Pseudomonas aeruginosa utilizes host polyunsaturated phosphatidylethanolamines to trigger theft-ferroptosis in bronchial epithelium.

Haider H. Dar, Yulia Y. Tyurina, Karolina Mikulska-Raminska, Indira Shrivastava, Hsiu-Chi Ting, Vladimir A. Tyurin, James Krieger, Claudette M. St. Croix, Simon Watkins, Erkan Bayir, Gaowei Mao , Catherine Ambruster, Alexandr Kapralov, Hong Wang, Mathew R. Parsek, Tamil S. Anthonymuthu, Abiola F. Ogunsola, Becca A. Flitter, Cody J. Freedman, Jordan R. Gaston, Ted Holman, Joseph M. Pilewski, Joel S. Greenberger, Rama K. Mallampalli, Yohei Doi, Janet S. Lee, Ivet Bahar, Jennifer Bomberger, Hülya Bayır and Valerian E. Kagan

Supplementary Information

Supplementary Table 1. PE-OX species identified in Human Bronchail Epithelial (HBE) cells treated with *P. aeruginosa* hyper-biofilm mutant (ΔwspF) supernatant. Related to Figure 2.

Plus 1 Oxygen	Plus 2 Oxygen	Plus 3 Oxygen	Plus 4 Oxygen
PE(34:2)+1O	PE(34:4)+2O	PE(38:4)+3O	PE(38:2)+4O
	PE(38:4)+2O	PE(36:5)+3O	PE(40:6)+4O
	PE(36:5)+2O		
	PE(38:5)+2O		
	PE(36:4)+2O		
	PE(34:3)+2O		
	PE(36:3)+2O		
	PE(44:5)+2O		
	PE(38:6)+2O		
	PE(40:6)+2O		

Supplementary Table 2. Identification of PE-OX species in CF lung samples infected with *P. aeruginosa.* Related to Figure 5.

Plus 1 Oxygen	Plus 2 Oxygen	Plus 3 Oxygen	Plus 4 Oxygen
PE(36:3)+1O	PE(34:3)+2O	PE(38:5)+3O	PE(40:2)+4O
PE(36:4)+1O	PE(36:4)+2O	PE(40:8)+3O	PE(40:7)+4O
PE(38:2)+1O	PE(36:5)+2O	PE(42:4)+3O	
PE(40:2)+1O	PE(38:4)+2O		
PE(42:4)+1O	PE(38:7)+2O		
PE(42:5)+1O	PE(40:7)+2O		

Suplementary Table 3. Identification of Pathogenic bacteria present in CF lung samples. Related to Figure 5.

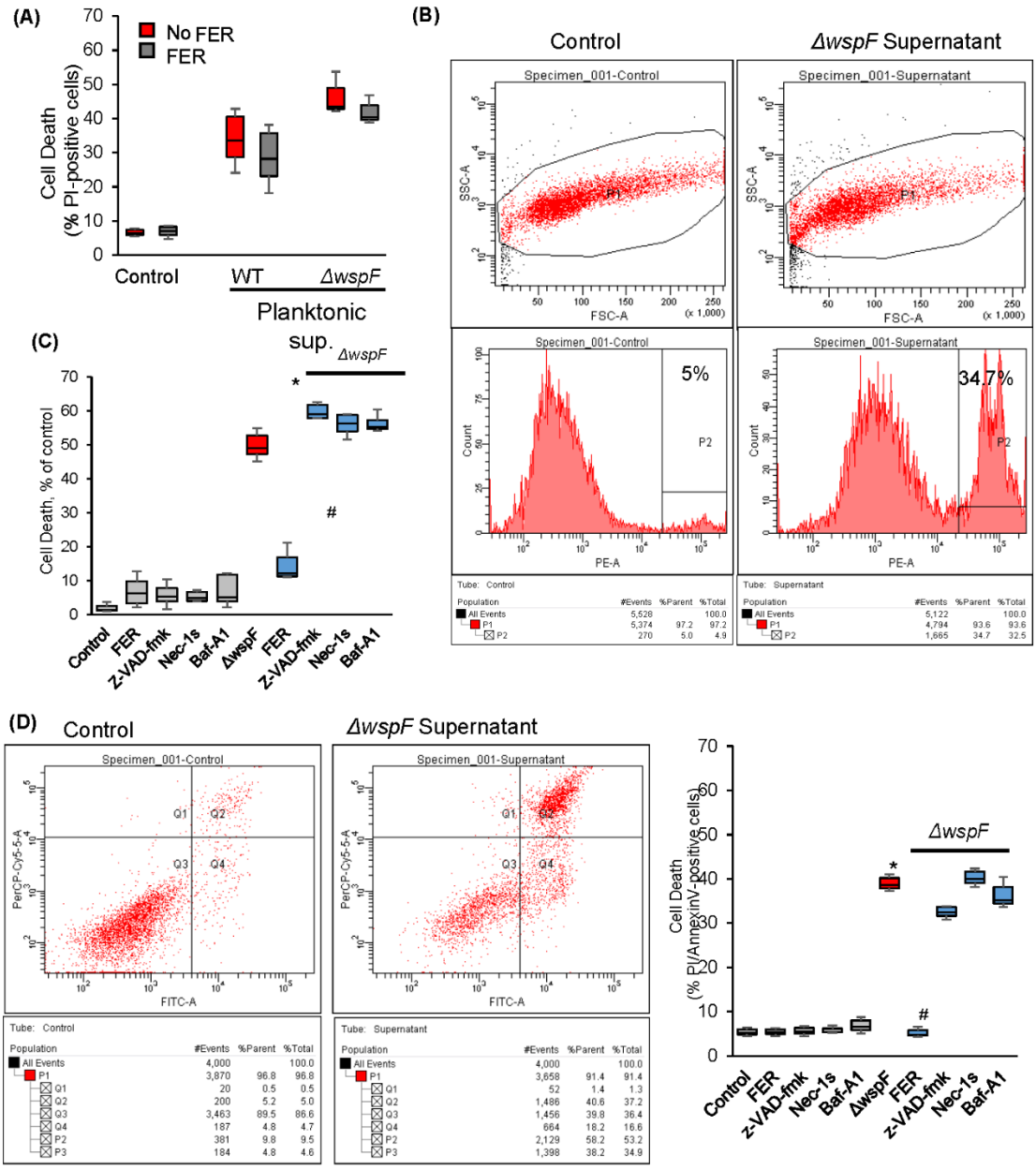
S. No.	Sample Name	Pathogenic bacteria
1	CF237	Achromobacter xylosoxidans
2	CF241	Pseudomonas aeruginosa
3	CF242	Pseudomonas aeruginosa, Stenotrophomas maltophilia
4	CF244	Burkholderia cenocepacia
5	CF245	Mycobacterium abscessus
6	CF246	Pseudomonas aeruginosa
7	CF247	Pseudomonas aeruginosa
8	CF248	Pseudomonas aeruginosa
9	CF249	Pseudomonas aeruginosa
10	CF250	Burkholderia cenocepacia

