Supplementary Information

TABLE S1 Primers used in this study.

Primer Name	Description	Primer Sequence
KA1061	5'-pUC19 *Lm*CFSAN*lit2* for	CGGTACCCGGGGATCCCTCCTTAGGCTATTC
KA1062	pUC19 *Lm*CFSAN*lit2* rev-3'	CCATGATTACGCCAAGCTTCATTATATTAAAAGG
KA1063	5'-pUC19 *Ef*TX1342*lit2* for	CGGTACCCGGGGATCCCGCTAGTGGGAACATC
KA1064	pUC19 *Ef*TX1342*lit2* rev-3'	CCATGATTACGCCAAGCTTCTCTCAAAGTCATTCC
KA1065	5'-*Lm*CFSAN*lit2* upstream	GACGGCCAGTGAATTCATGTCCGTGCCCTTTATG
KA1066	*Lm*CFSAN*lit2* upstream-3'	TAATAGAAATAGCATGC
KA1067	5'-*Lm*CFSAN*lit2* downstream	GCATGCTATTTCTATTAACTTTGACAACAATTCATCAT
KA1068	*Lm*CFSAN*lit2* downstream-3'	TGATTACGCCAAGCTGGACATTCTACTTGAGA
KA1155	5'-P_{xyl}	CAAGGAGGTGAATGTACAATGAAAAAAAGCATGC
KA1156	P_{xyl}-3'	CGGCCGGTACCGGATCCCTCCTTAGGCTATTC
TM1172	5'-pKFC ori QC for	GTGTATTAGCACCGTTATTATATCATG
TM1173	pKFC ori QC rev-3'	CTTTTTTCATCCTACCTTCTGTATCAG
TM1174	5'-pKFC plas for	GTAGGATGAAAAAAGAGCATTATCATATTC
TM1175	pKFC plas rev-3'	ACGGTGCTAATACACTTAACAAAATTTAG
KA1511	5'-*Lm*CFSAN*lit2* qPCR	GTCTCAGATCGCGGTATAAA
KA1512	*Lm*CFSAN*lit2* qPCR-3'	GCTACTCGTAGAAAGAAGCA
KA1515	5'-*Lm*CFSAN*copA* qPCR	TCTTTGCCGAACAACTACC
KA1516	*Lm*CFSAN*copA* qPCR-3'	TGCCATCACCAACGAATG
TM1554	*Ef*TX1342*lit2* T7-3'	GTTTATAATACGACTCACTATAGGGAGACAATCGTTGTTAGTG
TM1555	5'-*Ef*TX1342*lit2* T7	GTCTTTGCTTTGTCGCTTGC
TM1556	*Ef*TX1342*copB2* T7-3'	GTTTATAATACGACTCACTATAGGGAGAAGCATGTGCGCACCAG
TM1557	5'-*Ef*TX1342*copB2* T7	CCAATGTCAATTTGGCTACAG

