The phospholipid-repair system LplT/Aas in Gram-negative bacteria protects the bacterial membrane envelope from host phospholipase A\textsubscript{2} attack

Yibin Lin\textsuperscript{*,} Mikhail Bogdanov\textsuperscript{#}, Shuo Lu\textsuperscript{*,} Ziqiang Guan\textsuperscript{**,} William Margolin\textsuperscript{##,} Jerrold Weiss\textsuperscript{***}, Lei Zheng\textsuperscript{*}

From the Center for Membrane Biology, Department of Biochemistry and Molecular Biology, the University of Texas Health Science Center at Houston McGovern Medical School

Department of Biochemistry and Molecular Biology, University of Texas McGovern Medical School

Department of Biochemistry, Duke University School of Medicine

Inflammation Program and Departments of Internal Medicine and Microbiology, University of Iowa Carver College of Medicine

Running title: Resistance Mechanism of E. coli to host sPLA\textsubscript{2}

To whom correspondence should be addressed: Lei Zheng, Center for Membrane Biology, Department of Biochemistry and Molecular Biology, The University of Texas McGovern Medical School, 6431 Fannin Street, Houston, TX 77030, USA, Tel: (713) 500-6083; Fax: (713) 500-0545; E-mail: lei.zheng@uth.tmc.edu

Keywords: phospholipase A\textsubscript{2}, lysophospholipid, membrane envelope, outer membrane asymmetry, bacterial resistance, antimicrobial, lipid hydrolysis

ABSTRACT

Secretory phospholipases A\textsubscript{2} (sPLA\textsubscript{2}) are potent components of mammalian innate-immunity antibacterial mechanisms. sPLA\textsubscript{2} enzymes attack bacteria by hydrolyzing bacterial membrane phospholipids, causing membrane disorganization and cell lysis. However, most Gram-negative bacteria are naturally resistant to sPLA\textsubscript{2}. Here we report a novel resistance mechanism to mammalian sPLA\textsubscript{2} in Escherichia coli, mediated by a phospholipid repair system consisting of the lysophospholipid transporter LpIT and the acyltransferase Aas in the cytoplasmic membrane. Mutation of \textit{lplT} or \textit{aas} gene abolished bacterial lysophospholipid acylation activity and drastically increased bacterial susceptibility to the combined actions of inflammatory fluid components and sPLA\textsubscript{2}, resulting in bulk phospholipid degradation and loss of colony-forming ability. sPLA\textsubscript{2}-mediated hydrolysis of the three major bacterial phospholipids exhibited distinctive kinetics and deacylation of cardiolipin to its monoacyl-derivative closely paralleled bacterial death. Characterization of the membrane envelope in \textit{lplT}- or \textit{aas}-knockout mutant bacteria revealed reduced membrane packing and disruption of lipid asymmetry with more phosphatidylethanolamine present in the outer leaflet of the outer membrane. Moreover, modest accumulation of lysophospholipids in these mutant bacteria destabilized the inner membrane and rendered outer membrane-depleted spheroplasts much more sensitive to sPLA\textsubscript{2}. These findings indicated that LpIT/Aas inactivation perturbs both the outer and inner membranes by bypassing bacterial membrane maintenance mechanisms in order to trigger specific interfacial activation of sPLA\textsubscript{2}. We conclude that the LpIT/Aas system is important for maintaining the integrity of the membrane envelope in Gram-negative bacteria. Our insights may help inform new therapeutic strategies to enhance host sPLA\textsubscript{2} antimicrobial activity.

The ability of humans and other mammalian species to combat a wide array of potentially invasive bacterial species depends, in part, on diverse cellular and humoral antibacterial innate immune systems (1,2). Among the latter,
secretory phospholipases A₂ (sPLA₂) can act directly against many Gram-positive bacteria and against both Gram-positive and Gram-negative bacteria when present in concert with other cellular and humoral host defense systems (3-10). sPLA₂ catalyze the breakdown of membrane phospholipid (PL) by hydrolyzing the acyl ester bond at the sn-2 position, generating free fatty acid and detergent-like lysophospholipid (LPL) (11). sPLA₂ are present in various tissues, tears, and inflammatory fluids, including at high levels in blood plasma of patients with acute bacterial infections. Among the family of ten different sPLA₂ in humans, the Group IIA isoform (sPLA₂-IIA) is considered the most potent antibacterial (11). However, under at least certain circumstances (e.g., combined action with membrane attack complex (MAC)), other sPLA₂ including the “pancreatic” sPLA₂-IB can also act on Gram-negative bacteria and contribute to host antibacterial action (6,13,16,17).

sPLA₂ act most efficiently by binding to PL-rich interfaces from which individual PL molecules can diffuse into the active site pocket of the bound enzyme, be degraded, and the products replaced by another PL substrate molecule from this interface (namely interfacial activation mechanism) (14,18). sPLA₂, especially Group IIA, can penetrate through the cell wall of Gram-positive bacteria to directly bind to the membrane, resulting in the high susceptibility of many Gram-positive species (3). In contrast, Gram-negative bacteria exhibit much greater intrinsic resistance to sPLA₂, which has been attributed to the unique structure of their additional outer envelope layer, the outer membrane (OM) (3,19). The OM of Gram-negative bacteria is arranged with an asymmetric lipid distribution: phospholipids (mainly phosphatidylethanolamine, PE) comprise the inner leaflet of the membrane, while the outer leaflet contains the complex glycolipid lipopolysaccharide (LPS). The restriction of OM PLS to the inner leaflet of the OM precludes, under normal conditions, access of an extracellular sPLA₂ to the bacterial PL. The neutrophil-derived bactericidal/permability-increasing protein (BPI) and MAC facilitate sPLA₂ attack by disrupting the LPS-rich outer leaflet of the OM (1,6). However, even under these conditions, the bacterial potency of sPLA₂ towards many Gram-negative bacteria (e.g., E. coli, Salmonella typhimurium, Pseudomonas aeruginosa and Neisseria meningitidis) is still limited, requiring doses of sPLA₂ often beyond their physiological concentrations (1,6,13,20,21). Thus, this intrinsic resistance may help Gram-negative pathogens survive host antibacterial mechanisms and also limits potential therapeutic application of sPLA₂ towards Gram-negative bacteria-mediated infections.

Of note, Gram-negative bacteria uniquely contain a PL repair system comprised of two inner membrane (IM)-associated proteins: LPL transporter (LpIT) and acyltransferase-acyl carrier protein synthetase (Aas) (22) (Fig. 1a). LpIT promotes energy-independent trans-bilayer migration of each of the major LPL metabolites in Gram-negative bacteria (i.e. lysophosphatidylethanolamine (LPE), lysophosphatidylycerol (LPG), and lyso-cardiolipin) from the periplasmic to the cytoplasmic leaflet of the IM (23). Aas is a peripheral membrane protein on the cytoplasmic side of the IM (24), positioned to convert transported LPLs to their respective parent diacyl-PL species (PE or PG) or a triacyl form of cardiolipin (CL)(23). The potentially membrane-disruptive properties of LPLs raised the possibility that the LpIT/Aas protein system plays a housekeeping role to minimize accumulation of LPLs generated de novo as by-products of normal bacterial envelope modification processes, as in the biogenesis of OM lipoproteins (25,26). Deletion of lplT or aas gene, however, resulted in only a modest elevation of LPL in E. coli and no change in bacterial growth or viability (22,23,26). In contrast, OM-depleted spheroplasts generated from E. coli cells that lacked LpIT or Aas function exhibited increased sensitivity to venom-derived sPLA₂ (23), suggesting that LpIT/Aas provide a “self-defense” mechanism to counteract the membrane-disruptive effects of LPLs produced under host-stressed conditions that include activation of sPLA₂ action.