Supplementary Table 4. Bacterial strains, plasmids, and primers used for loxA complementation study. Related to Figure 1

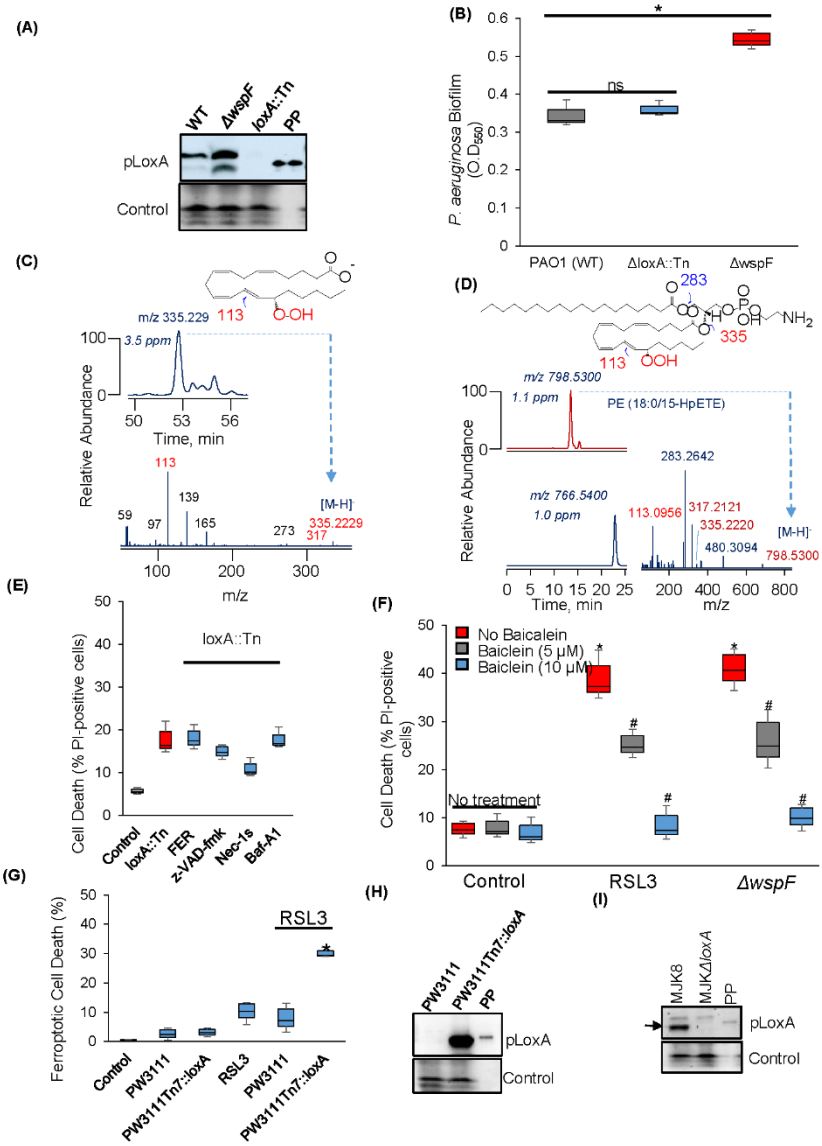
Strains	**Characteristics**	**Reference**
E. coli DH5α (Zymo5α)	F-φ80lacZΔM15 Δ(lacZYA-argF)U169 deoR nupG recA1 endA1 hsdR17(rK- mK+) phoA glnV44 (supE44) thi-1 gyrA96 relA1, λ–	Zymo Research
E. coli DH5α pENTR221L5L2::RBS-PA1169	E. coli harboring pDONR221P5P2 with an attL flanked, 2,070 bp fragment encoding a 12 bp synthetic ribosomal binding site and the 2,058 bp loxA ORF from P. aeruginosa PAO1, KmR	This study
E. coli DH5α pUC18miniTn7T::PBAD-PA1169	E. coli harboring a suicide plasmid for inserting arabinose-inducible PA1169 onto the P. aeruginosa chromosome at the attTn7 site, GmR	This study
P. aeruginosa PW3111	MPAO1 with a transposon inserted into PA1169 (loxA::Tn)	(1)
P. aeruginosa PW3111 Tn7:P_{BAD}-loxA	MPAO1 with a transposon inserted into PA1169 (loxA; PW3111) and complemented with arabinose-inducible loxA at attTn7 site, GmR	This study
Plasmids	**Characteristics**	
pUC18miniTn7T-Gm-GW	pUC18-miniTn7T-Gm with a Gateway destination cloning site, ApR, GmR, CmR	(4)
pTNS3	helper plasmid encoding tnsABCD, ApR	(2)
pENTR221L1L5r:araC-P_{BAD}	pDONR221P1P5r with an attL-flanked, 1,192-bp fragment encoding the araC repressor and the P_{BAD} promoter, KmR	(3)
pDONR221P5P2	Multisite Gateway donor vector with attP5 and attP2 recombination sites, CmR, KmR	Invitrogen
pENTR221L5L2::RBS-PA1169	pDONR221P5P2 with an attL flanked, 2,070 bp fragment encoding a 12 bp synthetic ribosomal binding site and the 2,058 bp loxA ORF from P. aeruginosa PAO1, KmR	This study
pUC18miniTn7T::P_{BAD}-PA1169	Suicide plasmid for inserting arabinose-inducible PA1169 onto the P. aeruginosa chromosome at the attTn7 site, GmR	This study
Primer	**Sequence**	**Reference**
attB5-RBS-loxA-F	ggggacaactttgtatacaaaagttgccag aggaggatattcATGAAACGCAGGAGTGTGC	This study
attB2-loxAdown-R	ggggaccactttgtacaagaaagctgggtaCGCCCCGTCCTCT TTCTC	This study
M13F (-21)	GTAAAACGACGGCCAG	Eurofins
M13R	CAGGAAACAGCTATGAC	Eurofins
pBAD-F	ATGCCATAGCATTTTTATCC	This study
pUC18BackboneR01	GGCCGATTCATTAATGCAGC	This study
PTn7R	CACAGCATAACTGGACTGATTTC	(4)
PglmS-down	GCACATCGGCGACGTGCTCTC	(4)