TXM1560	*Lm*CFSAN*lit2* T7-3'	GTTTATAATACGACTCACTATAGGGAGAGTCAAAGTAGAAACAC
TXM1561	5'-*Lm*CFSAN*lit2* T7	CAAAACTGGAGTAGATCTCAAG
TM1564	*Ef*TX1342*lit1* T7-3'	GTTTATAATACGACTCACTATAGGGAGAAAATAAAGGACACTAGG
TM1565	5'-*Ef*TX1342*lit1* T7	GCGTTTAAGGGAAACACTTGGAC
KA1566	*Lm*CFSAN*lgt2* T7-3'	GTTTATAATACGACTCACTATAGGGAGACAATTTCTCCATAGG
KA1567	5'-*Lm*CFSAN*lgt2* T7	GCTAATAGAAGAGCAAAGCGTG
TM1568	*Ef*TX1342*copA* T7-3'	GTTTATAATACGACTCACTATAGGGAGAGTCGCATGTGTCATTGG
TM1569	5'-*Ef*TX1342*copA* T7	CTGTGAACCAATTATCTGGTGTTC
KA1576	5'-*Ef*TX1342gyrA qPCR	TTTCCAACAGGCGGTTTAG
KA1577	*Ef*TX1342gyrA qPCR-3'	TCCATTCGGCATTTCAGTC
TM1594	5'-*Ef*TX1342*lit2* qPCR	GTCGCTTGCAATTTCAGTTAC
TM1595	*Ef*TX1342*lit2* qPCR-3'	GGATTGTTCAAGTAGTTCATC
TM1596	5'-*Ef*TX1342*lit1* qPCR	GGCTTTAAGTATCACGATCAC
TM1597	*Ef*TX1342*lit1* qPCR-3'	AATCAAGGTCACACGATCAAC
TM1598	5'-*Ef*TX1342*copA* qPCR	ACGATTGAAAAAGCTGTGAAC
TM1599	*Ef*TX1342*copA* qPCR-3'	ATAACCTGCATCCGTAACTG
KA1604	*Lm*CFSAN*copY* T7-3'	GTTTATAATACGACTCACTATAGGGAGACATTGAATTCCTCCG
KA1605	5'-*Lm*CFSAN*copY* T7	CAGGTATCGAATTCAGAG
KA1628	5'-*Lm*CFSAN*lgt1* T7	GCGCTAATTGGTGCAG
KA1629	*Lm*CFSAN*lgt1* T7-3'	GTTTATAATACGACTCACTATAGGGAGACTTCCAAATGAATACC
TM1786	5'-*Lm*CFSAN*lit2* P_{Gram+}	TAGAACTAGTGGATCAACGACGGCCAGTGAATTGACAAA AATTGGTATATATGATATAATATAATCAAGGAGTGATCTA
TM1787	*Lm*CFSAN*lit2* P_{Gram+}-3'	AGGGAACAAAAGCTGACCATCCCTCCTTAGG
TM1800	5'-*Lm*CFSAN*lit2* Ppen	AACAGCGCGTGTATTAGGAGTGATCTAATAT
TM1801	*Lm*CFSAN*lit2* Ppen-3'	ACGGCCAGTGAATTAAGCTAATTCCGGTGG
TM1841	5'-*Lm*CFSAN*copY* qPCR	CAGGTATCGAATTCAGAGTTAGATG
TM1842	*Lm*CFSAN*copY* qPCR-3'	CCAACTATTACGCTCTTGCATTC