To test this hypothesis, we used E. coli as a model to explore the role of LpIT/Aas in the resistance of intact Gram-negative bacteria to mammalian sPLA₂. Our findings demonstrate a remarkable role for cytoplasmic membrane LpIT/Aas in protecting E. coli intact bacteria from the restriction of OM
the attack of sPLA₂. Unexpectedly, our data indicated that the resistance mediated by LplT/Aas is not attributed to any direct repairing of sPLA₂-generated LPLs. Instead, LplT and Aas cooperatively function to maintain the ability of both the OM and IM to act as structural barriers to initial interfacial activation of sPLA₂. The conservation of the lplT/aas loci in many Gram-negative bacteria including those pathogenic species listed above raises the possibility of a conserved mechanism of resistance in Gram-negative microorganisms. This study provides the first evidence of a physiological role of LplT/Aas in bacterial defense and suggests new strategies for rendering Gram-negative bacterial pathogens more sensitive to host antimicrobial activities.

RESULTS

Coupling of LplT and Aas in phospholipid repair—In prokaryotes, functionally related genes are often co-transcribed in one bicistronic operon. The genes of aas and lplT are adjacent to each other in the same operon in the genomes of E. coli and many other Gram-negative bacterial species (22). Our previous spheroplast study was carried out using the E. coli BL21(DE3) strain (23). To avoid any genetic variation, our current study was performed in the background of a standard E. coli W3110 strain. Two single gene knockout strains, ΔlplT and Δaas, were generated using the λ red recombination approach (Fig. S1a, Table S1). Deletion of these genes did not affect bacterial growth in LB broth (Fig. S1b). Their loss of LPL acylation (ΔlplT and Δaas) activity or LPL transport (ΔlplT) was confirmed using the spheroplast-based [32P] LPE acylation and transport assays (Figs. 1b, 1c & S2a). The functional coupling of LplT and Aas was further demonstrated by treating ΔlplT spheroplasts with 1% Triton X-100 detergent (Figs. 1b & S2b). These assays are consistent with our previous observation (23) that LplT and Aas operate as a coupled functional unit which provides a unique cytoplasmic membrane PL repairing mechanism in E. coli.

Deletion of the lplT or aas genes increases sensitivity of E. coli to sPLA₂-dependent killing—E. coli, including rough strains (producing truncated LPS), are intrinsically resistant to sPLA₂, including all ten human isoforms tested at μM concentration (6,13,15). Exposure of E. coli to host non-enzymatic antibacterial proteins producing alterations of the OM (e.g., BPI; MAC) increases bacterial sensitivity to mammalian sPLA₂, including sPLA₂-IB (“pancreatic”). To test the possible role of LplT and/or Aas on the sensitivity of E. coli to sPLA₂, we incubated E. coli WT, ΔlplT and Δaas strains with purified porcine pancreatic sPLA₂ (referred to as psPLA₂) and/or a previously characterized rabbit inflammatory (ascitic) fluid (AF) that contains BPI, several other rabbit granulocyte-derived cationic antimicrobial peptides and proteins and each of the components of the complement system needed to form MAC (6,21,27,28). Under the conditions tested, WT E. coli was resistant to AF and/or psPLA₂, as judged by colony-forming units (CFU) (Fig. 2a). In sharp contrast, both ΔlplT and Δaas strains exhibited much greater sensitivity to combined treatment with AF + psPLA₂ (>95% of the mutant cells killed within 1 h) (Fig. 2a). The greater sensitivity of the mutant strains was also manifest kinetically, with nearly all of the mutant bacteria killed within 2 h, with a half time of approximately 30 min (Fig. 2b). Maximal killing of the mutant strains required the combined and dose-dependent presence of both AF and psPLA₂ (Figs. 2a,c,d) and the presence in AF of cationic antimicrobial proteins (e.g., BPI) (Fig. 2a). Virtually identical results were obtained using three different AF (Fig. S3), including one collected from a complement (C)6-deficient rabbit that is unable to form the membrane attack complex of complement (C5a-8)₉a (6). We found that both ΔlplT and Δaas cells in lag, logarithmic growth or stationary phase were highly prone to treatment with AF + psPLA₂ and their sensitivities were indistinguishable while the resistance of WT was apparent at each phase of growth (Fig. S4). We also tested a E. coli double knockout strain Δaas/lplT, in which the entire aas/lplT locus was deleted in the genome (Fig. S1). The increased sensitivity of the ΔlplT, Δaas and Δaas/lplT strains was nearly identical in all tested conditions (Figs. 2b-2d). These data strongly suggest that the susceptibility of E. coli to psPLA₂ in the presence of AF can be significantly enhanced by disrupting either the lplT or aas gene and thus imply an essential role of LplT/Aas in protecting the bacterium from sPLA₂ attack.
**LPL acylation confers bacterial resistance to sPLA₂**—We next asked whether the increased susceptibility of Δ*lplT* and Δ*aas* strains is caused by their loss of LPL acylation activity or by the gene deletions per se, given the fact that the membrane proteins LplT and Aas are also structural components of *E. coli* membrane envelope. To address this question, we generated two chromosomal single residue knock-in mutant strains, *lplT*D30A and *aas*H36A. D30 is a conserved residue localized on the 1st transmembrane domain of LplT and was predicted to be important for substrate binding (25). By substituting D30 with an alanine, the *lplT*D30A strain completely abrogated its LPE transport activity (Fig. 3a). The bifunctional Aas protein harbors two catalytic domains: an N-terminal acyltransferase PlsC domain and a C-terminal acyl-ACP synthetase ACS domain. H36 is an essential catalytic residue in the PlsC domain (29). Spheroplasts generated from *lplT*D30A or *aas*H36A cells lack the ability to acylate LPE (Figs. 3b & S2). We next examined these two knock-in strains for their sensitivity to psPLA₂. The results showed that both *lplT*D30A and *aas*H36A strains exhibited high sensitivity to psPLA₂ and the extent of their susceptibility was nearly identical to that observed in the knockout strains (Fig. 3c). Note that expression of both LplT*D30A and Aas*H36A proteins was similar to that of the respective WT proteins (Fig. 3d). Taken together, these results strongly support the notion that the LPL acylation function of LplT/Aas confers increased bacterial resistance to psPLA₂-dependent killing.

**PL degradation is facilitated by inactivation of LplT/Aas**—The increased susceptibility of Δ*lplT* and Δ*aas* strains may reflect increased PL hydrolysis mediated by psPLA₂. Under normal growth conditions, three major *E. coli* PLs (PE, PG and CL) are present in the IM, whereas PE is relatively enriched in the OM (30). To better characterize the action of psPLA₂, WT and mutant *E. coli* were metabolically pre-labelled with [³²P]Pi and then incubated for 2 h with AF ± psPLA₂. At the times indicated, lipids were extracted and resolved by thin-layer chromatography (TLC). The identity of each PL/LPL species was unambiguously determined by comparing its migration rate with that of PL/LPL standards on TLC (Fig. S5). Metabolic radiolabeling of the WT and mutant bacteria were generally similar, with PE representing 60-65 mol%, PG 20 mol%, CL 7-8 mol% and other components representing in toto <10 mol% of the total labeled PL pools (zero time in Fig. 4a & Table S2).