References

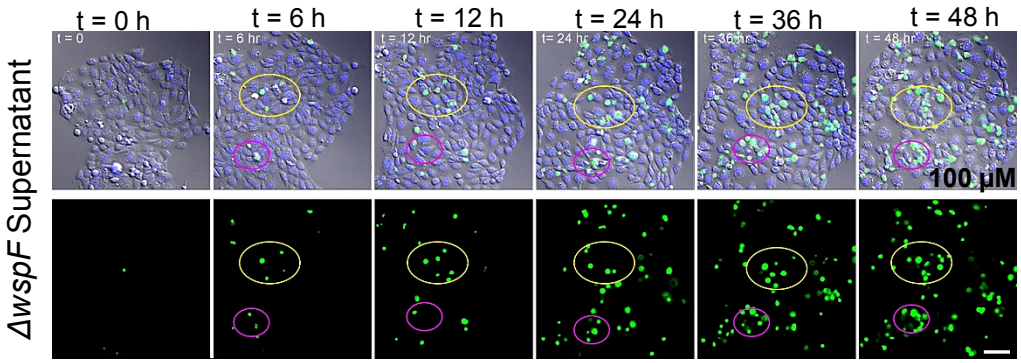
1. Jacobs MA, et al. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA*. 2003; 100(24):14339-44.
2. Choi KH, et al. Genetic Tools for Select-Agent-Compliant Manipulation of *Burkholderia pseudomallei*. *Applied and Environmental Microbiology*. 2008;74(16): 1064–1075.
3. Khakimova M, et al. The Stringent Response Controls Catalases in Pseudomonas aeruginosa and Is Required for Hydrogen Peroxide and Antibiotic Tolerance. *Journal of Bacteriology*. 2013;195(9):2011–2020.
4. Choi KH, and Schweizer HP. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. *Nature protocols.* 2006;1(1):153-61.



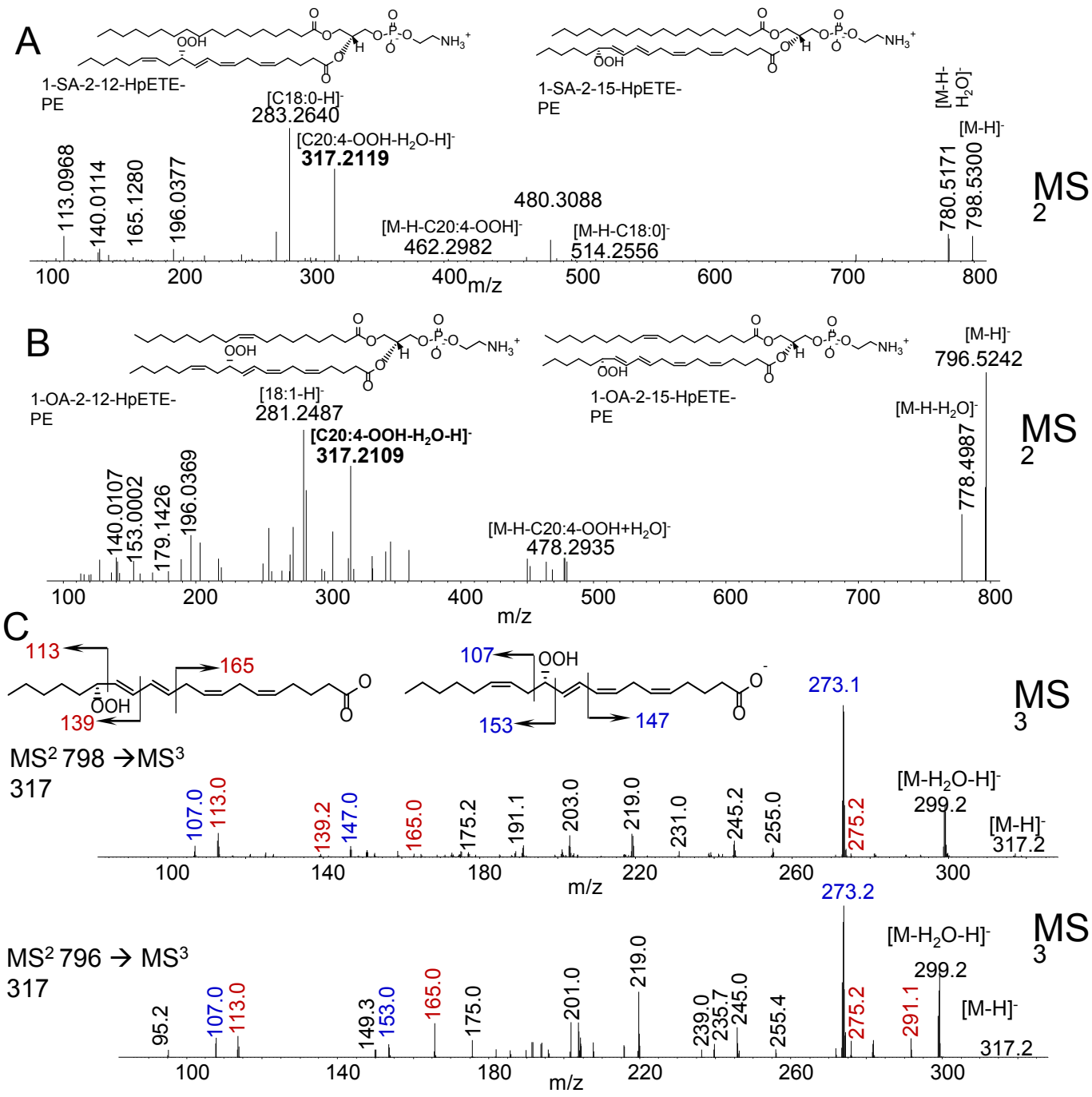
Supplementary Figure 1. *P. aeruginosa* supernatant induced cell death is rescued by ferrostatin-1. Related to Figure 1. **(A)** HBE cells were treated with *P. aeruginosa* WT *or ΔwspF* mutant supernatants (Sup.) collected in planktonic growth conditions in the presence or absence of a ferroptosis inhibitor, ferrostatin-1 (FER, 0.2 μM) for 20h at 37°C. Cell death was monitored after PI-staining by flow-cytometry. Data represent means±s.d., N=3. (**B**) Representative FACS plots showing the settings of gating used for cell death analysis using PI-staining. Control (Left panel) and *ΔwspF* supernatant (Right panel). (**C** & **D**) HBE cells were incubated with *ΔwspF* supernatants alone or with z-VAD-fmk (20 μM), Nec-1s (necrostatin-1s, 20 μM) or Baf-A1 (bafilomycin-A1, 1 nM). FER was used for comparative assessments of ferroptotic cell death ("positive control"). Cell death was assessed by MTT assay (**C**) or PI/Annexin V double-staining (**D**) (Right panel). Data represent mean±s.d., *p<0.05 vs. control (untreated); #p<0.05 vs. *ΔwspF* only N=3. (**D**) Representative PI/Annexin V double staining FACS plots for control and *ΔwspF* supernatant (Left panel).



