Knockout of *mlaA* increases *Escherichia coli* virulence in a silkworm infection model

Haruka Nasu*, Riko Shirakawa*, Kazuyuki Furuta, Chikara Kaito

Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Kita-ku, Okayama, Japan

* These authors contributed equally to this work.
* ckaito@okayama-u.ac.jp

Abstract

The *mlaA* gene encodes a lipoprotein to maintain an outer membrane lipid asymmetry in gram-negative bacteria. Although the role of *mlaA* in bacterial virulence has been studied in several bacterial species, there are no reports of its role in *E. coli* virulence. In this study, we found that knockout of *mlaA* in *E. coli* increased its virulence against silkworms. The *mlaA*-knockout mutant was sensitive to several antibiotics and detergents, but resistant to vancomycin and chlorhexidine. The *mlaA*-knockout mutant grew faster than the parent strain in the presence of silkworm hemolymph. The *mlaA*-knockout mutant also produced a larger amount of outer membrane vesicles than the parent strain. These findings suggest that *mlaA* knockout causes *E. coli* resistance to specific antimicrobial substances and increases outer membrane vesicle production, thereby enhancing *E. coli* virulence properties in the silkworm infection model.

Introduction

The outer membrane of gram-negative bacteria, including *E. coli*, has a lipid asymmetry, in which the outer leaflet comprises lipopolysaccharide (LPS) and the inner leaflet comprises phospholipids [1]. The outer leaflet has low fluidity and contributes to inhibit the invasion of various antibiotics and foreign chemicals into bacterial cells [2]. The outer membrane lipid asymmetry is maintained by the Mla system, phospholipase A (PldA), and LPS palmitoyltransferase (PagP) [3]. PldA and PagP contribute to maintain the lipid asymmetry by degrading phospholipids in the outer leaflet of the outer membrane. The Mla system consists of 6 proteins, MlaA, MlaB, MlaC, MlaD, MlaE, and MlaF, that transport phospholipids from the outer leaflet of the outer membrane into the inner membrane. MlaA is a lipoprotein located at the outer membrane that transfers phospholipids to MlaC, a periplasmic protein [4]. MlaC transfers phospholipids to the MlaFEDB complex in the inner membrane, and MlaFEDB then inserts the phospholipids into the inner membrane.

Knockout of the Mla system destroys the lipid asymmetry of the outer membrane and increases the sensitivity to various antimicrobial substances in many gram-negative bacteria. Knockout of MlaA causes bacterial sensitivity to various antimicrobial substances; sodium dodecyl sulfate (SDS)/EDTA in *E. coli* [4,5], hydrophobic antibiotics such as erythromycin, rifampicin, azithromycin, and polymyxin E in *Haemophilus influenzae* [6]; polymyxin B and
vancomycin in Neisseria gonorrhoeae [7]; and tetracycline, ciprofloxacin, chloramphenicol, cathelicidin, and LL-37 in P. aeruginosa [8,9]. Knockout of MlaC also causes sensitivity to macrolides and fluoroquinolones in Burkholderia species [10], and gentamycin, novobiocin, and rifampicin in Acinetobacter baumannii [11]. In contrast, however, the MlaA-knockout mutant of E. coli shows resistance to chlorhexidine [12]. E. coli mutants resistant to the antimicrobial peptide aenicin-3 carry mutations in the mlaA gene or the mlaBCDEF operon [13]. The characteristics of antibiotics for which knockout of the Mla system causes bacterial sensitivity or resistance, however, are unknown.

Knockout of MlaA exerts both negative and positive effects on the virulence properties of various bacterial species. In Shigella flexneri, knockout of MlaA decreases bacterial spreading among host cells [14,15]. In H. influenzae and H. parasuis, knockout of MlaA attenuates bacterial infectivity in host epithelial cells and decreases the bacterial burden in a mouse model [6,16]. In Burkholderia pseudomallei, knockout of MlaA decreases bacterial persistence in mouse spleen [17]. On the other hand, in N. gonorrhoeae and V. cholerae, knockout of MlaA increases the bacterial number in the mouse genital tract or gut [7,18]. In H. influenzae, A. baumannii, N. gonorrhoeae, and V. cholerae, knockout of MlaA increases the production of outer membrane vesicles (OMV), which have various functions in infectious processes, such as absorbing antimicrobial molecules and transporting toxins to host cells [7,18–21]. In P. aeruginosa, knockout of MlaA decreases bacterial virulence in a mouse lung infection model [9], but increases bacterial virulence in a fruit fly infection model [8]. How MlaA affects E. coli virulence, however, is unclear.