In the presence of AF + psPLA₂, no breakdown of PL nor accumulation of LPL was found in the WT parent strain (Fig. 4a). In contrast, massive PL degradation occurred in both Δ*aas* and Δ*lplT* strains under the same treatment conditions (not in the presence of AF only (Fig. S6)), reflected by accumulation of multiple LPLs (Fig. 4a). Overall, the kinetics of degradation of the various PL species were very similar in the Δ*aas*, Δ*lplT* and Δ*aas*/Δ*lplT* strains. The levels of LPL in the two mutant strains rose to nearly 45% of the total radiolabeled PL pool when nearly all bacteria were killed (Fig. 4b and Table S2). However, the catabolic kinetics of individual PL was remarkably different (Figs. 4c-4d). LPE was initially the most prominent LPL breakdown product representing 17 mol% of the total PL pool after just 10 min incubation. Most (>80%) of the treated mutant bacteria were viable at this time (Fig. 2b), consistent with psPLA₂ action initially limited to the OM. In contrast, accumulation of lyo-derivatives of PG and CL progressed more slowly and continuously throughout the 2 h incubation period, equaling (LPG) or exceeding (mono-acyl CL (MCL)) net accumulation of LPE at 2 h (Figs. 4c-d). Indeed, an acute induction of CL was apparent in both mutant strains when the mutant bacteria were treated with both AF + psPLA₂ (not AF alone (Fig. S6)), resulting in a nearly 2-fold increase in CL levels within the first 10 min of incubation (Fig. 4a). After 2 h of incubation, the level of MCL exceeded by nearly 3x the amount of CL initially present in the bacteria before treatment with AF + psPLA₂. psPLA₂-catalyzed de-acylation of CL is stepwise *in vitro* (Fig. S5)(23). However, only the mono-acyl form was detected in AF + psPLA₂-treated *E. coli* (Δ*lplT* and Δ*aas*) (Fig. 4a). In contrast to the kinetics of LPE accumulation, the rate and extent of CL hydrolysis much more closely paralleled loss of bacterial viability (Figs. S7a-b), consistent with psPLA₂ action in the IM. These results demonstrate that inactivation of LplT/Aas facilitates the PL hydrolytic activity of sPLA₂ on the bacterial envelope.
LplT/Aas prevent the binding of psPLA2 to the E. coli outer membrane—The lack of LPL accumulation in WT E. coli treated with AF + psPLA2 (Fig. 4a) could reflect either extremely efficient reacylation of psPLA2-triggered LPL or an unexpected effect of LplT/Aas on the resistance of E. coli to psPLA2 attack. To distinguish these two possibilities, the same reaction conditions were examined with [14C]-oleic acid labeled cells and 1.5% albumin to trap the released free fatty acids (and LPL) in the extracellular medium and thus preclude LPL reacylation. Consistent with the TLC assays, both ΔlplT and Δaas strains showed progressive PL degradation, with nearly 30% of radiolabeled bacterial fatty acids recovered in the extracellular medium after 2 h incubation, whereas there was little detectable fatty acid released from the WT bacteria (Fig. 5a). These findings thus indicate an unexpected effect of LplT/Aas on the resistance of E. coli to psPLA2 attack.

To further test this possibility, we also examined psPLA2 binding to the WT and mutant bacteria, since sPLA2 binding to target E. coli is an important determinant of its action (31,32). As shown in Fig. 5b, psPLA2 binding was detected to the mutant but not the WT bacteria expressing functional LplT and Aas and only when added together with AF. Thus, these results suggest that, surprisingly, the function of LplT and Aas maintains the resistance of E. coli to psPLA2 by occluding psPLA2 from the bacterial outer envelope.

Effects of LplT/Aas inactivation on the physical and compositional properties of the outer membrane—Enhanced psPLA2 binding and catalysis towards the mutant strains suggested that the natural resistance of the OM to sPLA2 depended on the functional integrity of LplT/Aas. If so, we predicted that the properties of the outer leaflet of the OM would be modified in the absence of functional LplT or Aas in a way conducive to sPLA2 action. We tested this hypothesis by comparing the interaction of the WT and mutant strains with two different probes of membrane outer leaflets: i) Nile Red-based NR12S whose amphiphilic anchor (Fig. 6a) precludes transbilayer migration of the dye (33); and ii) PE-specific probe Ro 09-0198 (cinnamycin)-PEG5000-Alexa488 (34) that is also restricted to the surface of the outer leaflet by virtue of the linkage of PEG5000 (Fig. 6c). Incubation of NR12S with WT, ΔlplT and Δaas E. coli resulted in both an increase in the maximum fluorescence of the dye in comparison to its properties in aqueous buffer and a blue shift of its emission spectrum (Fig. 6b), indicative of insertion of the dye into the (outer) membrane in each of these strains. However, in both of the mutant strains, the fluorescence intensity of the dye was substantially higher than in WT bacteria and the wavelength of their fluorescence maximum exhibited a slight red-shift (Fig. 6b). These findings are consistent with looser (less ordered) intermolecular packing within the outer leaflet of the two mutant strains.

One possible explanation for the different interactions of NR12S with the mutant bacteria is a role for LplT/Aas in maintaining the extreme lipid asymmetry of the OM of WT E. coli, restricting phospholipids (e.g. PE) to the inner leaflet and enriching the more ordered LPS in the outer leaflet. Binding of fluorescent Ro 09-0198-PEG5000-Alexa488 was significantly greater in the mutant vs. the WT strains (Fig. 6d), consistent with an increased amount of accessible PE on the surface of E. coli when the bacteria lack functional LplT or Aas. In contrast, no bacterial-bound fluorescence was detected when the probe was incubated with a PE-deficient E. coli strain AL95 (35), confirming that bacterial binding of Ro 09-0198-PEG5000-Alexa488 was PE-dependent. Pre-incubation of the mutant, but not WT bacteria with a low dose of AF further increased binding of the fluorescent Ro 09-0198 probe (Fig. 6d) whereas the cationic protein-depleted AF had no effect. These results strongly suggest that inactivation of LplT/Aas resulted in a less extreme lipid asymmetry of the OM, which was further amplified by superimposed OM-perturbing effects of the cationic components in AF (e.g. BPI).

Absence of LplT/Aas function does not promote activation of bacterial OM PagP or PldA—Two mechanisms known to contribute to the extreme lipid asymmetry of the OM are mediated by phospholipase A (PldA) and lipid A palmitoyltransferase (PagP), which are also potentially linked to the function of LplT/Aas. Both enzymes normally remain inactive in bacteria, but can be activated to generate LPL
when PL is present in the outer leaflet of the OM (36,37). There was no significant increase in LPL or free fatty acid in the ΔlplT/Δaas strains, even in the presence of AF (without psPLA2) (Table S2 & Fig. S8), indicating no activation of PldA under these conditions. PagP catalyzes palmitoylation of lipid A by using externalized PL as acyl donor to convert PE + hexa-acylated lipid A (LPS) to hepta-acylated LPS and LPL. However, lipid A released from the extracted LPS of both WT and mutant strains and analyzed by mass spectrometry revealed only hexa-acylated lipid A (Fig. S9) indicating that OM PagP is not activated in the absence of LplT/Aas.

**LplT/Aas protect bacterial IM from sPLA2 attack**—The extent of PL degradation by the combined action of AF + psPLA2 in the mutant strains suggests strongly that PL breakdown and accumulation of LPL under bactericidal conditions extend to the IM where essential energy-dependent and -generating machinery reside. We recently showed that OM- and cell wall-depleted spheroplasts generated from WT *E. coli* BL21(DE3) cells were resistant to added venom sPLA2 whereas the corresponding spheroplasts from mutant (ΔlplT or Δaas) bacteria were sensitive (23). These observations were confirmed and extended using spheroplasts generated from the WT and mutant W3110 strains used in this study. In contrast to the requirements for action on intact bacteria, psPLA2 added alone (i.e., without AF), even at relatively low enzyme concentrations, produced extensive PL degradation in ΔlplT or Δaas spheroplasts (Figs. 7a-c). By contrast, WT spheroplasts were highly resistant to the added psPLA2 even with albumin present to preclude recycling of the PL breakdown products. These findings indicate that the presence of LplT/Aas during bacterial growth and spheroplast generation markedly increases the resistance of the IM to psPLA2.

In the absence of added psPLA2, the spheroplasts from both the WT and mutant strains were stable in 0.5 M sucrose and equally able to regenerate CFU (Fig. 7a). Incubation with psPLA2 under these conditions caused >90% reduction in regeneration of CFU from the mutant spheroplasts but had no effect on the WT spheroplasts (Fig. 7a). In the absence of added psPLA2, the mutant spheroplasts were much more susceptible to hypo-osmotic shock (Fig. 7d). Taken together, these findings suggest that the function of LplT and Aas contributes to the stability of the IM, with vulnerability to psPLA2 a particularly sensitive marker of IM instability (see Discussion).

**Discussion**

Here, we demonstrate a novel role of LplT and Aas in the resistance of *E. coli* to host sPLA2. In the experiments shown, we purposely used low doses of AF to dilute out sPLA2-IIA present in AF (5) and to eliminate any psPLA2-independent killing of either WT or mutant strains. Under identical conditions in which no detectable PL degradation and LPL accumulation and no diminution in bacterial viability were seen in the WT strain, >90% of the mutant bacteria were killed (Figs. 2a, 4a). This degree of killing paralleled degradation of >40% of the pre-labeled PL and their accumulation as LPL, suggesting accumulation of LPL in the IM where they could exert toxic effects on the lamellar structure of bilayer and essential energy-generating and –dependent metabolic systems.