Supplementary Figure 2. pLoxA is required for *P. aeruginosa* supernatant induced ferroptosis. Related to Figure 1.
(**A**) Expression of pLoxA in the *ΔwspF* mutant. Western blots of pLoxA in lysates of WT, *ΔwspF* and *loxA*::Tn strains from biofilm cultures (Upper panel), PP is purified pLoxA protein. Control is loading control PA-I lectin. (**B**) *P. aeruginosa* wild type (WT), *ΔwspF* mutant or *ΔloxA*::Tn (PW3111) mutant were grown in LB broth for 12-14 h at 37°C, diluted (OD_{600} = 0.05) in MEM and plated in 96-well vinyl microtiter plates (100 µl per well). Bacteria were grown for 24 h at 37°C without agitation, the supernatants were removed and the bofilms were washed with water, stained with crystal violet (41%), and quantified at OD_{550}. Data represent mean ± s.d., *p<0.05 vs. WT, N = 3. (**C** and **D**) Typical LC-MS profiles of oxidized (Upper panel) and non-oxidized (Lower panel) AA (**C**) and AA-PE (**D**). (**E**) HBE cells were treated with *loxA*::Tn supernatants alone or with FER (0.2 µM), or with z-VAD-fmk (20 µM) or Nec-1s (necrostatin-1s, 20 µM) or Baf-A1 (bafilomycin-A1, 1 nM). Data presented are means±s.d., *p<0.05 vs. *loxA*::Tn, N=3 (**F**) HBE cells were treated with *ΔwspF* supernatants or RSL3 in the absence or presence of Baicalein (5.0 and 10.0 µM) for 20 h at 37°C. Cell death was estimated by flow cytometry. Data represent means ± s.d., *p<0.05 vs untreated control; #p<0.05 vs. RSL3 or *ΔwspF* supernatant, N = 3. (**G**) Bacterial cell-lysates (pLoxA deficient or complemented, 100 µg each) were incubated with SAPE (100 µM) for 30 min and then added to RSL3 (20 nM) pre-treated HBE cells with or without FER (0.2 µM). Ferroptotic cell death = Cell death induced in the absence of ferrostatin-1 – cell death protected by ferrostatin-1. Data represent means ± s.d., *p<0.05 vs. RSL3 + *loxA*::Tn, N = 3. (**H**) Western blot shows expression of pLoxA in complemented strain (Upper panel). PP is purified pLoxA. Control is loading control PA-I lectin (Lower panel). (**I**) Western blot of pLoxA in lysates of MJK8 WT and MJKΔloxA using antibody against pLoxA. PP is recombinant pLoxA. Lower panel: Loading control PA-I-lectin.

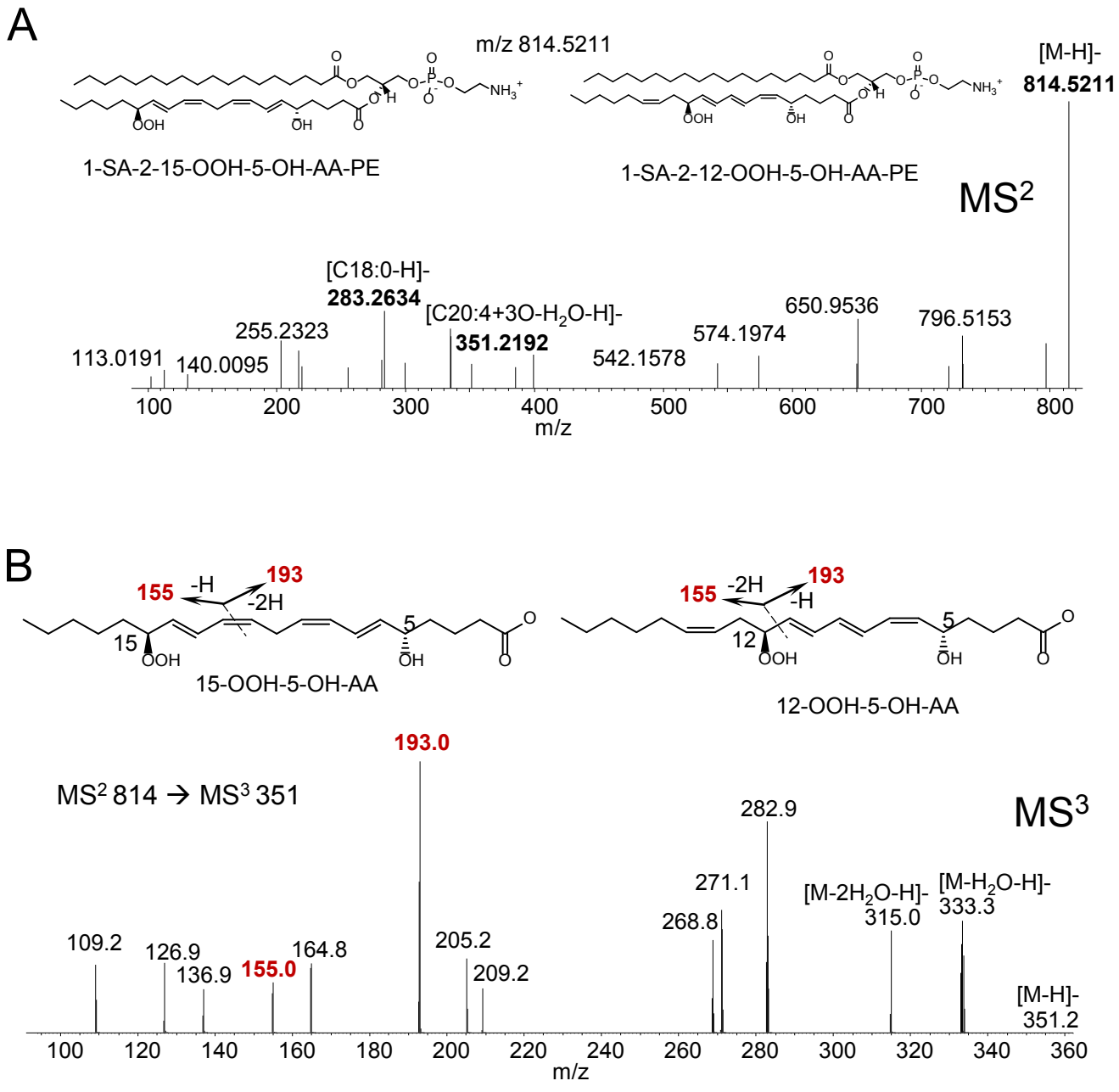


Supplementary Figure 3. Cell death induced by *ΔwspF* supernatants. Related to Figure 2. HBE cells were treated with *ΔwspF* supernatants in the presence of Sytox Green and monitored for 48h by live-cell imaging. Sytox Green is a membrane impermeant nuclear dye that will only be seen in cells with permeable plasma membranes (which is indicative of the early stages of cell death processes). Two areas in the image are selected (yellow and magenta circles) in each case over a 48h imaging cycle (imaging every 30 minutes) the evident cell death expands in a clonal way to include multiple cells within the delineated areas. Bar=100 microns