We previously investigated gene mutations that upregulate E. coli virulence properties using a silkworm infection model [22,23]. In the present study, we searched E. coli mutants with high virulence from among E. coli mutants resistant to vancomycin and found that knockout of MlaA upregulates E. coli virulence properties.

Results

Knockout of mlaA increases E. coli killing activity against silkworms

We previously revealed that amino acid substitutions in LptD or LptE, the LPS transporter subunits, as well as knockout of OpgG or OpgH, the synthetases for osmoregulated periplasmic glucan, cause E. coli resistance against vancomycin and increase E. coli killing activity against silkworms [22,23]. Based on this finding, we hypothesized the existence of E. coli genes whose knockout could lead to high vancomycin resistance as well as high killing activity against silkworms. By searching E. coli gene knockout mutants showing vancomycin resistance from a transposon mutant library, we identified 50 gene knockout mutants exhibiting vancomycin resistance (Table 1). We evaluated the killing activity of the vancomycin-resistant mutants against silkworms, and found that the mlaA-knockout mutant killed silkworms faster than the parent strain (Table 1, Fig 1). The increased killing activity of the mlaA-knockout mutant was blocked by introducing the intact mlaA gene (Fig 1). In addition, the mlaA-knockout mutant exhibited better growth than the parent strain in the presence of vancomycin, and the vancomycin resistance was decreased to the parent level by introducing the intact mlaA gene (Fig 2A). These results suggest that mlaA knockout leads to vancomycin resistance and increases E. coli virulence against silkworms.

Knockout of mlaA alters E. coli sensitivity to various antimicrobial molecules

Next, we examined whether mlaA knockout alters E. coli sensitivity to various antimicrobial molecules. The mlaA-knockout mutant showed less growth than the parent strain in the
Table 1. Silkworm-killing activities of transposon mutants exhibiting resistance against vancomycin.