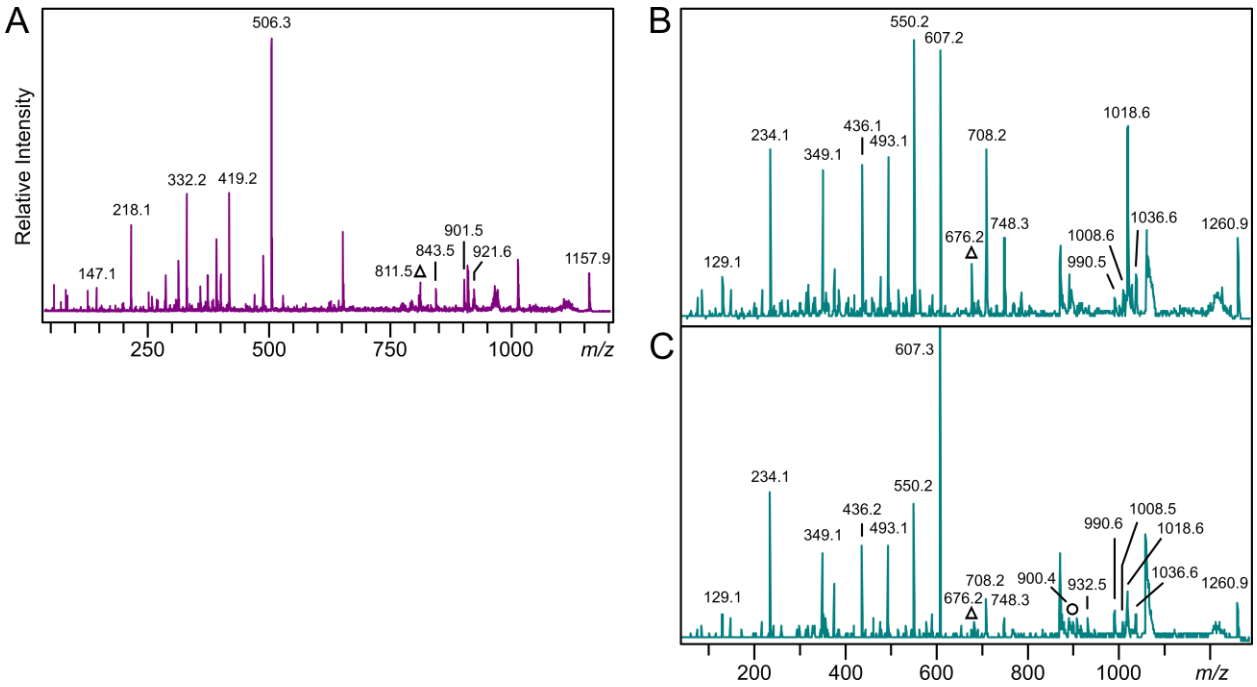

FIG S1 MALDI-TOF MS/MS spectrum of the protonated parent ions *m/z* 1157.9 and 1260.9. (A) MS/MS spectrum of the protonated parent ion *m/z* 1157.9 of Lpp purified from strain KA811 expressing *E. faecalis* TX1342 *lit2*. MS/MS spectra of the protonated parent ion *m/z* 1260.9 of the *L. monocytogenes* lipoprotein KO07_11695 from strain KA847 (ATCC 19115) (B), and KA849 (ATCC 19115 + pP_{xyl}*Lmlit2*) plus 2% xylose (C). The diagnostic dehydroalanyl ions for the diacylglycerol-modified (triangle) and the lyso (circle) lipopeptide are indicated.

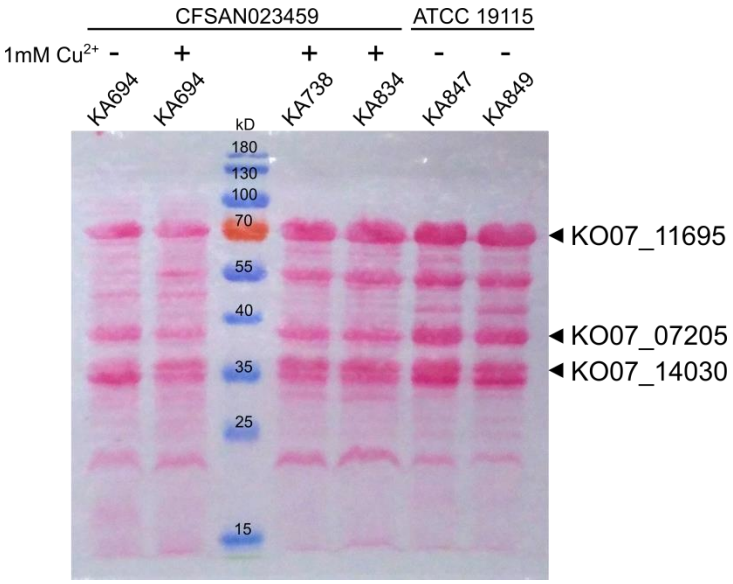

FIG S2 Lipoprotein profiles from *Listeria monocytogenes* CFSAN023459 and ATCC 19115. Using the Triton X-114 phase partitioning method, lipoproteins were enriched from the following *L. monocytogenes* strains grown in HTM+ with the indicated amount of $CuCl_2$: KA694 (CFSAN023459) without and with 1 mM $CuCl_2$, KA738 (CFSAN023459 Δ*lit2*) with 1 mM $CuCl_2$, KA834 (CFSAN023459 Δ*lit2* + p*lit2*) with 1 mM $CuCl_2$ plus 2% xylose, KA847 (ATCC 19115) in HTM+only, and KA849 (ATCC 19115 + p*xyllit2*) in HTM+ plus 2% xylose. The Triton X-114 phases were separated by SDS-PAGE, transferred to nitrocellulose membrane, and visualized by Ponceau S staining. The lipoproteins chosen for analysis by MALDI-TOF are indicated.