We began these studies anticipating that the absence of LplT/Aas function would increase bacterial susceptibility to added sPLA2 by allowing increased net accumulation of LPL produced during sPLA2 treatment. However, the absence of measurable PL degradation (Fig. 4a) in the WT strain expressing both LplT and Aas precluded testing a role of LplT/Aas-mediated PL repair during sPLA2 treatment on restraining the consequences of sPLA2 action. Ironically, this limitation of our experimental design made possible detection of an unexpected role of LplT/Aas function during bacterial growth on the physical properties and composition of the outer leaflet of the OM. The relevance of these effects to psPLA2 action is strongly supported by the correlation of these OM changes with the ability of psPLA2 to act on the mutant but not WT bacteria. This was most evident in the studies of the PE-specific/OM outer leaflet-restricted PE-specific probe Ro 09-0198-PEG5000-Alexa488 that showed maximal interactions with AF-treated mutant strains (Fig. 6d), matching the conditions most favorable for psPLA2 surface binding and action on the intact bacteria (Fig. 5). Although
other surface modifications yielding accessing phosphorylethanolamine are possible, we believe that the mobilization of increased outer leaflet phosphatidylethanolamine (Fig. 6d) is most consistent with changes promoting psPLA₂ binding and interfacial activation as well as the looser lipid packing revealed by the studies with NR12S (Fig. 6b).

The absence of detectable PldA or PagP activation under identical experimental conditions (Figs. S7-8) suggests that smaller perturbations in the lipid organization of the OM are sufficient for psPLA₂ activation in comparison to requirements for activation of these endogenous OM lipid-modifying enzymes. The absence of PldA and PagP activation may in fact be important, leaving accessible substrate PL available to the added sPLA₂. The ability of initially limited alterations of the OM leads to the massive net PL degradation observed (Figs. 4a, 5a) may be explained by the ability of products of sPLA₂ action (e.g., LPL), unlike those of PagP (e.g. hyper-acylated lipid A and LPL), to exacerbate membrane instability and thereby promote conditions favorable for further sPLA₂ action, especially in the setting of no LPL re-acylation (i.e., no LpIT/Aas function). Detergent-like LPL can disturb lamellar membrane structure, owing to its bending elasticity and local curvature-modulating features, promoting substrate binding and catalysis by sPLA₂ (18,38). Further perturbation of the OM would be expected to lead to greater mobilization of PE in the outer leaflet and activation of PagP, resulting in conversion of hexa- to hepta-acylated LPS, increased lipid packing in the outer leaflet, and resistance to membrane-active cationic antibacterial peptides and proteins including those present in AF (39). However, the rapid breakdown of PE by psPLA₂ could preclude this bacterial stress response. The apparent absence of PagP activity also suggests that inactivation of LpIT/Aas does not trigger the bacterial virulence regulatory system PhoP/PhoQ (40). The absence of induction of these protective bacterial responses may help further explain the remarkable sensitivity of the LpIT/Aas mutants to the combined action of host co-factors for sPLA₂ in AF and psPLA₂ that we observed.

The remarkable differences in psPLA₂ susceptibility of IM spheroplasts recovered from WT vs. ΔlplT and Δaas mutant strains (Fig. 7a) parallel relatively small differences in LPL accumulation in these IM-rich cell-free fractions (4 mol% from the WT strain and ca. 8 mol% in the mutant strains) (22,23). These findings thus also suggest that initially small lipid differences can be sufficient for much greater differences in sPLA₂ action, especially in the setting of absent LpIT/Aas function. The resistance of the WT spheroplasts to psPLA₂ was somewhat surprising given the sensitivity of cell-free membrane protoplasts from the Gram-positive bacteria Staphylococcus aureus and Bacillus subtilis (3). These findings suggest important differences in the sensitivity of phospholipids within the corresponding cytoplasmic (inner) membranes of Gram-negative and Gram-positive bacteria to host sPLA₂ that may contribute to the greater bactericidal potency of mammalian sPLA₂ toward Gram-positive bacteria.

More challenging is to understand how LpIT and Aas residing in or tightly associated with the IM can affect the changes in the OM. OM lipid asymmetry as an essential protective strategy of Gram-negative bacteria is believed to be facilitated by multiple mechanisms including the cytoplasmic Mla system, which promotes retrograde transport of diacyl PLs from OM to IM (41). The OM conformation is also mediated by the processes of membrane biogenesis including those for LPS, which also occur in the IM (19,30). Whether accumulation of LPL as a result of LpIT/Aas inactivation alters the conformation and function of these biogenetic systems involved in the envelope homeostasis is unknown. It has been shown that LPL directly modifies the activity of bacterial membrane proteins such as mechanosensitive channel MscL (42). Indeed, the acute accumulation of CL at the early stage of the psPLA₂ action (Fig. 4a and Table S2) suggested that bacterial de novo PL biosynthetic enzymes were stimulated by the accumulation of LPL. Characterization of the function and lipid requirement of membrane biosynthetic proteins in the ΔlplT and Δaas strains may help to understand the role of LpIT/Aas in maintaining bacterial OM lipid asymmetry.

In summary, we have demonstrated a new Gram-negative bacterial resistance mechanism against host sPLA₂ using E. coli and psPLA₂ that depends on the conserved LPL transport and
acylation system (LpIT/Aas) that is unique to Gram-negative bacteria. Given the conservation of this system in many other Gram-negative bacteria and the similar interfacial activation requirements of the various sPLA₂, we predict that the principles revealed in this study should apply to other strains and species of Gram-negative bacteria and other sPLA₂ including sPLA₂-IIA, a hallmark of mammalian inflammatory responses to infection (3,4,7,12). The continuing development and spread of antibiotic resistance genes among Gram-negative bacteria – normal members of the microbiome and pathogens alike – have increased interest in the design and development of compounds that rather than targeting bacterial functions crucial for viability per se instead target sites that would render host defense systems mobilized during infection and inflammation more effective (43). The fact that inactivation of LpIT and Aas does not effect on bacterial normal growth (Fig. S1b), but specifically disarm their resistance to sPLA₂-mediated host antibacterial attack fits well this description. Therefore, the experimental model described herein provides a template for testing LpIT/Aas as new drug targets dealing with Gram-negative bacterial resistance in the future.

Experimental procedures

Materials and strains—The rabbit AFs used in this study were previously isolated from sterile inflammatory peritoneal exudates in New Zealand white rabbits following intraperitoneal injection with oyster glycogen-saturated saline (6,27) and stored at -80°C prior to use. To deplete rabbit AF of cationic proteins, AF was incubated with CM-sephadex resin equilibrated in 0.9% (w/v) saline buffered with 2.5 mM Tris-HCl, pH7.5 for 1 h followed by recovery of the unbound fraction that contained greater than 95% of the total AF protein content but was fully (>98%) depleted of cationic proteins (28). sPLA₂ purified from porcine pancreas and Crotalus adamanteus venom were purchased from Sigma or Worthington. [³²P] PO₄ and [¹⁴C] oleic acid were purchased from MP Biochemicals or Perkins Elmer. [³²P] LPL was generated by digestion of [³²P] PL using venom PLA₂ as previously described (23). 1-oleoyl-2-hydroxy-sn-glycido-3-phosphoethanolamine (18:1 LPE) was a product of Avanti Polar Lipids. Anti-psPLA₂ antibody was purchased from Lifespan Biosciences. E. coli strains W3110, AL95 and plasmids used for bacterial genetics were gifts from Drs. William Dowhan or Jiqiang Ling (University of Texas, McGovern Medical School). NR12S (N-[3-[(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl]oxy]propyl]-N-methyl-N-(3-sulfopropyl)-1-dodecanaminium) was kindly provided by Dr. Andrey S. Klymchenko (University of Strasbourg). Ro 09-0198 and Alex488-polyethylene glycol 5000-NHS were purchased from Cayman or Nanocs.