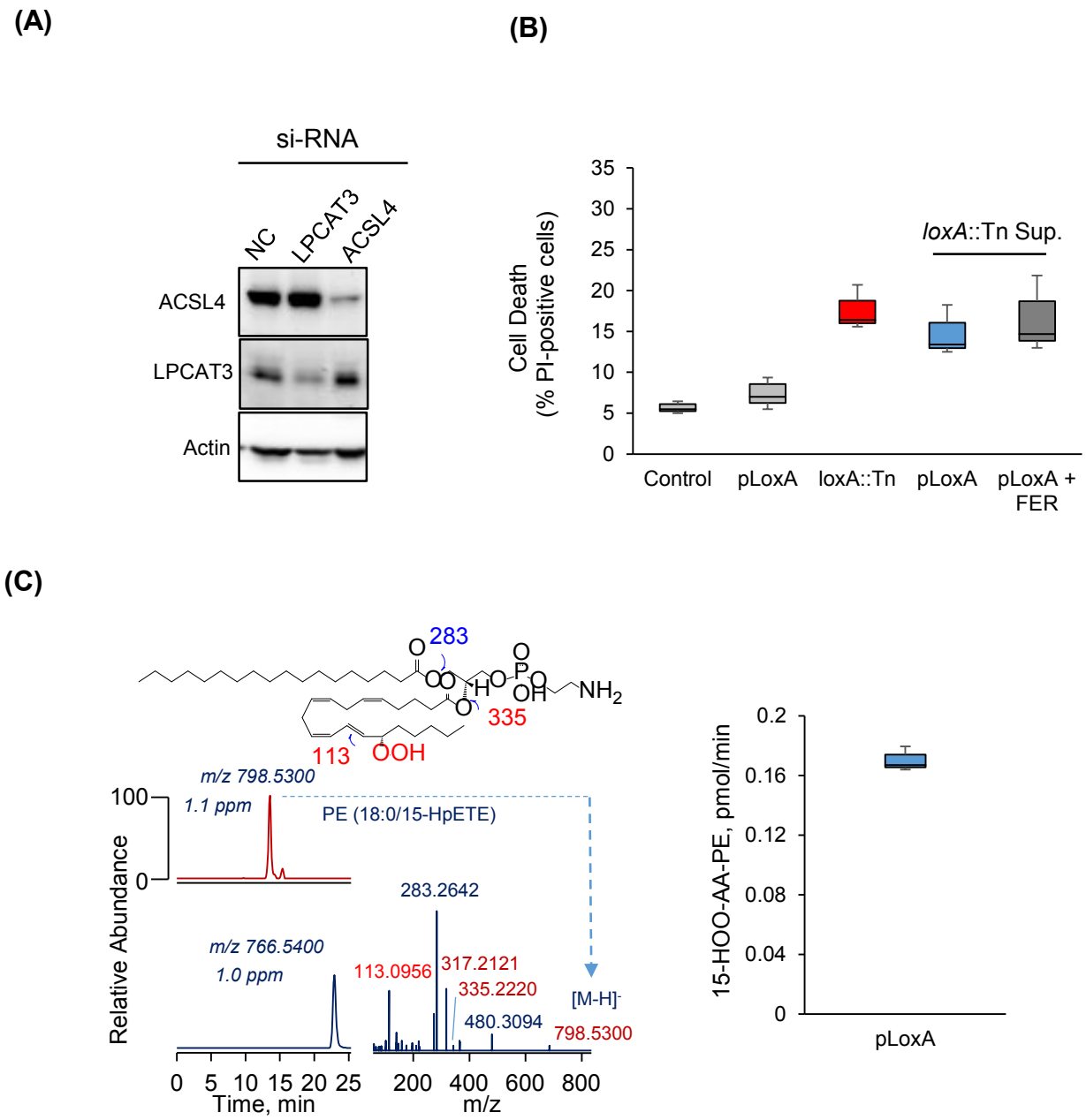


Supplemental Figure. 4. Identification of di-oxygenated PE species in HBE cells exposed to supernatants obtained from *ΔwspF* mutants. Related to Figure 2.

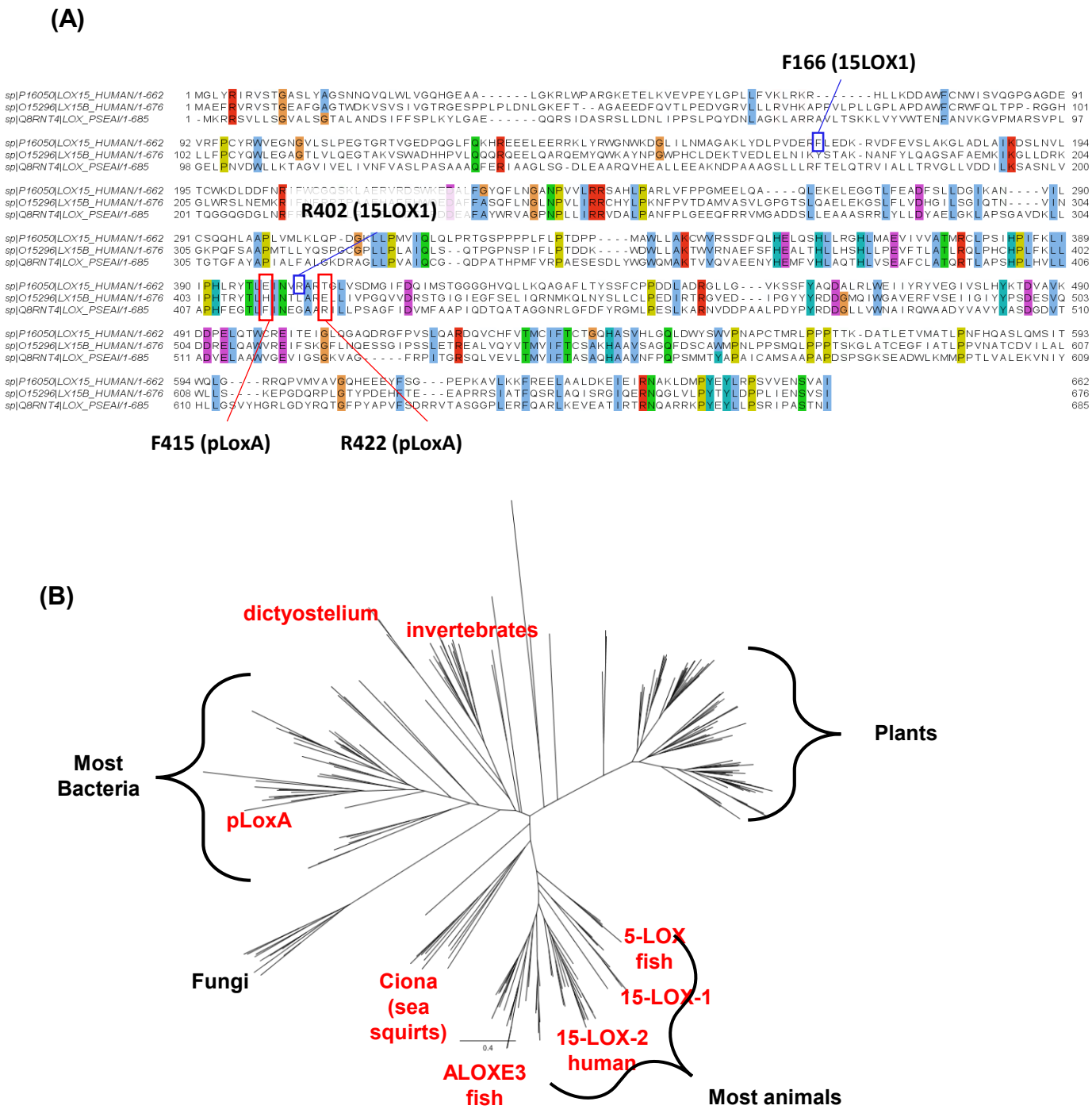
MS^2 profiles of di-oxygenated PE species (15-HOO-AA-PE and 12-HOO-AA-PE): (**A**) with m/z 798.5300 identified as 1-stearoyl-2-12-HpETE-*sn*-glycero-3-phosphoethanolamine (1-SA-2-12-HpETE-PE,) and 1-steraoyl-2-15-HpETE-*sn*-glycero-3-phosphoethanolamine (1-SA-2-15-HpETE-PE,) and (**B**) with m/z 796.5242 identified as 1-oleoyl-2-12-HpETE-*sn*-glycero-3-phosphoethanolamine (1-OA-2-12-HpETE-PE) and 1-oleoyl-2-15-HpETE-*sn*-glycero-3-phosphoethanolamine (1-OA-2-12-HpETE-PE). Inserts: Structural formulae of identified species; (**C**) MS^3 profiles of molecular ion with m/z 317.23 (m/z 335-H_2O) formed during MS^2 fragmentation of di-oxygenated PE species with m/z 798.5300 (upper panel), m/z 796.5242 (Lower panel). Insert: Structural formulae of identified di-oxygenated AA metabolites: 12-HOO-arachidonic acid (12-HpETE) and 15-HOO-arachidonic acid (15-HpETE). Characteristic fragments used for identification of 12-HpETE and 15-HOO-AA are shown in blue and red, respectively. Identification was performed based on MS^2 and MS^3 fragmentation analysis using a Tribrid™ Ion-trap/Orbitrap™ Fusion-Lumos™ mass spectrometer, (Thermo-Fisher Scientific, NJ).