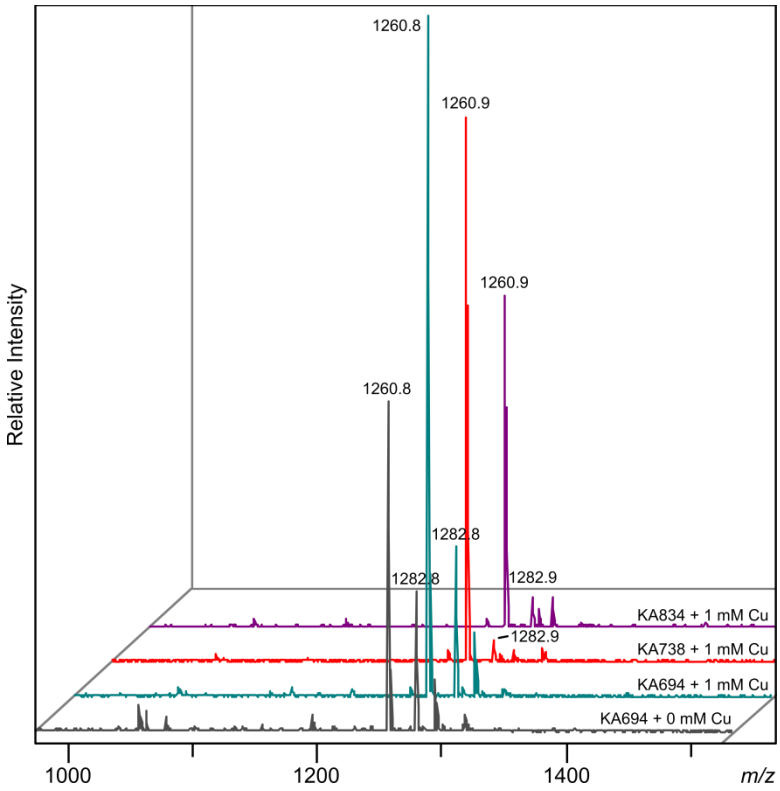

FIG S3 MALDI-TOF MS of KO07_11695, a predicted peptide ABC transporter substrate-binding lipoprotein. The stacked parent MS spectra of the *m/z* 1260 ion region corresponding to the *N*-terminal lipopeptide of KO07_11695 from *L. monocytogenes* CFSAN023459 are shown. The natural abundance of the sodium adduct *m/z* 1282 is also indicated. The lipoprotein was purified from KA694 (CFSAN023459) grown without and with 1 mM $CuCl_2$, KA738 (CFSAN023459 Δ*lit2*) grown with 1 mM $CuCl_2$, and KA834 (CFSAN023459 Δ*lit2* + *pxyllit2*) grown with 1 mM $CuCl_2$ plus 2% xylose.