| Strain ID | Gene | Product                                      | Survival (%) |
|-----------|------|----------------------------------------------|--------------|
| JD20172   | insH1| Transposase InsH for insertion sequence element IS5A | 100          |
| JD20241   | carB | Carbamoyl-phosphate synthase large chain      | 60           |
| JD20379   | acnB | Aconitate hydratase B                         | 20           |
| JD20391   | hpt  | Hypoxanthine phosphoribosyltransferase        | 40           |
| JD20727   | ybbO | Uncharacterized oxidoreductase                | 20           |
| JD20913   | segA | Endonuclease segA                             | 80           |
| JD21066   | ybiU | UTP-glucose-1-phosphate uridylyltransferase   | 100          |
| JD21400   | ycfL | Uncharacterized protein YcfL                  | 100          |
| JD21547   | rpsT | PTS system glucose-specific EIIC component    | 40           |
| JD21607   | guaB | Inosine-5’-monophosphate dehydrogenase        | 100          |
| JD21620   | ndk  | Nucleoside diphosphate kinase                 | 80           |
| JD21662   | mltA | Membrane-bound lytic murein transglycosylase A| 60           |
| JD21673   | nlpI | Lipoprotein NlpI                              | 60           |
| JD21732   | ybiB | Protein HtrL                                  | 100          |
| JD21864   | ybs3 | Probable L, D-transpeptidase YbiS             | 100          |
| JD22094   | opgG | Glucans biosynthesis protein G                | ND           |
| JD22095   | opgH | Glucans biosynthesis glucosyltransferase H    | ND           |
| JD22143   | yceG | Endolytic murein transglycosylase             | 80           |
| JD22152   | ycfL | Uncharacterized protein YcfL                  | 100          |
| JD22156   | lpoB | Penicillin-binding protein activator LpoB     | 80           |
| JD23323   | mlaA | Intermembrane phospholipid transport system lipoprotein MlaA | 0   |
| JD23420   | uraA | Uracil permease                               | 100          |
| JD23606   | proX | Glycine betaine/proline betain-binding periplasmic protein | 100  |
| JD23607   | ygaZ | Inner membrane protein YgaZ                   | 100          |
| JD23673   | ghB  | Glutathione synthetase                        | 100          |
| JD23934   | yghQ | Inner membrane protein YghQ                   | 100          |
| JD23935   | yghR | Uncharacterized ATP-binding protein YghR      | 80           |
| JD23938   | yghS | Uncharacterized ATP-binding protein YghS      | 100          |
| JD23939   | yghT | Uncharacterized ATP-binding protein YghT      | 100          |
| JD24024   | tolC | Outer membrane protein TolC                   | 100          |
| JD24230   | mlaC | Intermembrane phospholipid transport system binding protein MlaC | 60  |
| JD24231   | mlaD | Intermembrane phospholipid transport system binding protein MlaD | 40  |
| JD24242   | ybfI | Rnase adapter protein RapZ                    | 80           |
| JD24280   | sspA | Glutamyl endopeptidase                        | 100          |
| JD24335   | dusB | tRNA-dihydouridine synthaseB                  | 100          |
| JD24462   | waaY | Lipopolysaccharide core heptose(II) kinase RfaY| 80  |
| JD24466   | waaR | Lipopolysaccharide 1,2-glucosyltransferase    | 100          |
| JD24468   | waaB | Lipopolysaccharide 1,6-galactosyltransferase  | 100          |
| JD24476   | waaQ | Lipopolysaccharide core heptosyltransferase RfaQ | 100  |
| JD24492   | yicC | UPF0701 protein YicC                         | 100          |
| JD24700   | wecA | Undecaprenyl-phosphate alpha-N-acetylglucosaminly 1-phosphate transferase | 60  |
| JD25777   | slt  | Soluble lytic murein transglycosylase         | 20           |
| JD26183   | bioB | Biotin synthase                              | 80           |
| JD26764   | glf  | UDP-galactopyranose mutase                    | 60           |
| JD27118   | mltB | Membrane-bound lytic murein transglycosylase B| 100          |
| JD27649   | hycB | Formate hydrogenlyase subunit 2              | 100          |
| JD27708   | mlaE | Intermembrane phospholipid transport system permease protein MlaE | 60  |

(Continued)
The growth of the mlaA-knockout mutant was indistinguishable from that of the parent strain in the presence of tetracycline (Fig 2A). Consistent with a previous report [12], the mlaA-knockout mutant showed better growth than the parent strain in the presence of chlorhexidine (Fig 2A). The alteration of drug sensitivity in the mlaA-knockout mutant was restored to the parent strain level by introducing the intact mlaA gene (Fig 2A). These results suggest that mlaA knockout alters E. coli sensitivity to various antimicrobial molecules.

To clarify the molecular mechanisms by which mlaA knockout increases E. coli virulence against silkworms, we examined bacterial growth in the presence of silkworm hemolymph in which antimicrobial peptides were induced by injection of heat-killed bacteria [24]. The mlaA-knockout mutant grew faster than the parent strain in the presence of silkworm hemolymph (Fig 2B). The faster growth of the mlaA-knockout mutant in the presence of silkworm hemolymph was abolished by introducing the intact mlaA gene (Fig 2B). In contrast, in the absence of silkworm hemolymph, the growth of the mlaA-knockout mutant was indistinguishable from that of the parent strain (Fig 2C). These results suggest that the mlaA-knockout confers E. coli resistance to silkworm immune mechanisms.

