at 18°C. Cells were harvested and lysed by sonication, and the insoluble material was removed by centrifugation. The protein was purified using Ni-Sepharose affinity chromatography, the His tag was cleaved by treatment with His₆-tagged TEV protease (Sigma-Aldrich, manufacturer protocol), and the tag was removed by a second chromatographic step with the Ni-Sepharose resin in which the protease was also retained. The purified protein was concentrated using a 10-kDa-molecular-weight (MW)-cutoff Centricon device (Millipore, Bedford, MA, USA), dialyzed against 10 mM HEPES, 200 mM NaCl, pH 7.4, and quantified by absorbance at 280 nm using a molar absorption coefficient ($\epsilon_{280\text{nm}}$) of $28,000 \text{ M}^{-1} \text{ cm}^{-1}$ (calculated from aromatic residues using ExPasy ProtParam, available at <https://web.expasy.org/protparam/>).

Galleria mellonella rearing conditions. *G. mellonella* larvae were reared under laboratory standardized conditions. Briefly, for the constant supply of larvae, the whole insect life cycle was maintained. *G. mellonella* moths were incubated in ventilated cages where the oviposition took place. Eggs were collected and incubated at 25°C in cages containing a fresh mixture of the artificial diet, based on honey, glycerol, yeast, beeswax, and wheat bran (sterile at 1 atm of overpressure, for 15 min) (45). Batches of last-stage caterpillars were stored in petri dishes in the dark at 10°C and were used within a week of picking. Groups of 10 larvae were prepared for each condition. The larvae were incubated overnight (ON) at room temperature before conducting the experiment, with the aim of recovering active metabolism.

Galleria mellonella experiments. For infection experiments, each of the four variants of *E. coli* ATCC 25922 previously described was inoculated through the last left proleg of each larva with 10 μ l of bacterial suspension containing approximately 2×10^8 CFU/ml. One hour postinfection, 10 μ l of meropenem (10 mg/kg) or saline solution was administered to the last right proleg. Larvae were incubated at 37°C for 5 days, and dead larvae were recorded daily.

In the assays where the OMVs were injected together with the susceptible strain, a mixture of OMVs (OMVs-EF or OMVs-NDM-1) with a suspension of 2×10^8 CFU/ml (final inoculum) was inoculated into the last left proleg of the larva. Vesicles with NDM-1 were evaluated in four 2-fold serial dilutions ranging from 366 μ g/ml to 45.7 μ g/ml, resulting in four doses of 16.64, 7.32, 3.66, and 1.83 mg of vesicles per kilogram of larva. As 7.32 mg/kg of vesicles was able to provide resistance to meropenem administered in a single dose, we performed all subsequent experiments with this amount of OMVs-NDM-1.

When analyzing the OMVs-NDM-1 stability inside the hemolymph, vesicles carrying NDM-1 were administered to the larvae at three different time points before infection: 22, 6, or 0 h prior to bacterial inoculation. Then, infection and antibiotic treatment were conducted as described above. Each injection was performed into a different proleg with a final volume of 10 μ l, always starting at the last left proleg.

When comparing OMVs-NDM-1 with equivalent amounts of free NDM-1 soluble protein, the two forms of NDM-1 were administered to the larvae 6 h preinfection, in order to analyze the stability inside the hemolymph. Then, infection and antibiotic treatment were performed as described above. The amount of NDM-1 anchored to the OMVs was quantified by Western blotting, as well as the soluble protein.

MBL detection and cell β -lactamase activity measurements. NDM-1 protein levels were determined by SDS-PAGE followed by Western blotting with NDM-1 antibodies at a 1:1,000 dilution from a 700- μ g/ml solution (Instituto de Salud y Ambiente del Litoral, ISAL, UNL-CONICET, Argentina) and immunoglobulin G-alkaline phosphatase conjugates at a 1:3,000 dilution (Bio-Rad). Briefly, the samples were mixed with loading buffer and heated to denature the peptide structure. SDS-PAGE (14%) was used for

separation of the sample components and subsequently transferred onto a nitrocellulose membrane (GE). Western blotting with antibodies detecting GroEL was performed as a loading control (16). The amount of NDM-1 associated with OMVs was determined by densitometry (Image J software) from nitrocellulose membranes and converted to protein amounts through a calibration curve constructed under the same experimental conditions with quantified recombinant NDM-1.