Construction of E. coli knockout strains—Chromosomal in-frame mutants were generated in the background of E. coli W3110 strain using λ red homologous recombination approach as described (44). The primers used for mutagenesis are listed in Table S1. For gene knockout mutants, linear gene displacement cassettes containing a chloramphenicol resistance region and homologous sequences were PCR amplified from the plasmid template pKD3. The PCR products were transformed into competent cells of E. coli W3110 cells carrying the plasmid pKD46 by electroporation. λ red recombinase in the competent cells was induced by adding L-arabinose in the medium. Transformants were incubated in LB broth containing ampicillin and 10 mM L-arabinose for 2 h at 32 °C for homologous recombination and then selected on LB agar plates containing chloramphenicol at 30 °C overnight. Replacement of the target genes by the chloramphenicol resistance gene was verified by PCR. The temperature-sensitive plasmid pKD46 was cured by incubation at 37 °C in LB broth. Excision of resistance cassettes was carried out using the helper plasmid pCP20 at 30 °C. Temperature-sensitive plasmid pCP20 was further cured by overnight incubation at 42 °C in LB broth. Positive mutants were examined by the loss of chloramphenicol resistance and by PCR-based DNA sequencing.

Construction of E. coli knock-in strain—Chromosomal knock-in mutant strains were generated using the Kn-pBAD-ccdB selectable cassette in two steps. The heat-inducible plasmid pSIM6 was first transformed into the W3110 cells to prepare λ-Red induced cells. In the first step, a Kn-pBAD-ccdB cassette flanked by homologous sequences was amplified using PCR. The PCR product was transformed into the cells by
electroporation. Transformants were immediately suspended into 2 ml LB broth supplemented with 1% glucose and grown at 32 °C for 2 h to facilitate homologous recombination. Recombinants were selected on LB agar plates supplemented with ampicillin, kanamycin and 1% glucose at 30 °C overnight. Replacement of the target gene by the Kn-pBAD-ccdB cassette was verified by PCR and by comparing bacterial growth onto LB plates supplemented with 1% glucose or 1% arabinose. Prior to the second knock-in step, mutations corresponding to H36A and D30A was introduced into plasmids harboring the aas or lplT gene using site-directed mutagenesis. The PCR fragment containing aasH36A or lplTD30A mutation was transformed into the cells containing the Kn-pBAD-ccdB cassette. After electroporation, cells were incubated in 2 ml LB broth supplemented with 1% glucose at 32 °C for 2 h. Knock-in mutants were selected on LB agar plates containing ampicillin and 1% arabinose at 37 °C overnight. Replacement of the Kn-pBAD-ccdB cassette by the mutant DNA was verified by PCR and examined on LB plates supplemented with 1% arabinose. The pSIM6 plasmid was cured by growing positive candidates overnight at 37 °C in LB broth and then tested for sensitivity to ampicillin. Knock-in mutations were further confirmed by PCR-based DNA sequencing.

Bacterial cells used in all assays were prepared using similar approaches. Briefly, E. coli cells were grown overnight in LB broth at 37 °C and then diluted (1:100) into fresh medium for continuous growth from lag phase (OD600=0.1), to logarithmic phase (OD600=0.5) and finally stationary phase (OD600=4.0). All experiments were carried out using E. coli cells harvested at the logarithmic phase if not indicated.

**Preparation of E. coli spheroplasts**—E. coli cells were washed 3x and re-suspended in 10 mM HEPES (pH 7.5), 0.75 M sucrose, 10 mM MgSO4 and 2.5% (w/v) LiCl. After addition of 1 mg/ml lysozyme, cell suspensions were ice-chilled and then warmed to room temperature, followed by incubation with gentle shaking at 30 °C for 30 min. Intact spheroplasts were collected by centrifugation (3,000 × g for 10 min) at room temperature and suspended at 10 mg/ml total protein in the above buffer without LiCl. Spheroplast formation and stability were thoroughly monitored nephelometrically by comparing the OD600 of a 100 µl spheroplast solution to 2 ml of either plain water or a solution of 10 mM MgCl2/0.45 M sucrose respectively.

**LPL acylation Assay**—LPL acylation activity was measured by monitoring conversion of LPE to PE in spheroplasts as we previously described (23). [32P] LPE (~100,000 cpm) mixed with synthetic 18:1 LPE of 200 µM in ethanol was used as substrate. 10 µM (final concentration) of substrates was added into spheroplast solutions. At the indicated time, reactions were terminated by adding chloroform-methanol (1:2 v:v). The lipids were extracted and separated by TLC and analyzed using a phosphorimager. The acylation activity was expressed as % of LPE to PE conversion.

**LplT transport Assay**—LplT transport activity was measured by detecting import of [32P] LPE into spheroplasts using an approach we established previously (23). Briefly, 10 µM (final concentration) of substrates was added into 0.5 ml of spheroplast solutions. At the indicated time, samples were layered onto 0.15 ml of 22% perchloric acid and 0.50 ml of silicone oil and centrifuged at 14,000 × g for 5 min at room temperature to separate sphero-plasts from free LPE substrate. After centrifugation, the sample moves through silicone oil and the radioactivity of collected spheroplasts was measured using a liquid scintillation counter to calculate transport activity.

**Analysis of LplT and Aas mutant protein expression**—LplT and Aas proteins were overproduced using a T7 expression vector in E. coli BL21(DE3) strains as previously described (23). The genes encoding LplT or Aas from E. coli were cloned into a pET28a plasmid to fuse with a N-terminal His6 tag. The mutations were introduced into the respective vector using a standard site-directed mutagenesis method. Overproduction of WT and mutant proteins was performed in antoinduction medium (45) at 37°C overnight. Cells were harvested and ruptured by sonication. Cell lysates at similar protein concentrations were subjected to SDS-PAGE, transferred to immunoblots, and probed with anti-His antibody.

**Bacterial viability assay**—sPLA2-mediated...
bactericidal tests were performed using the protocol previously described with slight modifications (16,28). Prior to assays, E. coli cells were washed three times and suspended in a buffer containing 40% HBSS and 1.5% BSA. 100 µl of cell suspension (~10^8 cells) was mixed with rabbit AF and/or psPLA2 at 37 °C for indicated times. The reactions were terminated by diluting into 1 ml LB broth, and then 100 µl of a dilution was immediately spread on a LB agar plate. CFU was determined after 16 h incubation at 37 °C.

**TLC PL analysis**—PL analysis was carried out using [32P] labelled E. coli cells by TLC. To prepare [32P] bacteria, E. coli were grown in LB broth supplemented with 5 µCi/ml [32P] PO₄. The assay was performed using the same protocol for viability test as described above. At indicated time, the reactions were stopped by adding chloroform followed by lipid extraction using the acidic Bligh and Dyer method as described previously (23). Lipids were loaded onto Silica Gel G thin-layer plate pre-soaked with boric acid and developed with chloroform/methanol/ammonia/H₂O (60:37.5:1:3 v/v). The air-dried plate was exposed to a storage phosphor screen (Eastman Kodak) overnight. Individual PL was visualized and quantified using a phosphoimager (Bio-Rad) to calculate PL content expressed as mol % of the total PL pool.

**Free fatty acid release assay**—sPLA2 activity was determined by measuring [14C] fatty acid hydrolyzed from bacterial cells as previously described (46). [14C] labeled cells were prepared in LB broth containing 0.5 µCi/ml [1-14C] oleic acid. After labeling for 2 h at 37 °C, cells were pelleted and then incubated in fresh LB broth for additional 30 min at 37 °C. Cells were washed with 1% BSA in sterile PBS to remove unincorporated radioactive precursors and then suspended in PBS. The assay was carried out using the same protocol of viability test as described above. The reactions were stopped by pelleting intact cells. The supernatant containing radiolabeled free fatty acid and lyso compounds was collected and quantified by a liquid scintillation counter.

**sPLA2 binding Assay**—100 µl of E. coli cells (~10^8) in a buffer containing 40% HBSS, and 1.5% BSA was incubated with 6 units of psPLA2 in the presence or absence of 20 µl AF at 37 °C for 30 min. Cells were pelleted and washed 3x with the same buffer and then analyzed by Western blot using anti-psPLA2 antibody.