**Knockout of mlaA increases OMV production**

Knockout of mlaA leads to increased production of OMVs in N. gonorrhoeae, H. influenza, V. cholerae [7,20]. We examined whether mlaA knockout increases OMV production in E. coli.

| Strain ID | Gene | Product | Survival (%) |
|-----------|------|---------|--------------|
| JD27710   | mlaF | Intermembrane phospholipid transport system ATP-binding protein MlaF | 80 |
| JD27958   | citC | Citrate[pro-3S]-lyase ligase | 80 |

Transposon mutants exhibiting resistance to vancomycin are listed. Bacterial solutions (2 x 10⁹ CFU) were injected into silkworms (n = 5) and percent survival at 2 days post infection was measured and is presented as “Survival”. Percent survival of the parent strain was 100%. ND, not determined.

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Fig 2. The mlaA-knockout mutant exhibits various sensitivities to antibiotics and resistance to silkworm antimicrobial substances. (A) *E. coli* overnight culture of the Parent/pMW118, mlaA::Tn/pMW118, or mlaA::Tn/pMW118-mlaA strain was 10-fold serially diluted; spotted onto LB agar plates supplemented with or without vancomycin, levofloxacin, chloramphenicol, tetracycline, CTAB, cholic acid, or chlorhexidine; and incubated at 37˚C. To examine the sensitivity to oxacillin and ampicillin, the mlaA deletion mutant (markerless deletion mutant of mlaA) transformed with pMW218 (ΔmlaA/pMW218) and the mlaA deletion
mutant transformed with pMW218-mlaA (ΔmlaA/pMW218-mlaA) were used. (B) E. coli strains of the Parent/pMW118, mlaA::Tn/pMW118, or mlaA::Tn/pMW118-mlaA were aerobically cultured in LB medium and silkworm hemolymph was added to the bacterial culture at 40 min after the bacterial inoculation. The vertical axis represents the OD600 value of the bacterial culture, and the horizontal axis represents the culture time. The means ± standard errors from 5 independent experiments are shown. Star indicates Student t-test p value less than 0.05 between the Parent/pMW118 vs. mlaA::Tn/pMW118, and between mlaA::Tn/pMW118 vs. mlaA::Tn/pMW118-mlaA. (C) E. coli strains of the Parent/pMW118, mlaA::Tn/pMW118, or mlaA::Tn/pMW118-mlaA were aerobically cultured in LB medium and the OD600 values of the cultures were measured.

SDS-polyacrylamide gel electrophoresis analysis revealed that the amounts of proteins with about 33 kDa increased in the OMV fraction of the mlaA-knockout mutant compared with the parent strain (Fig 3A, S1 Raw images). Western blot analysis revealed that the amounts of OmpA and LPS, which are components of OMV, increased in the OMV fraction of the mlaA-knockout mutant compared with the parent strain (Fig 3B and 3C, S1 Raw images). The increase in OmpA and LPS was decreased by introducing the intact mlaA gene (Fig 3B and 3C, S1 Raw images). These results suggest that mlaA knockout increases OMV production.

Knockout of pldA does not affect E. coli killing activity against silkworms

PldA and PagP degrade phospholipids of the outer membrane and contribute to maintain the lipid asymmetry of the outer membrane independently of the Mla system [3]. Double knockout of mlaA and pldA leads to a higher accumulation of phospholipids in the outer membrane

Fig 3. Knockout of mlaA increases OMV production. (A) Culture supernatants of the Parent/pMW118, mlaA::Tn/pMW118, or mlaA::Tn/pMW118-mlaA strains were ultracentrifuged and the precipitates were electrophoresed in a SDS polyacrylamide gel. The gel was stained by Coomassie brilliant blue. (B, C) The OMV fraction obtained in (A) was subjected to Western blot analysis using an anti-OmpA antibody (B) or an anti-LPS antibody (C). Lower graph indicate the relative band intensity compared with that in the parent strain. Data shown are means ± standard errors from three independent experiments. The asterisk represents a p value less than 0.05 (Student’s t test).
than the respective single knockouts [25]. We examined whether double knockout of mlaA and pldA, or mlaA and pagP increases vancomycin resistance. The growth of single knockout mutants of pldA or pagP was indistinguishable from that of the parent strain in the presence of vancomycin (Fig 4A). Double knockout mutants of mlaA and pldA showed slightly better growth than the respective single knockout mutants in the presence of vancomycin (Fig 4A). Colony forming unit assay confirmed the different sensitivity to vancomycin between the mlaA-knockout mutant and the mlaA/pldA double knockout mutant (Fig 4B). The growth of double knockout mutants of mlaA and pagP was similar to that of the mlaA-knockout mutant in the presence of vancomycin (Fig 4A). These results suggest that knockouts of mlaA and pldA additively increase vancomycin resistance.