β -Lactamase activity of living cells and OMVs was measured in a Jasco V-670 spectrophotometer at 30°C in reaction buffer (10 mM HEPES, 200 mM NaCl, pH 7.4), in 0.1-cm cuvettes, using 400 μ M meropenem as a substrate. Meropenem hydrolysis was monitored at 300 nm ($\Delta\epsilon_{300\text{nm}} = -6,500 \text{ M}^{-1} \text{ cm}^{-1}$). Living cells were prepared with the aim of mimicking the expression conditions encountered during *G. mellonella* coinfection experiments. Considering an average volume of hemolymph within each larva of approximately 50 μ l (25), the estimated final concentration of IPTG in the hemolymph after infection was 1.7 μ M. In this way, fresh LB supplemented with 20 μ M IPTG was inoculated with a 1:50 dilution of an ON culture of *E. coli* (pMBLe, pMBLe-NDM-1, or pMBLe-NDM-1 C26A) or *E. coli* Δ degP (pMBLe or pMBLe-NDM-1) induced with 20 μ M IPTG and grown with shaking at 37°C until reaching an OD₆₀₀ of 1. Cells were harvested, resuspended in fresh LB supplemented with 1.7 μ M IPTG, and grown for 2 h at 37°C. Cells from 1 ml of culture were pelleted, washed twice with reaction buffer to remove OMVs, and resuspended in (300 \times OD₆₀₀) μ l of the same buffer. β -Lactamase activity was measured from 5 μ l of cell suspension in 300 μ l of reaction buffer.

***E. coli* and *P. aeruginosa* coinfection.** In a first step, *P. aeruginosa* was transformed with a kanamycin-resistant vector in order to have a differential growth of *E. coli* and this strain. The selection of *P. aeruginosa* resistant clones was carried out by plating in LB agar medium with 1 mg/ml kanamycin, and on the other hand, all the *E. coli* variants were selected by plating on LB agar supplemented with 200 μ g/ml gentamicin transformed with pMBLe vectors that confer gentamicin resistance, which allowed us to quantify the bacterial burden without the interference of the bacterial flora from the larval gut.

To prepare the bacterial inoculum, strains were grown ON and then a 1:50 subculture was carried out until exponential phase (OD₆₀₀ of 1). The cultures were washed twice with physiological solution and resuspended to approximately 4×10^8 CFU/ml by OD₆₀₀ measurement. In the case of *E. coli*, all steps were carried out in the presence of 20 μ M IPTG. *E. coli* cultures were used at this inoculum, and instead, for *P. aeruginosa*, 10-fold dilutions were made in order to achieve approximately 4×10^5 CFU/ml. Equal volumes of *E. coli* and *P. aeruginosa* cultures were mixed, and 10 μ l of the bacterial mix with 20 μ M IPTG was injected through the last left proleg. Following this procedure, each larva was infected with 10^6 CFU of *E. coli* (each of the four different variants) and 10^2 CFU of *P. aeruginosa*, and subsequently meropenem was administered (2 mg/kg) 1 h postinfection. There was also a control group coinfecting with *E. coli* (nonexpressing NDM-1 strain), which received saline instead of meropenem.

Afterward, the larvae were incubated at 37°C for 24 h. After incubation, larvae from each group were immersed in 70% ethanol and vortexed for 30 s. Then, they were allowed to dry on a cooling gel covered with absorbent paper, and each caterpillar was placed in a 1.5-ml microtube with 300 μ l of saline solution. Each larva was homogenized with a microcentrifuge tube pestle, and an additional 300 μ l of saline solution was added. Then, 30 μ l of each homogenate was taken and 1:10 serial dilutions were made. Finally, each dilution was plated by the drop method in LB agar with gentamicin (200 μ g/ml) or kanamycin (1 mg/ml) for differential selection of coinfecting strains, in triplicate. The plates were incubated at 37°C for 24 h, and then the CFU/larva count was determined. Data are presented as scattered plot graphs with average and standard error.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.3 MB.

FIG S2, TIF file, 0.1 MB.

TABLE S1, DOCX file, 0.01 MB.

TABLE S2, DOCX file, 0.01 MB.

TABLE S3, DOCX file, 0.01 MB.

TABLE S4, DOCX file, 0.01 MB.

ACKNOWLEDGMENTS

We thank Alejandro M. Viale (IBR, CONICET-UNR, Argentina) for GroEL antibodies and Marina Avecilla for excellent technical assistance.

This research was supported by grants from the National Institutes of Health (R01AI100560 to R.A.B. and A.J.V., R01AI063517 to R.A.B., and R01AI072219 to R.A.B.) and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) to A.J.V. and to P.C.M. This study was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, award number 1101BX001974 to R.A.B. from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development, and the Geriatric Research Education and Clinical Center VISN 10 to R.A.B. L.J.G., P.C.M., and A.J.V. are staff members of CONICET, and M.M.B.M. is a recipient of a postdoctoral fellowship from CONICET.

M.M.B.M. and P.C.M. performed the microbiological and larva infection experiments. L.J.G. performed the molecular biology, strain engineering, and vesicle preparations. A.J.V., P.C.M., and L.J.G. conceived the project and designed the experiments, and M.M.B.M., R.A.B., A.J.V., P.C.M., and L.J.G. wrote the paper, discussed the results, and commented on the manuscript.

We declare no competing interests.

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