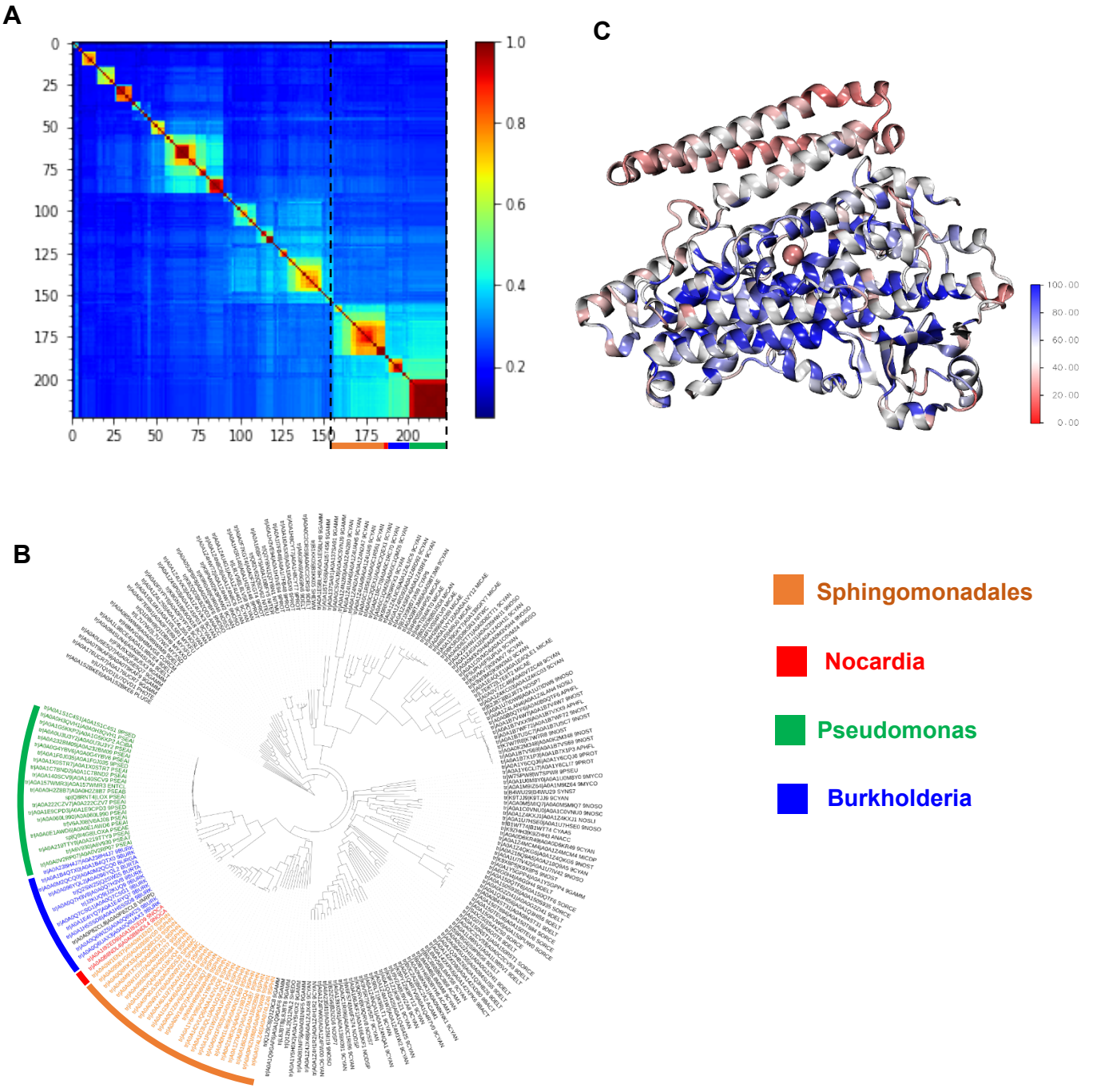
Supplemental Figure 5. Identification of tri-oxygenated PE species in HBE cells exposed to supernatants obtained from *ΔwspF* mutants. Related to Figure 2 (**A**) MS^2 profiles of tri-oxygenated PE species (1-stearoyl-2-12-hydroperoxy-5-hydroxy-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (1-SA-2-15-OOH-5-OH-AA-PE) and 1-stearoyl-2-15-hydroperoxy-5-hydroxy-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (1-SA-2-12-OOH-5-OH-AA-PE) with m/z 814.5211. Insert: (**A**) Structural formulae of identified PE species. (**B**) MS^3 profiles of molecular ion with m/z 351.23 formed during MS^2 fragmentation of di-oxygenated PE species with m/z 814.5211. Insert: Structural formulae of 12-hydroperxy-5-hydroxy-arachidonic acid (12-OOH-5-OH-AA) and 15-hydroperxy-5-hydroxy-arachidonic acid (15-OOH-5-OH-AA). Identification was performed based on both MS^2 and MS^3 fragmentation analysis using a Tribrid™ Ion-trap/Orbitrap™ Fusion-Lumos™ mass spectro-meter.