**NR12S fluorescence assay**—1 ml of E. coli cells were washed 3x buffer containing 0.1 M HEPES pH 7.5 and 100 mM KCl and suspended in 100 µl of same buffer. Cells were incubated with 20 nM NR12S (3 mM in DMSO stock) at room temperature for 30 min. Cells were washed 3x with the same buffer. Fluorescence spectra were measured on a fluorescence spectrophotometer at the excitation wavelength of 530 nm and the emission was recorded within the range between 560 nm and 700 nm wavelength.

**Ro 09-0198-based fluorescence assay**—To prepare a PE-specific fluorescent dye, Ro 09-0198 was conjugated with Alex488-polyethylene glycol 5000-NHS via amine-NHS chemistry. Briefly, the two compounds were mixed in a molar ratio of 1:2 in a buffer of 50 mM NaHCO₃ overnight at 4 °C. The reaction was stopped by adding 1 M lysine for 3 h. Prior to assays, E. coli cells were washed and then suspended in PBS saline. 20 µl of AF, cationic protein-depleted AF or buffer was added into 0.5 ml of cells (OD₆₀₀ = 1) for 30 min and then washed 2x with PBS supplemented with 2% LiCl. 1 µM of the Ro 09-0198 conjugate was mixed with bacteria for 30 min at 37 °C. Cells were washed 3x with buffer to remove unbound conjugates. Fluorescence signals were measured at the following wavelengths: excitation at 490 nm; emission at 520 nm using a fluorescence spectrophotometer.

**LC-ESI MS analysis of lipid A**—To isolate lipid A, 50 ml E. coli cells were washed twice with 50 ml PBS saline and then suspended in PBS (0.8 ml). Chloroform (1.0 ml) and methanol (2.0 ml) were added and the mixtures were vigorously shaked at room temperature for 30 min to make the one-phase Bligh-Dyer mixture (chloroform:methanol:water = 1:2:0.8) and extract and remove PL. Mixtures were then centrifuged at 5,000 rpm for 30 min. Pellets were washed once with fresh one-phase Bligh-Dyer solution (3.5 ml) and centrifuged as above. Resulting pellets were suspended in 1.0 ml of sodium acetate (12.5 mM, pH 4.5) and heated at 100 °C for 30 min. After cooling to room temperature, the suspension was
acidified to pH 1 with HCl and converted to a two-phase Bligh-Dyer mixture ((chloroform:methanol:PBS = 2:2:1.8 v/v) by adding 0.8 ml of PBS, chloroform and methanol (2 ml each). The lower (organic) phase of each mixture was retrieved after phase partitioning via centrifugation at 5,000 rpm for 30 min and used for analysis.

Normal phase LC-ESI MS was performed using an Agilent 1200 Quaternary LC system coupled to a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). A Unison UK-Amino column (3 µm, 25 cm × 2 mm) (Imtakt USA, Portland, OR) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v/v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 µl/min. The post-column splitter diverted ~10% of the LC flow to the ESI source of the TF5600 mass spectrometer, with MS settings as follows: Ion spray voltage (IS) = -4500 V, Curtain gas (CUR) = 20 psi, Ion source gas 1 (GS1) = 20 psi, De-clustering potential (DP) = -55 V, and Focusing Potential (FP) = -150 V. Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using Analyst TF1.5 software (Sciex, Framingham, MA).

**Spheroplasts stability test**—Spheroplasts generated from 10⁹ E. coli cells using the lysozyme-lithium method as above were suspended in 100 µl solution containing different concentrations of sucrose and then kept at 25 °C for 10 min. 900 µl LB broth supplemented with 0.45 M sucrose was added to spheroplasts. 3 µl of each samples was spotted on LB agar plate and incubated overnight at 37°C.

**Acknowledgments:** We thank William Dowhan for providing E. coli strains and the access to phosphoimager, Jiqiang Ling for providing plasmids and helps for bacterial genetic mutations, Andrey Klymchenko for providing the NR12S dye. This work was supported by NIH grants R01GM097290 and R01GM098572 to LZ, H2020-MSCA-RISE-2015-690853 to MB, and NIH grant R01GM61074 to WM.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions:** YL performed most experiments; MB assisted on lipid analysis and fluorescent assays; SL assisted on the Ro 09-0198 assay; ZQ performed MS analysis; WM helped on bacterial analyses; JW and LZ designed experiments and wrote the manuscript; all authors contributed to data analysis and the manuscript.

**Abbreviations:**
sPLA₂: secretory phospholipase A₂
LpIT: Lysophospholipid Transporter
Aas: 2-acyl lyso-PE acyltransferase/acyl-acyl carrier protein synthetase
OM: outer membrane
IM: inner membrane
LPS: lipopolysaccharide
MAC: membrane attack complex
BPI: bactericidal/permeability-increasing protein
PL: phospholipids
LPL: lysophospholipids
AF: ascitic fluid
psPLA2: pancreatic phospholipase A2
PE: phosphatidylethanolamine
PG: phosphatidylglycerol
CL: cardiolipin
LPE: lysophosphatidylethanolamine
LPG: lysophosphatidylglycerol
MCL: monoacylated cardiolipin
CFU: colony-forming units
Pi: inorganic phosphate
TLC: Thin-layer chromatography
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Figure Legends

Fig. 1 LplT/Aas-mediated LPL acylation in E. coli. a) Cartoon model showing lysophospholipid transporter LplT importing LPE from the periplasm across the cytoplasmic membrane for acylation by acyltransferase Aas to generate PE. b) LPE acylation assay of E. coli W3110 WT, ∆lplT, ∆aas strains. The activities were calculated based on conversion (%) of [32P] LPE to [32P] PE shown on TLC images in Fig. S2. c) LPE transport assays using spheroplasts generated from E. coli W3110 WT, ∆lplT, ∆aas cells. The transport activity of LplT in each sample was calculated based on the acquired radioactivity in the spheroplasts.

Fig. 2 Deletion of lplT or aas gene increases susceptibility of E. coli to psPLA2. a) Viability tests of E. coli W3110 WT, ∆lplT and ∆aas strains treated with 12 units of psPLA2 20 µl AF or 20 µl cationic protein-depleted AF for 90 min. b) Time-course viability assays of E. coli W3110 WT, ∆lplT, ∆aas and ∆aas/lplT strains (+ 12 units psPLA2, + 20 µl of AF). c-d) Titration experiment of E. coli viability: c) in the presence of 12 units of psPLA2 and indicated volume of AF; d) in the presence of 20 µl of AF and psPLA2 as indicated. The reaction time was 90 min. All data are expressed as CFU (%) compared to untreated WT control at zero time.

Fig. 3 E. coli resistance is determined by the function of LplT and Aas. a) LPE acylation assay of E. coli W3110, lplT^{D30A} and aas^{H36A} strains. The activities were calculated based on conversion (%) of LPE to PE shown on TLC images in Fig. S2. b) LPE transport assays using spheroplasts generated from E. coli W3110 WT, lplT^{D30A} and aas^{H36A} cells. The transport activity of LplT in each sample was calculated based on the acquired radioactivity in the spheroplasts. c) Viability tests of E. coli W3110 WT, lplT^{D30A} and aas^{H36A} strains (+ 12 units of psPLA2, +/- 20 µl AF) for 90 min. Data are expressed as CFU (%) compared to untreated WT control. d) Western blot analysis using anti-His antibody to examine LplT^{WT}, LplT^{D30A}, Aas^{WT} or Aas^{H36A} protein expressed in E. coli. Cell lysates containing the same amount of total protein were loaded for each sample.

Fig. 4 Inactivation of LplT/Aas facilitates psPLA2-mediated PL degradation in E. coli. a) TLC images of PL extracted from [32P] labeled E. coli cells of W3110 E. coli WT, ∆lplT, ∆aas and ∆aas/lplT strains. The cells were treated with 12 units of psPLA2 and 20 µl of AF for indicated times. b) Kinetics of PL degradation in ∆lplT, ∆aas and ∆aas/lplT cells. PL degradation is expressed as mol% of net LPL (LPE + LPG + MCL) compared to total PL in each sample. Degradation (mol%) of each individual PL of ∆lplT (c) and ∆aas (d) was obtained from image a and Table S2.