Based on these observations, we examined the effect of pldA knockout on E. coli virulence against silkworms. The pldA single knockout mutant killed silkworms with a similar time course as the parent strain (Fig 4C). The mlaA/pldA double knockout mutant killed silkworms with a similar time course as the mlaA single knockout mutant (Fig 4C). Thus, the pldA knockout does not increase silkworm killing activity.

Discussion

The findings of the present study revealed that knockout of mlaA, which maintains the lipid asymmetry of the outer membrane leads to increased virulence of E. coli against silkworms. The mlaA-knockout mutant exhibited increased OMV production, resistance to vancomycin, and increased killing activity against silkworms. Thus, this study unveiled a novel function of mlaA in E. coli virulence properties.

Knockout of mlaA increases bacterial virulence not only in E. coli, but also in N. gonorrhoeae, V. cholerae, and P. aeruginosa [7,8,18]. In contrast, knockout of mlaA attenuates bacterial virulence in S. flexneri, H. influenzae, H. parasuis, and B. pseudomallei [6,14–16]. Thus, the effect of the mlaA knockout on bacterial virulence differs between bacterial species. A previous study reported that in N. gonorrhoeae, knockout of mlaA sensitizes bacteria to antimicrobial peptides (defensin and polymyxin), vancomycin, and ampicillin, but increases OMV production; the authors speculated that the increased OMV could be advantageous for bacteria to survive in the mouse lower genital tract [7]. In V. cholerae, knockout of mlaA increases OMV production and alters the lipid composition of the outer membrane, which could be beneficial for bacteria adaptation in host environment containing antimicrobial peptides and bile acids [18]. In P. aeruginosa, knockout of mlaA increases bacterial virulence via ZnuA, which functions in Zn incorporation [8]. The effect of the mlaA knockout on OMV production was not examined in P. aeruginosa. Thus, the increased OMV production in the mlaA-knockout mutant is conserved among E. coli, N. gonorrhoeae, and V. cholerae, and could underlie the increased virulence of the mlaA-knockout mutant. It should also be noted that the animal infection models were different among the studies of different bacterial species, and the host environment could be differently involved in the bacterial virulence phenotypes of these mlaA-knockout bacteria.

The mlaA-knockout mutant of E. coli exhibited sensitivity to many antimicrobial molecules, including levofloxacin, chloramphenicol, oxacillin, CTAB, and cholic acid, but was resistant to vancomycin (Fig 2), chlorhexidine [12], and arenicin-3 [13]. Because the conserved chemical structure or conserved target molecules between vancomycin, chlorhexidine, and arenicin-3 is not known, it is difficult to understand the mechanism by which the mlaA-knockout mutant exhibits resistance to these 3 antimicrobial molecules, while it exhibits sensitivity to other antimicrobial molecules. We speculate that there are 3 possible reasons, as follows: (i) Because OMV have the capacity to absorb various antimicrobial molecules, the
Fig 4. Knockout of pldA does not affect silkworm-killing activity. (A) *E. coli* overnight culture of the Parent, *mlaA::Tn*, ΔpldA, ΔpagP, *mlaA::Tn/ΔpldA*, *mlaA::Tn/ΔpagP*, ΔpldA/ΔpagP, or *mlaA::Tn/ΔpldA/ΔpagP* strain was 10-fold serially diluted, spotted onto LB agar plates supplemented with or without vancomycin (400 μg/ml), and incubated at 37°C. (B) *E. coli* overnight culture of the Parent, *mlaA::Tn*, ΔpldA, or *mlaA::Tn/ΔpldA* strain was 10-fold serially diluted, spread onto LB agar plates supplemented with or without vancomycin (400 μg/ml), and incubated at 37°C. The appeared colonies were counted and CFU/ml was calculated. Data shown are means ± standard errors from three
increased OMV production in the mlaA knockout mutant may contribute to the resistance to these antimicrobial molecules. (ii) The mlaA knockout increases the amount of phospholipids in the outer leaflet of the outer membrane, which could alter the permeability of antimicrobial molecules and affect bacterial sensitivity to antimicrobial molecules. (iii) MlaA may transport phospholipids bound to antimicrobial molecules from the outer leaflet of the outer membrane to the inner membrane. The knockout of mlaA may block the transport of antimicrobial molecules from the outer membrane to the inner membrane. These possibilities should be addressed in future studies.

By constructing multiple gene deletion mutants of pldA, pagP, and mlaA, which respectively maintain the lipid asymmetry of the outer membrane, we revealed that the mlaA/pldA double knockout mutant, compared with the mlaA-knockout mutant, had increased resistance to vancomycin. We also revealed that, compared with the mlaA-knockout mutant, the mlaA/pldA double knockout mutant did not have increased killing activity in silkworms. Thus, pldA has a role with mlaA in vancomycin resistance, but no role in the silkworm-killing activity. Our analysis suggests the mlaA is the main factor among pldA, pagP, and mlaA that affects the vancomycin resistance and virulence of E. coli.

MlaA is attracting attention as a drug target, because mlaA knockout increases the sensitivity of many bacteria to various antibiotics. The present study, however, demonstrated that the mlaA knockout confers E. coli resistance to vancomycin and exhibits high virulence in silkworms. Investigating the molecular mechanisms of how the mlaA knockout upregulates bacterial virulence will help to elucidate the biological significance of the Mla system, and contribute to the evaluation of MlaA as a drug target.

Materials and methods

Bacteria and culture condition

E. coli KP7600 strain and its gene-knockout mutants were cultured on LB agar medium and the bacterial colonies were aerobically cultured in LB liquid medium at 37˚C. E. coli strains transformed with pCP20 or pMW118 were cultured on LB agar plates containing 100 μg/ml ampicillin. The details of the bacterial strains and plasmids used in this study are provided in Table 2.

Silkworm infection experiment

Third instar silkworms (Fu/Yo X Tsukuba/Ne) were purchased from Ehime Sansyu (Ehime, Japan). The silkworms were fed an artificial diet (Silkmate, Nosan, Japan) and maintained at 27˚C. Fifth instar silkworms were fed an antibiotic-free artificial diet (Sysmex) for 1 day and used for the infection experiment. E. coli overnight culture was centrifuged at 4050 g for 10 min, and the precipitated cells were suspended in 0.9% NaCl. Silkworms were injected with the bacterial solution using 1-ml syringes equipped with a 27-gauge needle via the intra-hemolymph route [27] and maintained at 37˚C. Silkworm survival was measured every ~12 h after the injection. The OD_{600} values of the bacterial solutions were measured before the injection to confirm that the number of bacteria did not differ between samples. The number of bacteria was determined by plating bacterial solution on LB agar plates.

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Genetic manipulation

To construct gene-knockout mutants of mlaA, pldA, and pagP, transduction using a phage P1 vir was performed. First, the pldA knockout strain (N0001) or pagP knockout strain (N0002) were constructed by transduction from JW3794 or JW0617 to KP7600 strain. After the transduction step, the kanamycin resistance marker was removed by transformation with pCP20 expressing FLP recombinase, and pCP20 was removed by culturing the bacteria at 43˚C. Next, the pldA/mlaA double knockout strain (N0003) or pagP/mlaA double knockout strain (N0004) were constructed by transduction from JD23323 to the pldA or pagP markerless knockout strains. Third, the pldA/pagP double knockout strain was constructed by transduction from JW0617 to the pldA markerless knockout strain. The kanamycin resistance marker was removed from the pldA/pagP double knockout strain by transformation with pCP20, and the transduction was performed from JD23323 to the markerless pldA/pagP double knockout strain, resulting in the mlaA/pldA/pagP triple knockout strain. The gene knockouts in the mutant strains were confirmed by PCR. To construct a plasmid carrying the mlaA gene, the DNA fragment encoding the mlaA gene was amplified by PCR using primer pairs (forward, 5’-TCTTCTAGACCGCAGTACCGTTATTTTTC-3’, reverse, 5’-GGTTGTTACCTGTTCCGATCATCAGGTT-3’) from genomic DNA of KP7600 as a template. The amplified DNA fragment was cloned into XbaI and KpnI sites of pMW118 or pMW218, resulting in pMW118-mlaA or pMW218-mlaA.