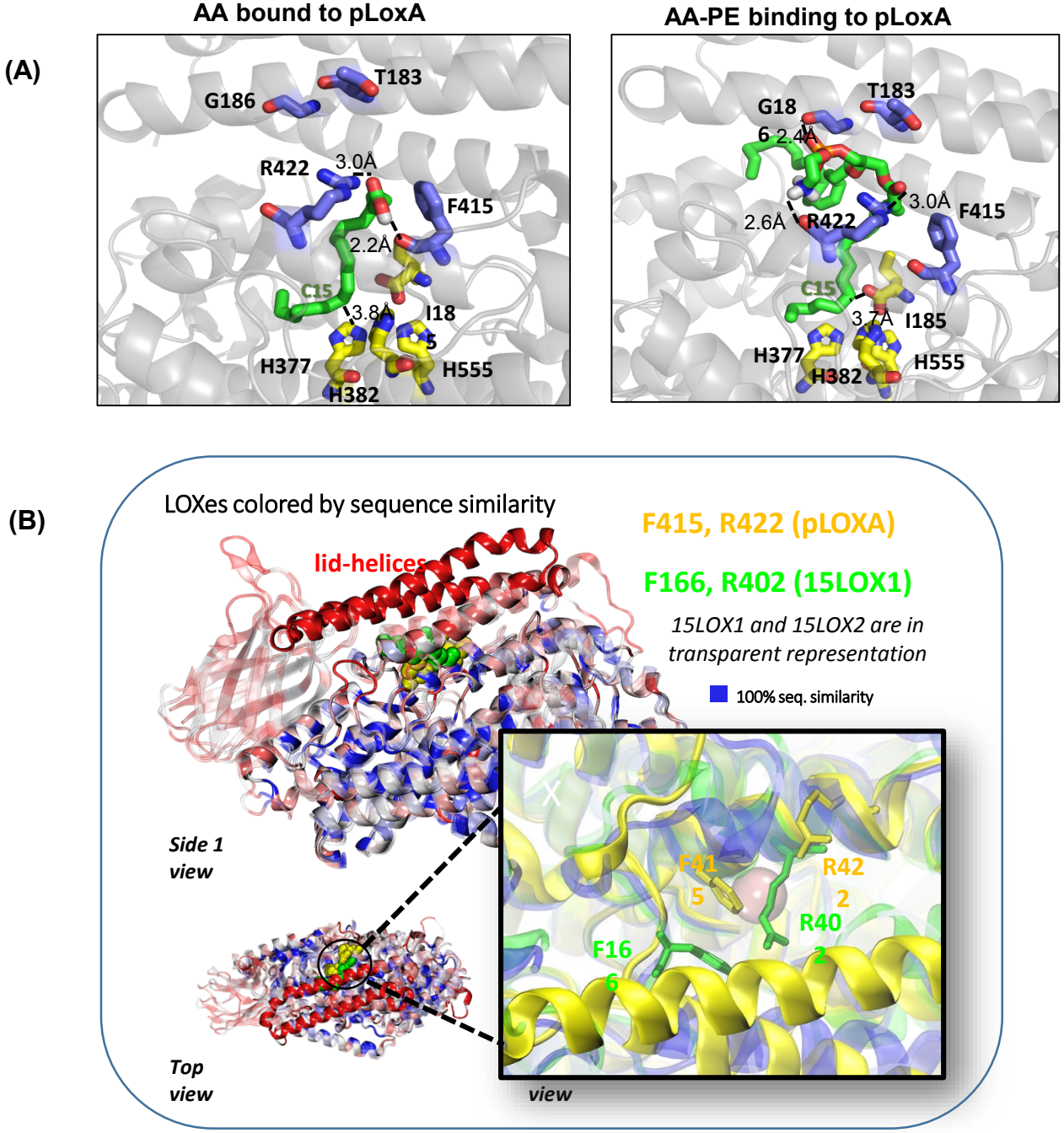
Supplementary figure 6. *P. aeruginosa* induced ferroptosis is affected by manipulations of (phospho)lipids in HBE cells. Related to Figure 3. (**A**) HBE cells were transfected with siRNA negative control (NC) or siRNA against *ACSL4* or *LPCAT3* for 12h. Transfected cells were trypsinzed and plated in 6-cm culture dish. After 50h, samples were collected and processed for Western blotting using anti-ACSL4 and anti-LPCAT3 antibodies. (**B**) HBE cells (untreated controls) or incubated with recombinant purified pLoxA (10.0 µg) only or pLoxA plus *loxA*::Tn supernatant for 20h at 37°C. Cell death was estimated using flow cytometry. Data represent means±s.d., N=3. (**C**) Accumulation of 15-HOO-AA-PE species in DOPC/AA-PE liposomes catalyzed by pLoxA. (Left panel) Typical LC-MS profiles of oxidized (Upper panel) and non-oxidized (Lower panel) AA-PE and MS^2 of oxidized AA-PE are shown. Possible formula of oxidized AA-PE is inserted. (Right panel) Quantitative assessment of 15-HOO-AA-PE molecular species. Data represent means±s.d., N=3.



Supplementary Figure 7. Evolutionary analysis of LOX sequences. Related to Figure 4. (**A**) Alignment of three sequences (two mammalian and the *P. aeruginosa*) obtained using Clustalx. Conserved residues are highlighted; those involved in AA binding are indicated. (**B**) Phylogenetic tree (undirected graph) obtained from the sequence-comparison map presented in Figure **4A.**