Fig. 5 LplT/Aas prevent AF-dependent binding of psPLA2 to E. coli. a) Free fatty acid release assay of E. coli W3110 WT, ∆lplT and ∆aas cells. ~10^6 E. coli cells labelled with [14C] oleic acid were incubated with 20 µl AF and/or 12 units of psPLA2. The radioactivity in the supernatant fraction was determined by scintillation counting. PL degradation is expressed as the % of total [14C] radioactivity in the reaction that
is recovered as the products of hydrolysis. b) Western blot of psPLA2 pull down assay of W3110 WT, ΔlplT, Δaas and Δaas/ΔlplT cells in the presence of psPLA2 and +/- AF. psPLA2 bound with the cells was detected using anti-psPLA2 antibody.

**Fig. 6 Alterations of the outer membrane in ΔlplT and Δaas strains.** a) Chemical structure of amphiphilic fluorescent probe NR12S; b) emission spectra of fluorescent probe NR12S in W3110 WT, ΔlplT and Δaas cells after excitation at 530 nm; c) assay of bacterial OM asymmetry by determining PE at the bacterial surface; d) changes in fluorescent intensity following the addition of the Ro 09-0198-PEG5000-Alexa488 conjugate into W3110 WT and mutant cells are shown.

**Fig. 7 LplT/Aas protect the IM from sPLA2 attack.** a) Susceptibility of spheroplasts generated from E. coli W3110 WT, ΔlplT or Δaas cells after treated with 12 units of psPLA2. Data are expressed as % of recovered CFU compared to untreated spheroplasts from the WT control. b) free fatty acids release assay of W3110 WT, ΔlplT or Δaas spheroplasts (+/- 1.5% BSA). c) psPLA2 titration experiment for free fatty acid release assay. Spheroplasts made from [14C] oleic acid-labelled E. coli cells were incubated with indicated concentrations of psPLA2 at 37 °C for 20 min. d) Stability test of WT, ΔlplT or Δaas spheroplasts (For details see Materials and Methods).

**Reference**

1. Elsbach, P., Weiss, J., and Levy, O. (1994) Integration of antimicrobial host defenses: role of the bactericidal/permeability-increasing protein. *Trends Microbiol* **2**, 324-328
2. Nauseef, W. M. (2007) How human neutrophils kill and degrade microbes: an integrated view. *Immunological Reviews* **219**, 88-102
3. Weiss, J. P. (2015) Molecular determinants of bacterial sensitivity and resistance to mammalian Group IIA phospholipase A2. *Biochimica et biophysica acta* **1848**, 3072-3077
4. Nevalainen, T. J., Graham, G. G., and Scott, K. F. (2008) Antibacterial actions of secreted phospholipases A2. Review. *Biochim Biophys Acta* **1781**, 1-9
5. Weinrauch, Y., Elsbach, P., Madsen, L. M., Foreman, A., and Weiss, J. (1996) The potent anti-Staphylococcus aureus activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. *Journal of Clinical Investigation* **97**, 250-257
6. Madsen, L. M., Inada, M., and Weiss, J. (1996) Determinants of activation by complement of group II phospholipase A2 acting against Escherichia coli. *Infection and Immunity* **64**, 2425-2430
7. Wu, Y., Raymond, B., Goossens, P. L., Njamkepo, E., Guiso, N., Paya, M., and Touqui, L. (2010) Type-IIA secreted phospholipase A2 is an endogenous antibiotic-like protein of the host. *Biochimie* **92**, 583-587
8. Birts, C. N., Barton, C. H., and Wilton, D. C. (2010) Catalytic and non-catalytic functions of human IIA phospholipase A2. *Trends in Biochemical Sciences* **35**, 28-35
9. Weinrauch, Y., Elsbach, P., Madsen, L. M., Foreman, A., and Weiss, J. (1996) The potent anti-Staphylococcus aureus activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. *The Journal of clinical investigation* **97**, 250-257
10. Femling, J. K., Nauseef, W. M., and Weiss, J. P. (2005) Synergy between extracellular group IIA phospholipase A2 and phagocyte NADPH oxidase in digestion of phospholipids of Staphylococcus aureus ingested by human neutrophils. *J Immunol* **175**, 4653-4661
11. Burke, J. E., and Dennis, E. A. (2009) Phospholipase A2 structure/function, mechanism, and signaling. *Journal of Lipid Research* **50**, S237-S242
Resistance mechanism of E. coli to host sPLA2

12. Grönroos, J. O., Laine, V. J. O., and Nevalainen, T. J. (2002) Bactericidal group IIA phospholipase A2 in serum of patients with bacterial infections. Journal of Infectious Diseases 185, 1767-1772
13. Degousee, N., Ghomashchi, F., Stefanski, E., Singer, A., Smart, B. P., Borregaard, N., Reithmeier, R., Lindsay, T. F., Lichtenberger, C., Reinisch, W., Lambeau, G., Arm, J., Tischfield, J., Gelb, M. H., and Rubin, B. B. (2002) Groups IV, V, and X phospholipases A2s in human neutrophils: role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. The Journal of biological chemistry 277, 5061-5073
14. Lambeau, G., and Gelb, M. H. (2008) Biochemistry and Physiology of Mammalian Secreted Phospholipases A2. Annual Review of Biochemistry 77, 495-520
15. Koduri, R. S., Grönroos, J. O., Laine, V. J. O., Le Calvez, C., Lambeau, G., Nevalainen, T. J., and Gelb, M. H. (2002) Bactericidal Properties of Human and Murine Groups I, II, V, X, and XII Secreted Phospholipases A2. Journal of Biological Chemistry 277, 5849-5857
16. Weinrauch, Y., Abad, C., Liang, N. S., Lowry, S. F., and Weiss, J. Mobilization of potent plasma bactericidal activity during systemic bacterial challenge. Role of group IIA phospholipase A2. The Journal of clinical investigation 102, 633-638
17. Weiss, J., Wright, G., Bekkers, A. C. A. P. A., Van den Bergh, C., and Verheij, H. M. (1991). J. Biol. Chem. 266, 4162
18. Berg, O. G., Gelb, M. H., Tsai, M.-D., and Jain, M. K. (2001) Interfacial Enzymology: The Secreted Phospholipase A2-Paradigm. Chemical Reviews 101, 2613-2654
19. Henderson, J. C., Zimmerman, S. M., Crofts, A. A., Boll, J. M., Kuhns, L. G., Herrera, C. M., and Trent, M. S. (2016) The Power of Asymmetry: Architecture and Assembly of the Gram-Negative Outer Membrane Lipid Bilayer. Annu Rev Microbiol 70, 255-278
20. Weiss, J., Inada, M., Elsbach, P., and Crowl, R. M. (1994) Structural determinants of the action against Escherichia coli of a human inflammatory fluid phospholipase A2 in concert with polymorphonuclear leukocytes. Journal of Biological Chemistry 269, 26331-26337
21. Qu, X. D., and Lehrer, R. I. (1998) Secretory phospholipase A2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears. Infect Immun 66, 2791-2797
22. Harvat, E. M., Zhang, Y. M., Tran, C. V., Zhang, Z., Frank, M. W., Rock, C. O., and Saier, M. H., Jr. (2005) Lysoosphospholipid flipping across the Escherichia coli inner membrane catalyzed by a transporter (LpIT) belonging to the major facilitator superfamily. The Journal of biological chemistry 280, 12028-12034
23. Lin, Y., Bogdanov, M., Tong, S., Guan, Z., and Zheng, L. (2016) Substrate Selectivity of Lysoosphospholipid Transporter LpIT Involved in Membrane Phospholipid Remodeling in Escherichia coli. The Journal of biological chemistry 291, 2136-2149
24. Jackowski, S., Jackson, P. D., and Rock, C. O. (1994) Sequence and function of the aas gene in Escherichia coli. The Journal of biological chemistry 269, 2921-2928
25. Zheng, L., Lin, Y., Lu, S., Zhang, J., and Bogdanov, M. (2017) Biogenesis, transport and remodeling of lyso phospholipids in Gram-negative bacteria. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1862, 1404-1413
26. Hsu, L., Jackowski, S., and Rock, C. O. (1991) Isolation and characterization of Escherichia coli K-12 mutants lacking both 2-acyl-glycerophosphoethanolamine acyltransferase and acyl-acyl carrier protein synthetase activity. The Journal of biological
Resistance mechanism of E. coli to host sPLA2