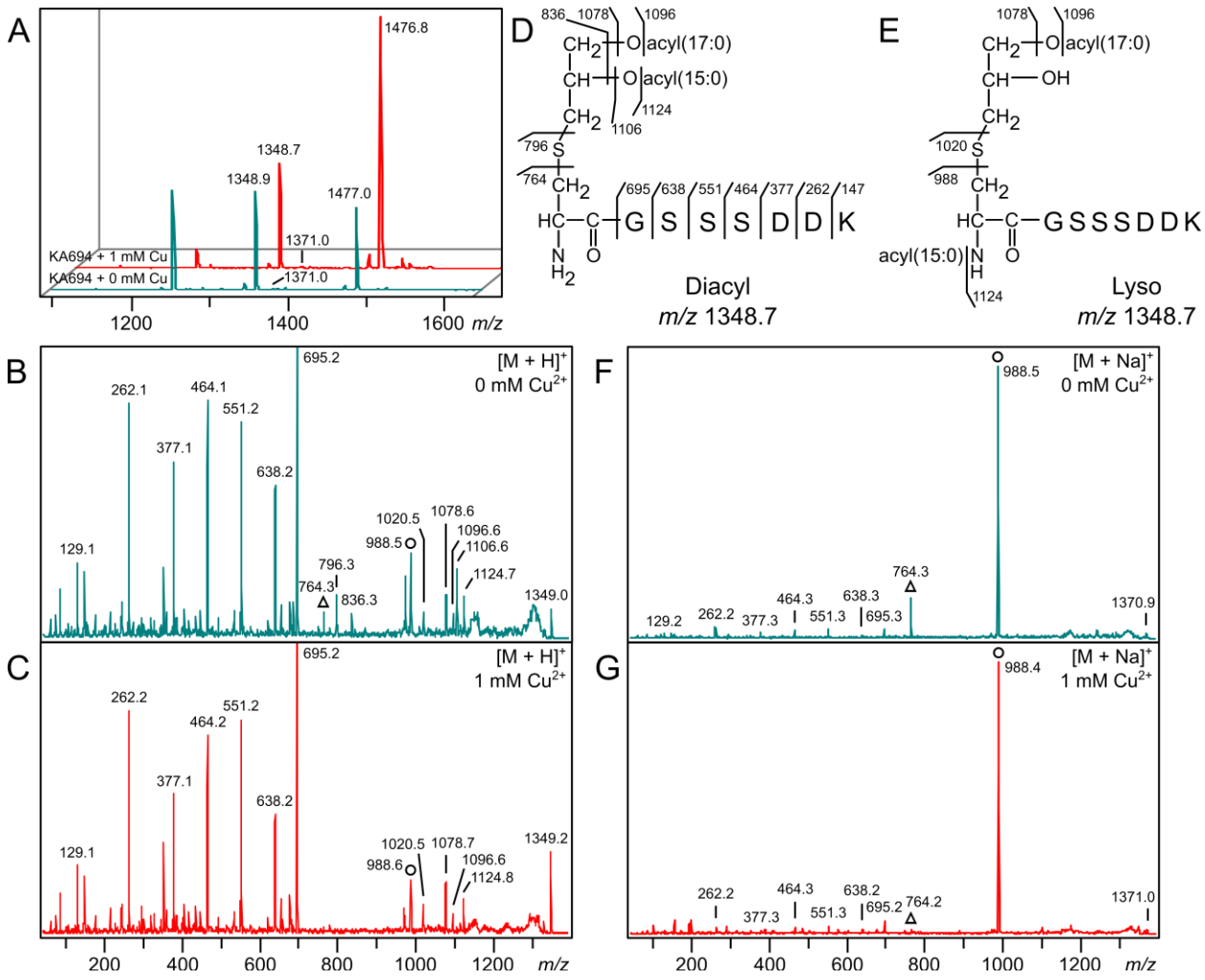

FIG S4 MALDI-TOF MS and MS/MS of KO07_07205, a predicted PnrA-like lipoprotein. (A) The stacked parent MS spectra of the *m/z* 1348 ion region corresponding to the *N*-terminal lipopeptide of KO07_07205 from *L. monocytogenes* CFSAN023459 (KA694) grown with (red traces) and without (turquoise traces) 1 mM $CuCl_2$ are shown. The natural abundance of the sodium adduct *m/z* 1370 is also indicated. (B, F) The MS/MS spectra of the protonated *m/z* 1349.0 (B) and the sodiated *m/z* 1370.9 (F) parent ions reveal both diacylglycerol-modified and lyso-form lipopeptides when KA694 is grown without $CuCl_2$. (C, G) The MS/MS spectra of the protonated *m/z* 1349.2 (C) and the sodiated *m/z* 1371.0 (G) parent ions reveal near-conversion to the lyso form when exogenous $CuCl_2$ is added to the growth media. (D, E) The elucidated structures of the diacylglycerol-modified (D) and the lyso-form (E) N-terminal lipopeptides are shown. The diagnostic dehydroalanyl ions for the diacylglycerol-modified (triangle) and the lyso (circle) lipopeptide are indicated.

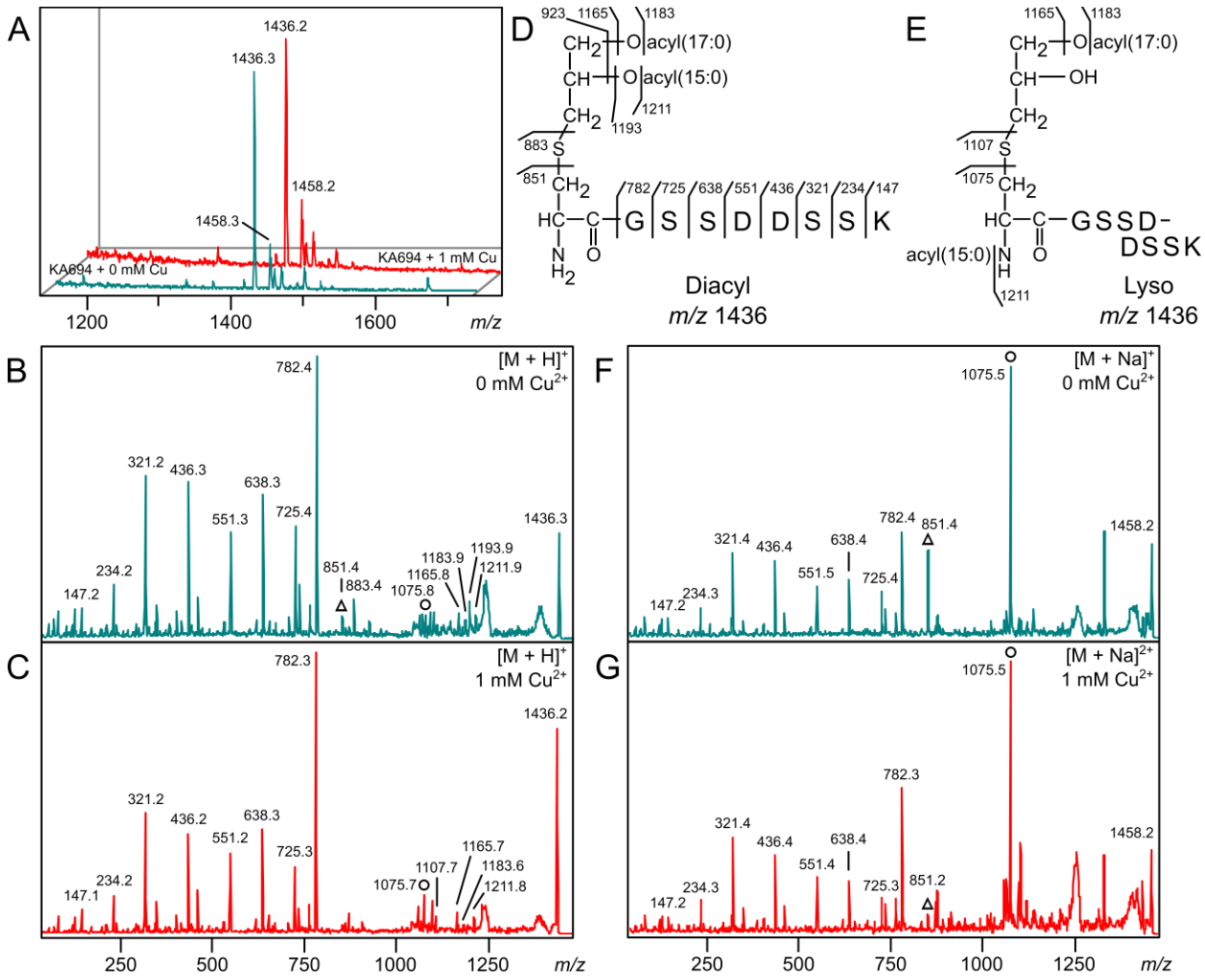

FIG S5 MALDI-TOF MS and MS/MS of KO07_14030, a predicted FMN-binding lipoprotein. (A) The stacked parent MS spectra of the *m/z* 1436 ion region corresponding to the *N*-terminal lipopeptide of KO07_14030 from *L. monocytogenes* CFSAN023459 (KA694) grown with and without 1 mM $CuCl_2$ are shown. The natural abundance of the sodium adduct *m/z* 1458 is also indicated. (B, F) The MS/MS spectra of the protonated m/z 1436.3 (B) and the sodiated m/z 1458.2 (F) parent ions reveal both diacylglycerol-modified and lyso-form lipopeptides when KA694 is grown without copper. (C, G) The MS/MS spectra of the protonated m/z 1436.2 (C) and the sodiated m/z 1458.2 (G) parent ions reveal near-conversion to the lyso form when exogenous $CuCl_2$ is added to the growth media. (D, E) The elucidated structures of the diacylglycerol-modified (D) and the lyso-form (E) N-terminal lipopeptides are shown. The diagnostic dehydroalanyl ions for the diacylglycerol-modified (triangle) and the lyso (circle) lipopeptide are indicated.

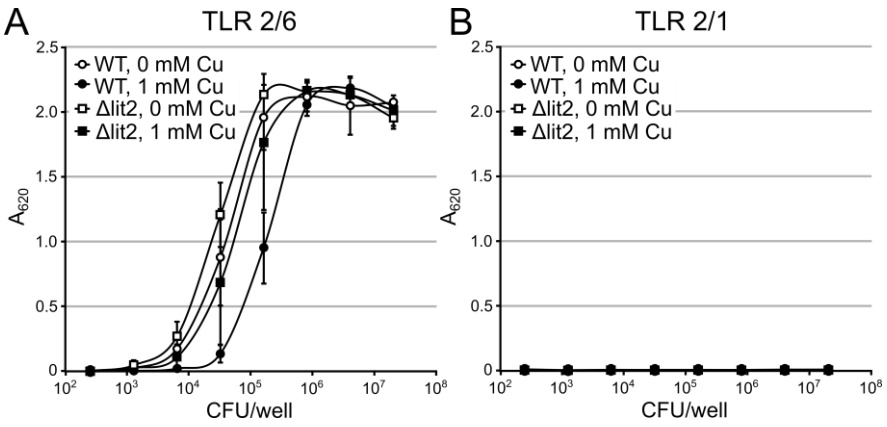

Fig S6 TLR2 response to whole bacteria. (A) HEK-Blue-TLR2/6 cells were exposed to 5-fold dilutions of heat-inactivated, whole bacteria cells of *L. monocytogenes* CFSAN023459 (WT) grown with or without 1 mM $CuCl_2$, as well as the derivative Δ*lit2* cells grown with or without 1 mM $CuCl_2$. (B) HEK-Blue-TLR2/1 cells were exposed to the same bacterial cell preparations as in panel A. The data are shown as the mean ± the standard deviation of three biological replicates.

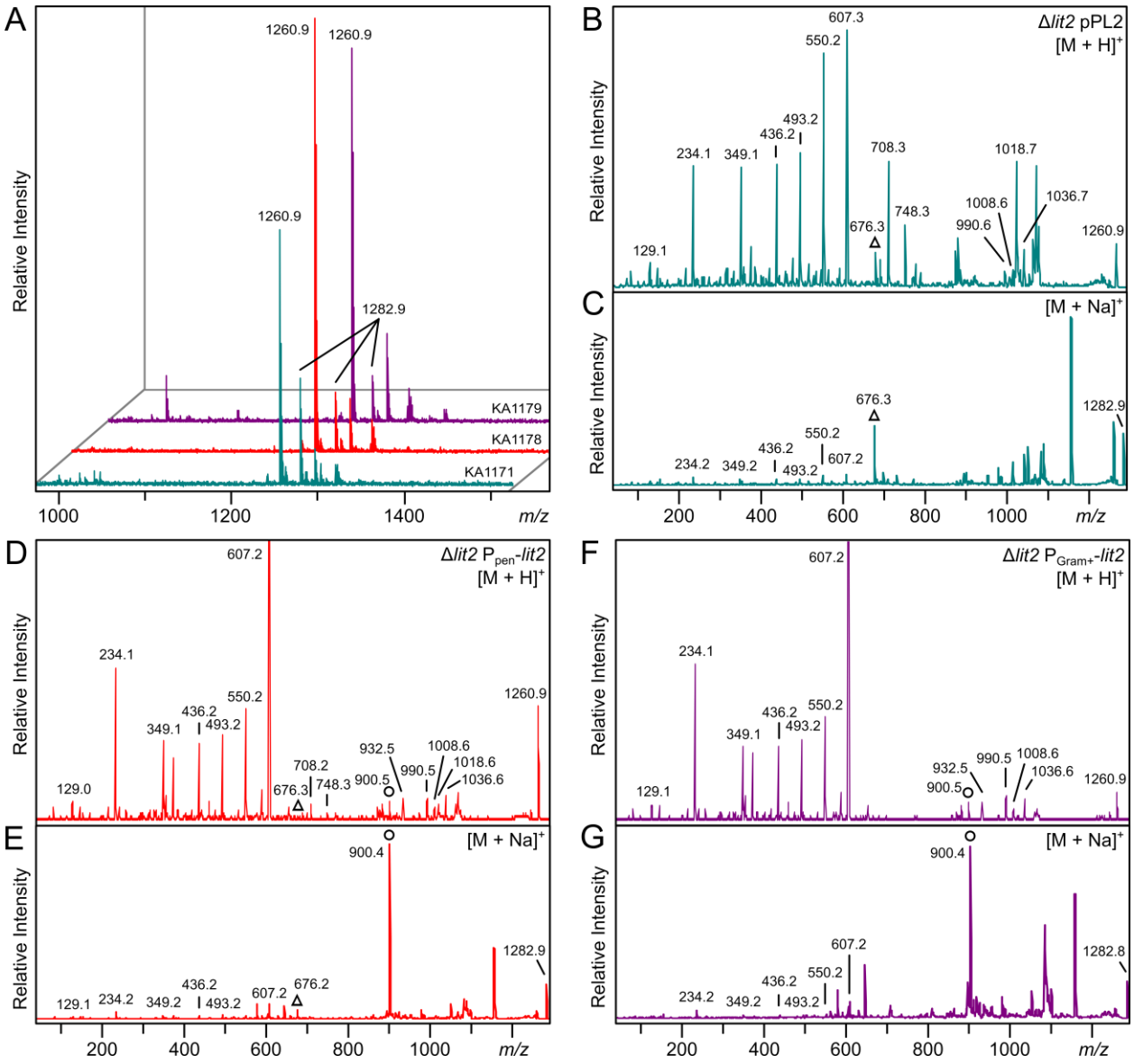

FIG S7 MALDI-TOF MS and MS/MS of KO07_11695 from strains KA1171, KA1178, and KA1179. (A) The stacked parent MS spectra of the *m/z* 1260 ion region corresponding to the *N*-terminal lipopeptide of KO07_11695 from *L. monocytogenes* CFSAN023459 are shown. The natural abundance of the sodium adduct *m/z* 1282 is also indicated. (B, C) The MS/MS spectra of the protonated *m/z* 1260 (B) and sodiated *m/z* 1282 (C) parent ions of strain KA1171 (Δ*lit2 attB*::pPL2). (D, E) The MS/MS spectra of the protonated *m/z* 1260 (D) and sodiated *m/z* 1282 (E) parent ions of strain KA1178 (Δ*lit2 attB*::pPL2-P_{pen}-*lit2*). (F, G) The MS/MS spectra of the protonated *m/z* 1260 (F) and sodiated *m/z* 1282 (G) parent ions of strain KA1179 (Δ*lit2 attB*::pPL2-P_{Gram+}-*lit2*). The elucidated structures of the diacylglycerol-modified and the lyso-form *N*-terminal peptides are shown in Fig. 2 (E and G, respectively). The diagnostic dehydroalanyl ions for the diacylglycerol-modified (triangle) and the lyso (circle) lipopeptide are indicated.

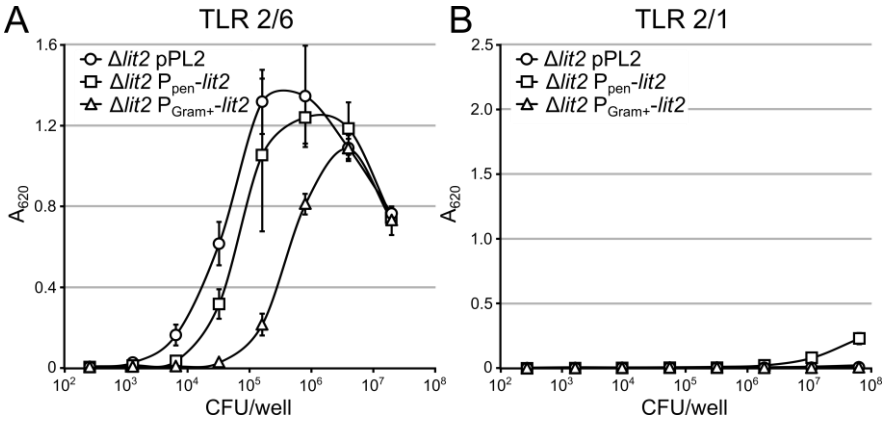

Fig S8 TLR2 response to whole bacteria. (A) HEK-Blue-TLR2/1 cells were exposed to 5-fold dilutions of heat-inactivated, whole bacteria cells of the *L. monocytogenes* CFSAN023459 Δ*lit2* derivative strains with the pPL2 empty vector, or *lit2* under the control of the P_{pen} or P_{Gram+} promoters, integrated into the chromosome. (B) HEK-Blue-TLR2/6 cells were exposed to the same bacterial cell preparations as in panel A. The data are shown as the mean ± the standard deviation of three biological replicates.

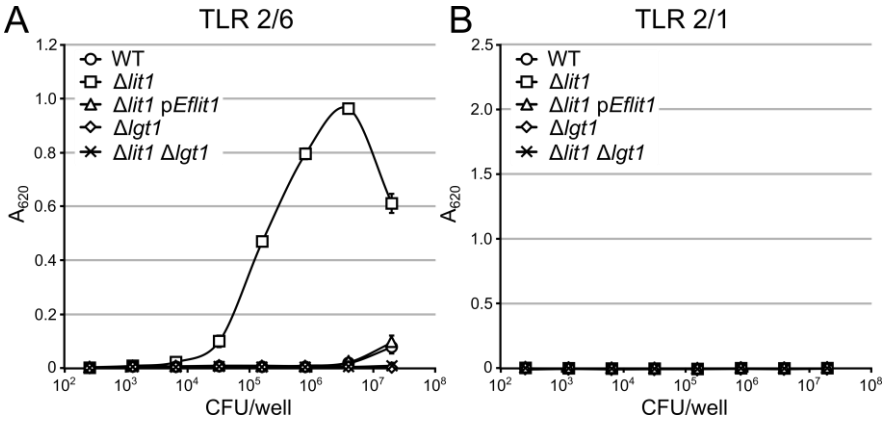

Fig S9 TLR2 response to whole bacteria. (A) HEK-Blue-TLR2/6 cells were exposed to 5-fold dilutions of heat-inactivated, whole bacteria cells of *Enterococcus faecalis* ATCC 19433 and the indicated derivatives. (B) HEK-Blue-TLR2/1 cells were exposed to the same bacterial cell preparations as in panel A. The data are shown as the mean ± the standard deviation of three biological replicates.