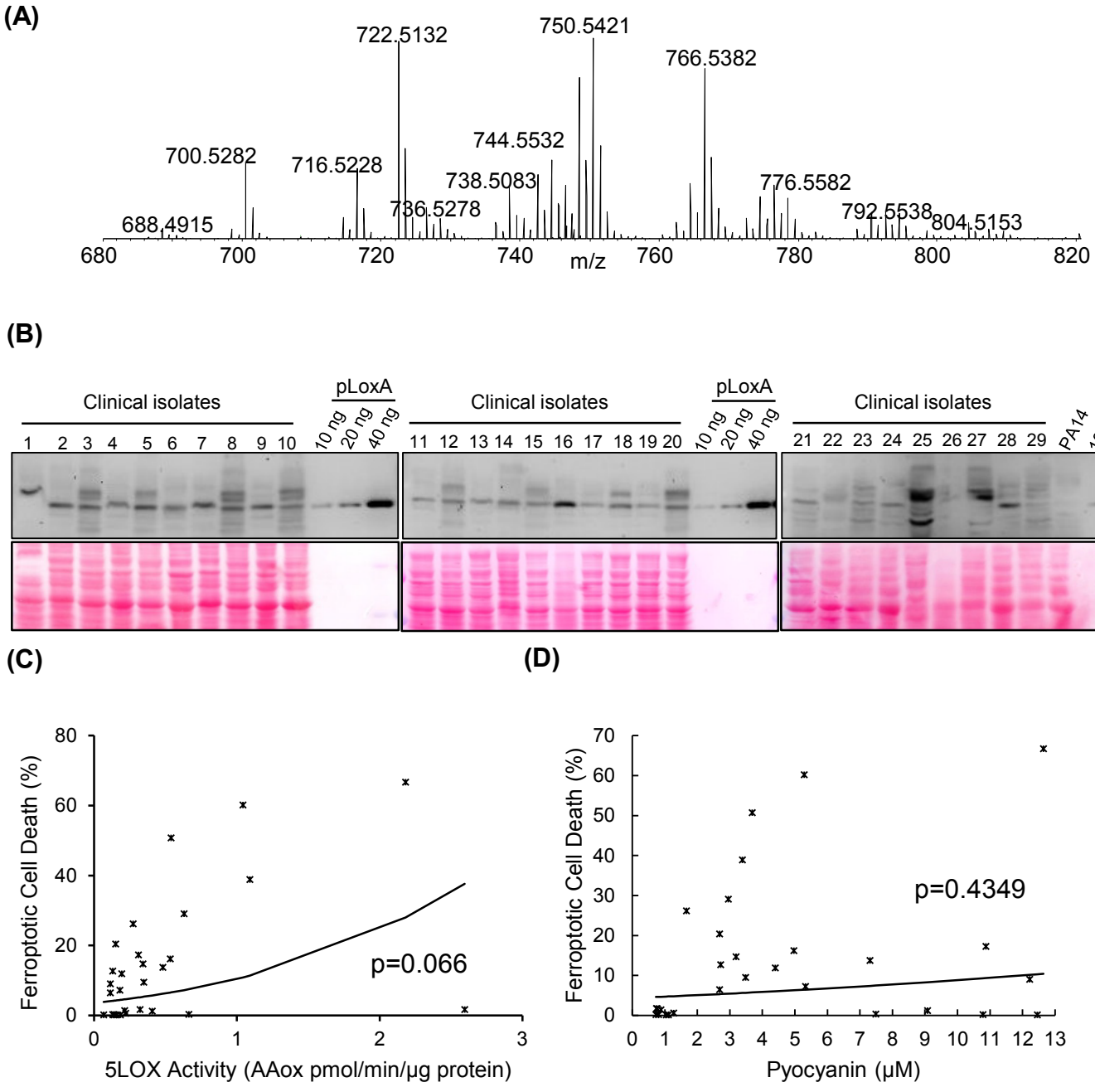


Supplementary Figure 8. Evolutionary analysis of lipoxygenases (comparable to pLoxA) among bacteria. Related to Figure 4. (**A**) The panel displays the pairwise sequence identity matrix derived from the MSA of 223 bacterial lipoxygenases. High sequence identity is shown in brown/red/orange, intermediate similarity in yellow/green, and low identity in blue. (**B**) Phylogenetic tree based on panel A. High sequence identity groups are colored: *Pseudomonas* (green), *Burkholderia* (blue), *Nocardia* (red) and *Sphingomonadales* (orange). (**C**) pLoxA crystal structure color-coded by percent sequence similarity (using BLOSUM100) among bacterial sequences. Regions showing relatively conserved sequences are in blue, those with intermediate sequence similarity are white, and low sequence similarity, red.

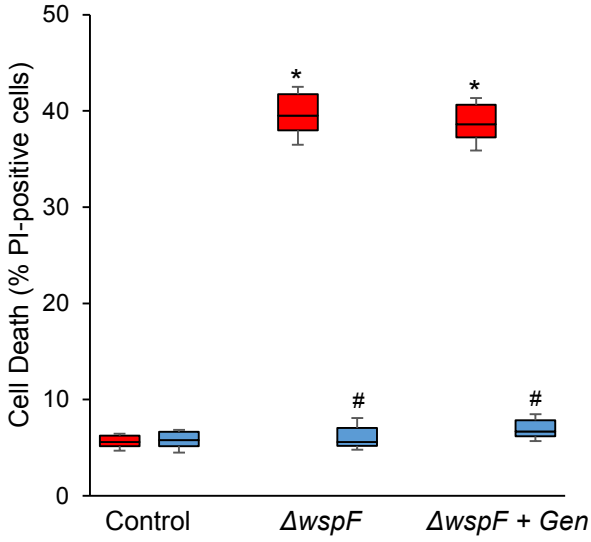


Supplementary Figure 9. Computational modeling of pLoxA structure and molecular dynamics. Related to Figure 4. (**A**) Close up view of the binding pose and coordinating residues for the two ligands, Binding of AA (left) panel and AA-PE (right) panel illustrate the difference between the two poses. In the AA-PE binding more ligand-protein interactions are seen, especially between the PE head-group and the protein, which are not present in the AA binding. The average energies indicate AA-PE binding is more efficient than AA to PLoxA. The closest distances between some ligand and protein atoms are indicated and labeled. (**B**) Location of ligand-binding site in the structure, and comparison of a pair of Arg-Phe, represented by both bacterial and mammalian LOXes.

Supplementary Figure 10



Supplementary Figure 10. *P. aeruginosa* clinical isolates from patients with persistent lower respiratory infection induce ferroptosis. Related to Figure 5. (**A**) Typical mass spectrum of PE from a patient with CF. (**B**) Representative Western blot of clinical isolates. Samples were prepared from clinical isolates grown under biofilm conditions and probed with anti-pLoxA antibody (Upper panel) or stained with Ponseau S (Lower panel). (**C**) Dependence of the ferroptotic death of HBE cells on the 5LOX activity of clinical isolates (n=29) and (**D**) the contents of pyocyanin in the supernatants of clinical isolates (n=29).



Supplementary Figure 11. Cell death induced by *ΔwspF* supernatant is not affected by presence of bacteria in the supernatant. HBE cells were treated with *ΔwspF* supernatant alone or with Gentamycin (20 µg/ml) in the presence or absence of ferrostatin-1(FER). Cells were incubated for 20h and then used for cell death analysis. Data represents mean±s.d., *p<0.05 vs. Control; #p<0.05 vs. *ΔwspF* or *ΔwspF*+Gen. N=3.

Supplementary Movie. Mobility of pLoxA lid helices (anm_movie_top). The movie shows the motion observed along ANM mode 1 viewed from top. The green structure represents the ANM model, developed along mode 1 from pdb 5IR5 (blue). The movie shows how along ANM mode 1, the pLoxA is able to move from lid-closed (blue, 5IR5) to lid-open (orange, 5IR4), clearly showing mobility of the lid helices.