Evaluation of bacterial resistance to antimicrobial substances

To measure bacterial resistance to antibiotics and detergents, autoclaved LB agar medium was mixed with antibiotics or detergents and poured into square plastic dishes (Eiken Chemical,
E. coli overnight cultures were serially diluted 10-fold in a 96-well microplate and 5 μl of the diluted bacterial solution was spotted onto the LB agar plates supplemented with drugs. The plates were incubated at 37°C for 1 day and colonies were photographed using a digital camera.

Bacterial resistance to silkworm hemolymph was measured according to our previous method [24]. Briefly, fifth instar silkworms were injected with heat-killed E. coli KP7600 cells and the hemolymph was collected at 1 day after inoculation. The collected hemolymph was frozen in liquid nitrogen and stored at -80°C. Overnight cultures (10 μl) of E. coli strains (Parent/pMW118, mlaA::Tn/pMW118, or mlaA::Tn/pMW118-mlaA) were inoculated into fresh LB medium (1 ml) and aerobically cultured at 37°C. The silkworm hemolymph (27 μl) was added to the culture at 40 min after the inoculation and the OD₆₀₀ was measured every 1 h.

**Preparation of OMV**

E. coli overnight culture (1 ml) was inoculated into 100 ml of LB medium in a flask and aerobically cultured at 37 °C for 24 h. The culture was centrifuged at 4450 g for 10 min, and the supernatant was filtered through a 0.22-μm polyvinylidene difluoride membrane (Millipore). The supernatant was centrifuged at 45,000 g for 3 h and the precipitate was dissolved with SDS sample buffer. The sample was electrophoresed in 15% SDS polyacrylamide gel and the gel was stained with Coomassie brilliant blue.

**Western blot analysis**

The OMV samples were electrophoresed in 15% SDS polyacrylamide gel, and blotted to a PVDF membrane (Immobilon-P, Millipore). The membrane was treated with TBST (20 mM Tris-HCl [pH7.6], 150 mM NaCl, 0.12% Tween20) containing 5% skim milk for 1 h. The membrane was treated with a TBST buffer containing 1:5000 anti-OmpA IgG (111120, Antibody research corp., MO, USA) or 1:10000 anti-LPS core (WN1 222–5, Hycult Biotech, Uden, The Netherlands) for 1 h at room temperature. After washing with TBST, the membrane was treated with TBST containing 1:5000 anti-rabbit IgG conjugated with horseradish peroxidase HRP or 1:5000 anti-mouse IgG conjugated with HRP for 1 h at room temperature. After washing with TBST, the membrane was treated with a HRP substrate (Western Lightning Plus-ECL, Perkin Elmer). The signal was visualized using ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan). The band intensity was measured by Image J software [28].

**Statistical analysis**

Differences of the growth curves in the presence of silkworm hemolymph were assessed using the Student t test in Microsoft Excel for Mac (version 16.56). Statistical analyses of the survival curves of silkworms were performed using the log rank test with GraphPad PRISM software (version 5.0c).

**Supporting information**

S1 Raw images. Original uncropped images for SDS polyacrylamide gel electrophoresis and western blots.

(PDF)

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Author Contributions

Funding acquisition: Chikara Kaito.
Investigation: Haruka Nasu, Riko Shirakawa, Chikara Kaito.
Methodology: Chikara Kaito.
Project administration: Chikara Kaito.
Resources: Chikara Kaito.
Supervision: Chikara Kaito.
Writing – original draft: Chikara Kaito.
Writing – review & editing: Riko Shirakawa, Kazuyuki Furuta, Chikara Kaito.

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