27. Zarember, K., Elsbach, P., Shin-Kim, K., and Weiss, J. (1997) p15s (15-kD antimicrobial proteins) are stored in the secondary granules of Rabbit granulocytes: implications for antibacterial synergy with the bactericidal/permeability-increasing protein in inflammatory fluids. *Blood* **89**, 672-679

28. Weinrauch, Y., Foreman, A., Shu, C., Zarember, K., Levy, O., Elsbach, P., and Weiss, J. (1995) Extracellular accumulation of potently microbicidal bactericidal/permeability-increasing protein and p15s in an evolving sterile rabbit peritoneal inflammatory exudate. *The Journal of clinical investigation* **95**, 1916-1924

29. Heath, R. J., and Rock, C. O. (1998) A Conserved Histidine Is Essential for Glycerolipid Acyltransferase Catalysis. *Journal of Bacteriology* **180**, 1425-1430

30. Kamp, J. A. F. O. d. (1979) Lipid Asymmetry in Membranes. *Annual Review of Biochemistry* **48**, 47-71

31. Weiss, J., Inada, M., Elsbach, P., and Crowl, R. M. (1994) Structural determinants of the action against Escherichia coli of a human inflammatory fluid phospholipase A2 in concert with polymorphonuclear leukocytes. *The Journal of biological chemistry* **269**, 26331-26337

32. Forst, S., Weiss, J., Maraganore, J. M., Heinrikson, R. L., and Elsbach, P. (1987) Relation between binding and the action of phospholipases A2 on Escherichia coli exposed to the bactericidal/permeability-increasing protein of neutrophils. *Biochimica et biophysica acta* **920**, 221-225

33. Kucherak, O. A., Oncul, S., Darwich, Z., Yushchenko, D. A., Arntz, Y., Didier, P., Mely, Y., and Klymchenko, A. S. (2010) Switchable nile red-based probe for cholesterol and lipid order at the outer leaflet of biomembranes. *Journal of the American Chemical Society* **132**, 4907-4916

34. Machaidze, G., and Seelig, J. (2003) Specific binding of cinnamycin (Ro 09-0198) to phosphatidylethanolamine. Comparison between micellar and membrane environments. *Biochemistry* **42**, 12570-12576

35. Bogdanov, M., Heacock, P. N., and Dowhan, W. (2002) A polytopic membrane protein displays a reversible topology dependent on membrane lipid composition. *The EMBO journal* **21**, 2107-2116

36. Dekker, N. (2000) Outer-membrane phospholipase A: known structure, unknown biological function. *Mol Microbiol* **35**, 711-717

37. Jia, W., Zoeiby, A. E., Petruzzello, T. N., Jayabalasingham, B., Seyedirashti, S., and Bishop, R. E. (2004) Lipid Trafficking Controls Endotoxin Acylation in Outer Membranes of Escherichia coli. *Journal of Biological Chemistry* **279**, 44966-44975

38. Tatulian, S. A. (2001) Toward understanding interfacial activation of secretory phospholipase A2 (PLA2): membrane surface properties and membrane-induced structural changes in the enzyme contribute synergistically to PLA2 activation. *Biophys J* **80**, 789-800

39. Bishop, R. E. (2005) The lipid A palmitoyltransferase PagP: molecular mechanisms and role in bacterial pathogenesis. *Mol Microbiol* **57**, 900-912

40. Groisman, E. A. (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* **183**, 1835-1842
Resistance mechanism of E. coli to host sPLA₂

41. Malinverni, J. C., and Silhavy, T. J. (2009) An ABC transport system that maintains lipid asymmetry in the Gram-negative outer membrane. *Proceedings of the National Academy of Sciences* **106**, 8009-8014

42. Lundbaek, J. A., and Andersen, O. S. (1994) Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. *The Journal of general physiology* **104**, 645-673

43. Sachdeva, S., Palur, R. V., Sudhakar, K. U., and Rathinavela, T. (2017) E. coli Group 1 Capsular Polysaccharide Exportation Nanomachinery as a Plausible Antivirulence Target in the Perspective of Emerging Antimicrobial Resistance. *Front Microbiol* **8**, 70

44. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006 0008

45. Studier, F. W. (2005) Protein production by auto-induction in high density shaking cultures. *Protein expression and purification* **41**, 207-234

46. Wright, G. C., Weiss, J., Kim, K. S., Verheij, H., and Elsbach, P. (1990) Bacterial phospholipid hydrolysis enhances the destruction of Escherichia coli ingested by rabbit neutrophils. Role of cellular and extracellular phospholipases. *The Journal of clinical investigation* **85**, 1925-1935
Resistance mechanism of E. coli to host sPLA2

Fig. 1
Resistance mechanism of E. coli to host sPLA₂

Fig. 2

(a) Resistance mechanism of E. coli to host sPLA₂

(b) Time course of CFU (%)

(c) Effect of cationic protein-depleted AF

(d) Effect of 20 μL AF

psPLA₂ - + + + - +
AF - + - + -
W3110 ΔiplT Δaas Δaas/iplT
Fig. 3

**Resistance mechanism of E. coli to host sPLA$_2$**

(a) PE conversion (%)

(b) LPE transport (nmol/g/h)

(c) CFU (%)

(d) SDS-PAGE gel

---

W3110

lipT$^{D304A}$

aas$^{H36A}$

psPLA$_2$

AF
Resistance mechanism of E. coli to host sPLA$_2$

Fig. 4

(a) Representative images of TLC analysis showing the lipid composition of WT, ΔplT, Δaas, and Δaas/ΔplT strains. The bands correspond to various lipids: CL, PE, PG, MCL, LPE, and LPG.

(b) Graph showing the survival cells (%) as a function of LPL/Total PL (%) over time (min).

(c) Graph showing the mol % of MCL, LPE, and LPG over time (min).

(d) Graph showing the mol % of MCL, LPE, and LPG over time (min).
Fig. 5

(a) Resistance mechanism of E. coli to host sPLA₂

(b) Staining of protein samples with psPLA₂

| psPLA₂ | AF | control |
|--------|----|---------|
| +      | +  | +       |
| +      | -  | +       |
| +      | +  | -       |
| +      | -  | -       |
| +      | +  | -       |
| +      | +  | -       |

W3110, ΔlipT, Δaas, Δaas/ΔlipT
Fig. 6

(a) NR12S

(b) Ex: 530 nm
Fluorescence intensity (a.u.)

- W3110
- ΔplT
- Δaas
- Buffer

Wavelength (nm)

(c) Alexa 488
PEG 5K
Cinnamycin
PE

OM

(d) Fluorescence intensity (a.u.)

- Buffer only
- + Cationic-depleted AF
- + AF

W3110 ΔplT Δaas AL95 (-PE)
Resistance mechanism of E. coli to host sPLA2

Fig. 7

(a) CFU (%) of W3110, ΔlpIT, and Δaas with -psPLA2 and +psPLA2.

(b) % ¹⁴C release over time for W3110, ΔlpIT, and Δaas with BSA.

(c) % ¹⁴C release as a function of PLA2 units for W3110, ΔlpIT, and Δaas.

(d) Sucrose adsorption assay for W3110, ΔlpIT, and Δaas.
The phospholipid-repair system LpIT/Aas in Gram-negative bacteria protects the bacterial membrane envelope from host phospholipase A2 attack

Yibin Lin, Mikhail Bogdanov, Shuo Lu, Ziqiang Guan, William Margolin, Jerrold P. Weiss and Lei Zheng

*J. Biol. Chem. published online January 18, 2018*

Access the most updated version of this article at doi: [10.1074/jbc.RA117.001231](http://dx.doi.org/10.1074/jbc.RA117.001231)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts