Supplementary Information

Insights into bacterial lipoprotein trafficking from a structure of LolA bound to the LolC periplasmic domain

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Supplementary information includes:

Figs. S1 to S9
Tables S1 to S5
Supplementary methods
Captions for movies 1 to 3

Other supplementary materials for this manuscript:

Movies 1 to 3

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Supplemental Figures

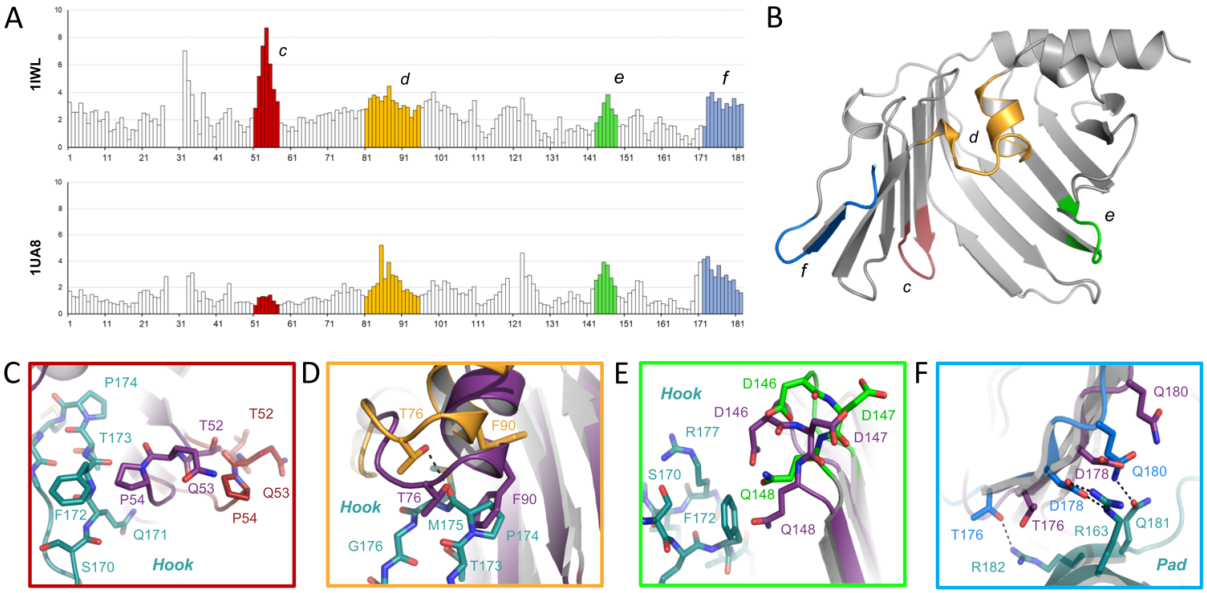


Figure S1. Comparison of LolA in isolation and in complex with LolC. (A) Rmsd plots for superpositions of LolA in complex with LolC (6F3Z) with structures of LolA in isolation (1IWL and 1UA8). Four regions with significant conformational differences are highlighted. (B) Structure of LolA colour-coded as per the rmsd plot. (C-F) Close-up views of LolA conformational differences in each region. Isolated LolA (1IWL) is shown in purple and the LolA-LolC complex is shown with LolC in teal and LolA coloured as in (B).

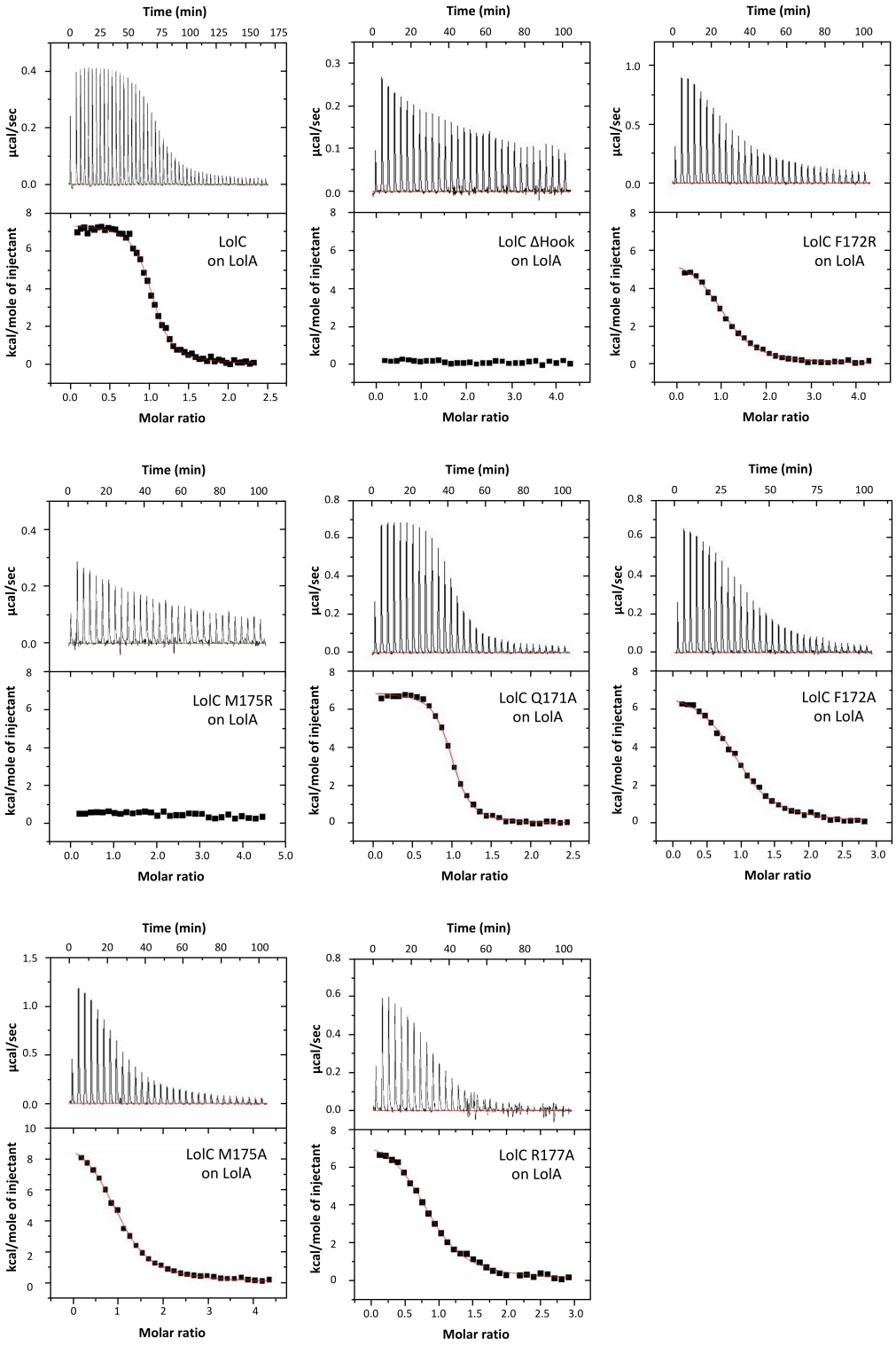


Figure S2. ITC titrations for LolA using wild-type or variant LolC periplasmic domain constructs. For each titration, a representative thermogram is shown in the upper part of the panel and fitted plot of background-subtracted heats of injection is shown immediately beneath. Values of affinities and thermodynamic parameters for all repeats are given in **Table S2**.

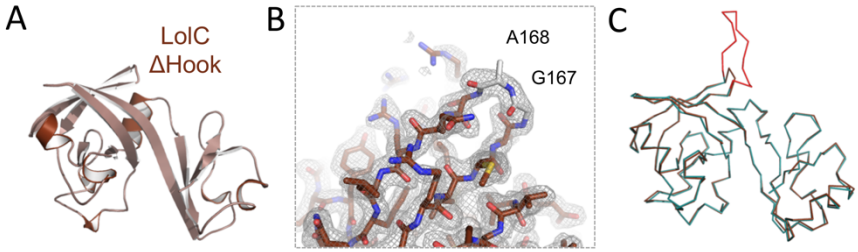
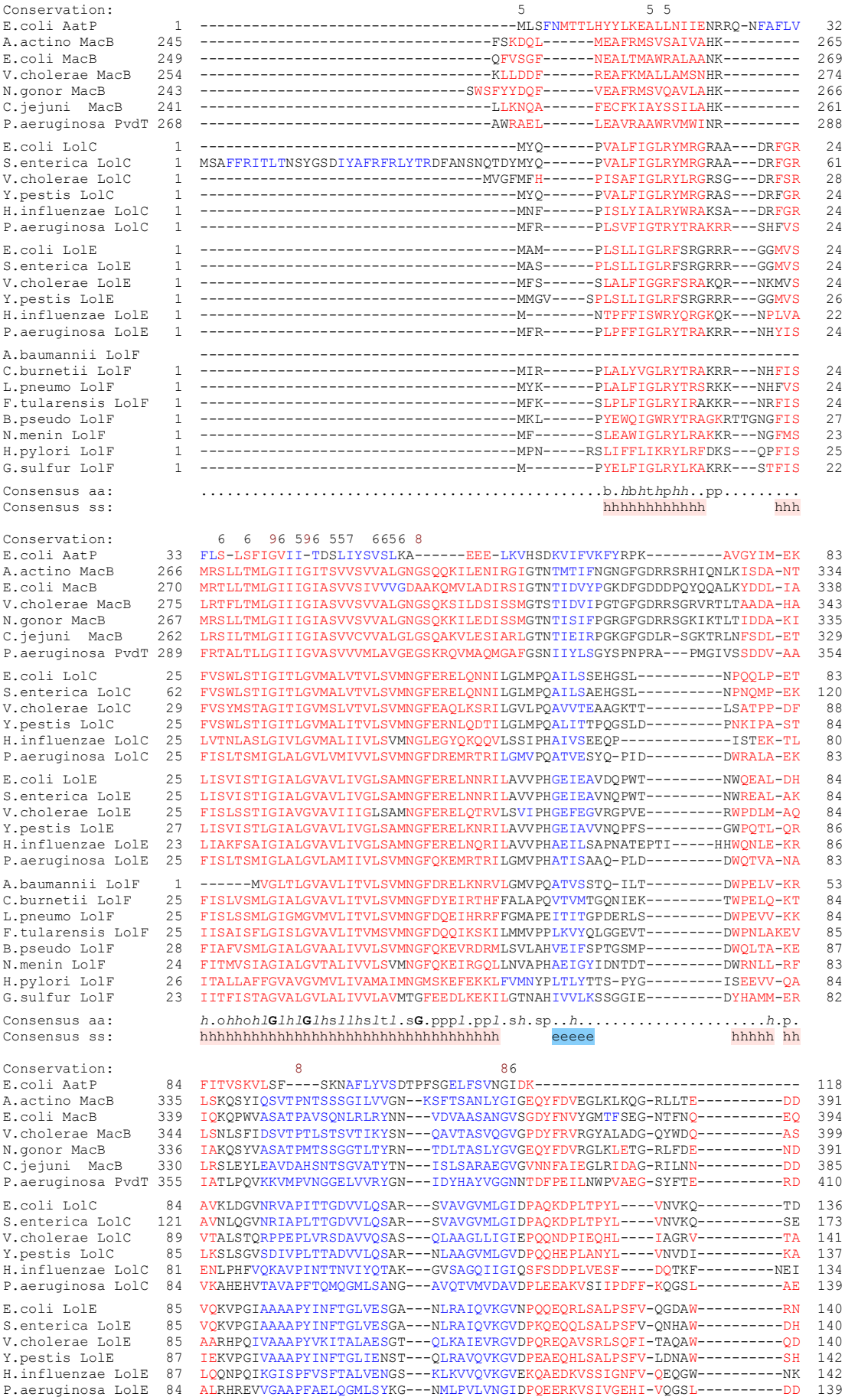


Figure S3. Removing the Hook from LolC does not disrupt its structure. (A) Crystal structure of the LolC ΔHook periplasmic domain construct. (B) Close-up view of the LolC ΔHook structure showing electron density for the linker residues (light gray) replacing the truncated Hook and surrounding β-strands. The mesh represents a weighted $2|F_o|-|F_c|$ electron density map contoured at 1 sigma. (C) Alignment of LolC ΔHook (brown) and wild-type periplasmic domains (teal). Hook shown in red.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C.jejuni MacB 241
P.aeruginosa PvdT 268
E.coli LolC 1
S.enterica LolC 1
V.cholerae LolC 1
Y.pestis LolC 1
H.influenzae LolC 1
P.aeruginosa LolC 1
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S.enterica LolE 1
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Y.pestis LolE 1
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P.aeruginosa LolE 1
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C.burnetii LolF 1
L.pneumo LolF 1
F.tularensis LolF 1
B.pseudo LolF 1
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H.pylori LolF 1
G.sulfur LolF 1
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C.burnetii LolF 25
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H.pylori LolF 26
G.sulfur LolF 23
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Consensus ss:
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N.gonor MacB 336
C.jejuni MacB 330
P.aeruginosa PvdT 355
E.coli LolC 84
S.enterica LolC 121
V.cholerae LolC 89
Y.pestis LolC 85
H.influenzae LolC 81
P.aeruginosa LolC 84
E.coli LolE 85
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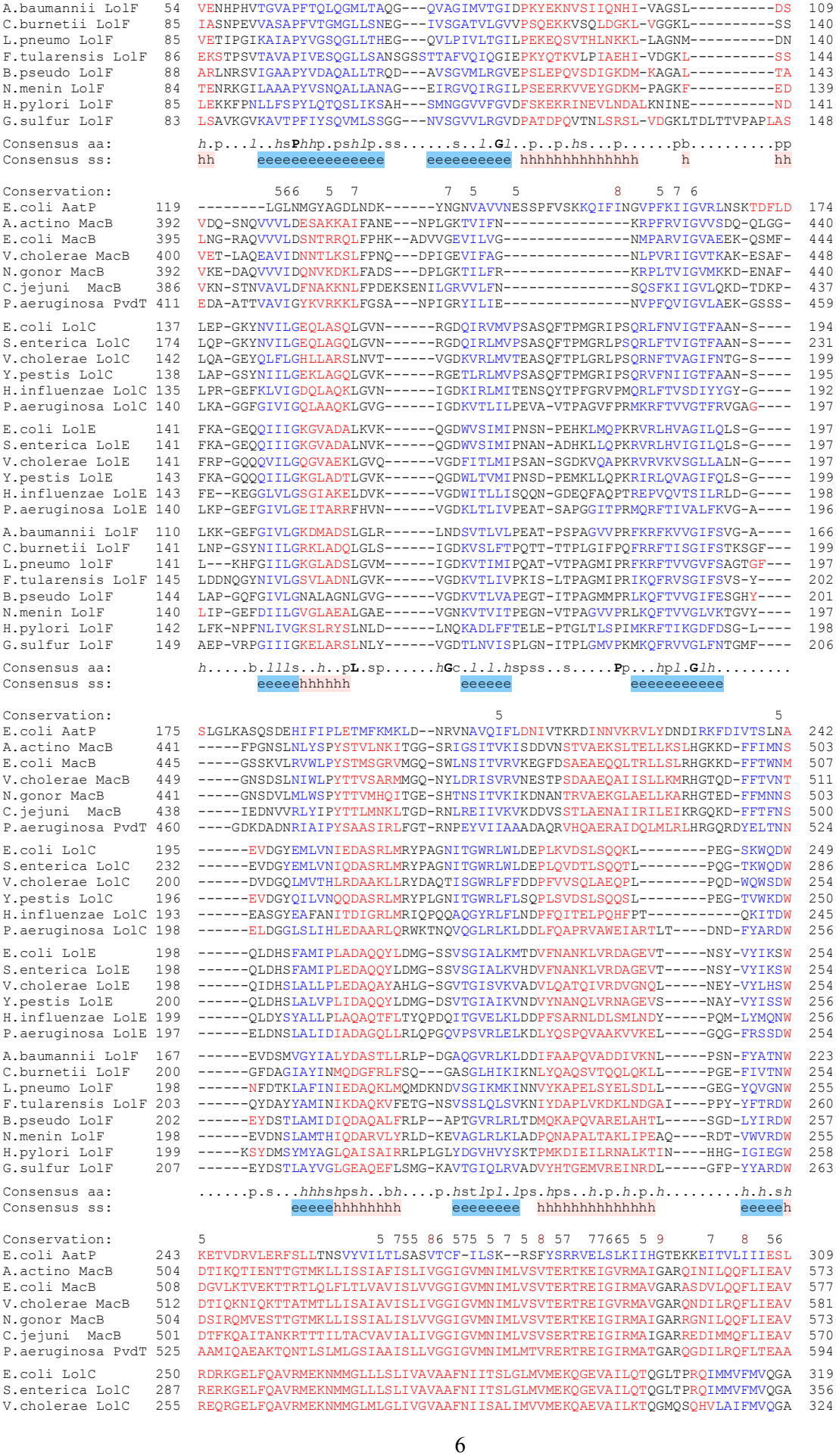
H.influenzae LolE 87
P.aeruginosa LolE 84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L.pneumo LolF 85 VETIPGIKAIAPYVGSQGLLTHEG---QVLPIVLTGILPEKEQSVTHLNKKL-LAGNM---------DN 140
F.tularensis LolF 86 EKSTPSVTAVAPIVESQGLLSANSGSSTTAFVQIQGIEPKYQTKVLPIAEHI-VDGKL---------SS 144
B.pseudo LolF 88 ARLNRSVIGAAPYVDAQALLTRQD---AVSGVMLRGVEPSLEPQVSDIGKDM-KAGAL---------TA 143
N.menin LolF 84 TENRKGILAAAPYVSNQALLANAG---EIRGVQIRGILPSEERKVVEYGDKM-PAGKF---------ED 139
H.pylori LolF 85 LEKKFPNLLFSPYLQTQSLIKSAH---SMNGGVVFGVDFSKEKRINEVLNDALKNINE---------ND 141
G.sulfur LolF 83 LSAVKGVKAVTPFIYSQVMLSSGG---NVSGVVLRGVDPATDPQVTNLSRSL-VDGKLTDLTTVPAPLAS 148
Consensus aa:
Consensus ss:
Conservation:
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A.actino MacB 392 VDQ-SNQVVVLDESAKKAIFANE---NPLGKTVIFN---------------KRPFRVIGVVSDQ-QLGG- 440
E.coli MacB 395 LNG-RAQVVVLDSNTRRQLFPHK--ADVVGEVILVG---------------NMPARVIGVAEEK-QSMF- 444
V.cholerae MacB 400 VET-LAQEAVIDNNTLKSLFPNQ---DPIGEVIFAG---------------NLPVRIIGVTKAK-ESAF- 448
N.gonor MacB 392 VKE-DAQVVVIDQNVKDKLFADS---DPLGKTILFR---------------KRPLTVIGVMKKD-ENAF- 440
C.jejuni MacB 386 VKN-STNVAVLDFNAKKNLFPDEKSENILGRVVLFN---------------SQSFKIIGVLQKD-TDKP- 437
P.aeruginosa PvdT 411 EDA-ATTVAVIGYKVRKKLFGSA---NPIGRYILIE---------------NVPFQVIGVLAEK-GSSS- 459
E.coli LolC 137 LEP-GKYNVILGEQLASQLGVN------RGDQIRVMVPSASQFTPMGRIPSQRLFNVIGTFAAN-S---- 194
S.enterica LolC 174 LQP-GKYNVILGEQLAGQLGVN------RGDQIRLMVPSASQFTPMGRLPSQRLFTVIGTFAAN-S---- 231
V.cholerae LolC 142 LQA-GEYQLFLGHLLARSLNVT------VGDKVRLMVTEASQFTPLGRLPSQRNFTVAGIFNTG-S---- 199
Y.pestis LolC 138 LAP-GSYNIILGEKLAGQLGVK------RGETLRLMVPSASQFTPMGRIPSQRVFNIIGTFAAN-S---- 195
H.influenzae LolC 135 LPR-GEFKLVIGDQLAQKLGVN------IGDKIRLMITENSQYTPFGRVPMQRLFTVSDIYYGY-G---- 192
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E.coli LolE 141 FKA-GEQQIIIGKGVADALKVK------QGDWVSIMIPNSN-PEHKLMQPKRVRLHVAGILQLS-G---- 197
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P.aeruginosa LolE 140 LKP-GEFGIVLGEITARRFHVN------VGDKLTLIVPEAT-SAPGGITPRMQRFTIVALFKVG-A---- 196
A.baumannii LolF 110 LKK-GEFGIVLGKDMADSLGLR------LNDSVTLVLPEAT-PSPAGVVPRFKRFKVVGIFSVG-A---- 166
C.burnetii LolF 141 LNP-GSYNIILGRKLADQLGLS------IGDKVSLFTPQTT-TTPLGIFPQFRRFTISGIFSTKSGF--- 199
L.pneumo lolF 141 L---KHFGIILGKGLADSLGVM------IGDKVTIMIPQAT-VTPAGMIPRFKRFTVVGVFSAGTGF--- 197
F.tularensis LolF 145 LDDNQGYNIVLGSVLADNLGVK------VGDKVTLIVPKIS-LTPAGMIPRIKQFRVSGIFSVS-Y---- 202
B.pseudo LolF 144 LAP-GQFGIVLGNALAGNLGVG------VGDKVTLVAPEGT-ITPAGMMPRLKQFTVVGIFESGHY---- 201
N.menin LolF 140 LIP-GEFDIILGVGLAEALGAE------VGNKVTVITPEGN-VTPAGVVPRLKQFTVVGLVKTGVY---- 197
H.pylori LolF 142 LFK-NPFNLIVGKSLRYSLNLD------LNQKADLFFTELE-PTGLTLSPIMKRFTIKGDFDSG-L---- 198
G.sulfur LolF 149 AEP-VRPGIIIGKELARSLNLY------VGDTLNVISPLGN-ITPLGMVPKMKQFRVVGLFNTGMF---- 206
Consensus aa:
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A.actino MacB 441 -----FPGNSLNLYSPYSTVLNKITGG-SRIGSITVKISDDVNSTVAEKSLTELLKSLHGKKD-FFIMNS 503
E.coli MacB 445 -----GSSKVLRVWLPYSTMSGRVMGQ-SWLNSITVRVKEGFDSAEAEQQLTRLLSLRHGKKD-FFTWNM 507
V.cholerae MacB 449 -----GNSDSLNIWLPYTTVSARMMGQ-NYLDRISVRVNESTPSDAAEQAIISLLKMRHGTQD-FFTVNT 511
N.gonor MacB 441 -----GNSDVLMLWSPYTTVMHQITGE-SHTNSITVKIKDNANTRVAEKGLAELLKARHGTED-FFMNNS 503
C.jejuni MacB 438 -----IEDNVVRLYIPYTTLMNKLTGD-RNLREIIVKVKDDVSSTLAENAIIRILEIKRGQKD-FFTFNS 500
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S.enterica LolC 232 ------EVDGYEMLVNIQDASRLMRYPAGNITGWRLWLDEPLQVDTLSQQTL--------PQG-TKWQDW 286
V.cholerae LolC 200 ------DVDGQLMVTHLRDAAKLLRYDAQTISGWRLFFDDPFVVSQLAEQPL--------PQD-WQWSDW 254
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E.coli LolE 198 ------QLDHSFAMIPLADAQQYLDMG-SSVSGIALKMTDVFNANKLVRDAGEVT-----NSY-VYIKSW 254
S.enterica LolE 198 ------QLDHSFAMIPLEDAQQYLDMG-SSVSGIALKVHDVFNANKLVRDAGEVT-----NSY-VYIKSW 254
V.cholerae LolE 198 ------QIDHSLALLPLEDAQAYAHLG-SGVTGISVKVADVLQATQIVRDVGNQL-----NEY-VYLHSW 254
Y.pestis LolE 200 ------QLDHSLALVPLIDAQQYLDMG-DSVTGIAIKVNDVYNANQLVRNAGEVS-----NAY-VYISSW 256
H.influenzae LolE 199 ------QLDYSYALLPLAQAQTFLTYQPDQITGVELKLDDPFSARNLDLSMLNDY-----PQM-LYMQNW 256
P.aeruginosa LolE 197 ------ELDNSLALIDIADAGQLLRLQPGQVPSVRLELKDLYQSPQVAAKVVKEL-----GQG-FRSSDW 254
A.baumannii LolF 167 ------EVDSMVGYIALYDASTLLRLP-DGAQGVRLKLDDIFAAPQVADDIVKNL-----PSN-FYATNW 223
C.burnetii LolF 200 ------GFDAGIAYINMQDGFRLFSQ---GASGLHIKIKNLYQAQSVTQQLQKLL-----PGE-FIVTNW 254
L.pneumo LolF 198 ------NFDTKLAFINIEDAQKLMQMDKNDVSGIKMKINNVYKAPELSYELSDLL-----GEG-YQVGNW 255
F.tularensis LolF 203 ------QYDAYYAMINIKDAQKVFETG-NSVSSLQLSVKNIYDAPLVKDKLNDGAI----PPY-YFTRDW 260
B.pseudo LolF 202 ------EYDSTLAMIDIQDAQALFRLP--APTGVRLRLTDMQKAPQVARELAHTL-----SGD-LYIRDW 257
N.menin LolF 198 ------EVDNSLAMTHIQDARVLYRLD-KEVAGLRLKLADPQNAPALTAKLIPEAQ----RDT-VWVRDW 255
H.pylori LolF 199 -----KSYDMSYMYAGLQAISAIRRLPLGLYDGVHVYSKTPMKDIEILRNALKTIN----HHG-IGIEGW 258
G.sulfur LolF 207 ------EYDSTLAYVGLGEAQEFLSMG-KAVTGIQLRVADVYHTGEMVREINRDL-----GFP-YYARDW 263
Consensus aa:
Consensus ss:
Conservation:
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V.cholerae MacB 512 DTIQKNIQKTTATMTLLISAIAVISLIVGGIGVMNIMLVSVTERTREIGVRMAVGARQNDILRQFLIEAV 581
N.gonor MacB 504 DSIRQMVESTTGTMKLLISSIALISLVVGGIGVMNIMLVSVTERTKEIGIRMAIGARRGNILQQFLIEAV 573
C.jejuni MacB 501 DTFKQAITANKRTTTILTACVAVIALIVGGIGVMNIMLVSVSERTREIGIRMAIGARREDIMMQFLIEAV 570
P.aeruginosa PvdT 525 AAMIQAEAKTQNTLSLMLGSIAAISLLVGGIGVMNIMLMTVRERTREIGIRMATGARQGDILRQFLTEAA 594
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S.enterica LolC 287 RERKGELFQAVRMEKNMMGLLLSLIVAVAAFNIITSLGLMVMEKQGEVAILQTQGLTPRQIMMVFMVQGA 356
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H.influenzae LolC 246  RVQKGEFFQAVRMEKNMMGLLISLIIVVAISNIVTSLSLMVVDKQGEIAILQTQGLTKSQVRSVFIYQGL  315
P.aeruginosa LolC 257  TRSHGNLYQAIRMEKTMIGLLLLLIVAVAAFNIISTLVMVVTDKKSDIAILRTLGATPGQIMATFMVQGT  326

E.coli LolE        255  IGTYGYMYRDIQMIRAIMYLAMVLVIGVACFNIVSTLVMAVKDKSGDIAVLRTLGAKDGLIRAIFVWYGL  324
S.enterica LolE    255  IGTYGYMYRDIQMIRAIMYLAMILVIGVACFNIVSTLVMAVKDKSGDIAVLRTLGAKDGLIRAIFVWYGL  324
V.cholerae LolE    255  QQKYGFLYRDIQLVRTIMYLVMVLVIGVASFNIVSTLMMAVKDRAGEIAILRTMGATDGLIKRIFVWQGV  324
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P.aeruginosa LolE  255  TRTQGSLFNAMKMEKTMIGLLLLLIIAVAAFNIIATLIMVVADKRTDIAILRTLGATPRQIMAIFMVQGT  324

A.baumannii LolF   224  TYTHGNLFNAIQMEKTLVGLLLVLIIVVAAFNIVSSLVMVVTDKKSDIAILRTLGASPSMITKIFMVQGT  293
C.burnetii LolF    255  TEQFGSFFKAIAMEKTIMFVILLLIVGVAIFNLVSTLVMVVNDKRADIAILRTLGASPRTIMSIFVIQGA  324
L.pneumo LolF      256  TQQFGAFFEAVKMEKTMMFMILLLIIAVAAFNLVSSLVMVVNDKQAEIAILRTIGATPSTILWVFIVQGM  325
F.tularensis LolF  261  TDENKSFFDALKMEKTMMFFILLLIITVAVFNLLSSLVMVVTDKRSDIAILRTMGMSSRQIITVFIYQGF  330
B.pseudo LolF      258  TQQNKTWFSAVQIEKRMMFIILTLIIAVAAFNLVSSLVMTVTNKQADIAILRTLGAQPGSIMKIFVVQGV  327
N.menin LolF       256  TFSNRSYFEAVELEKRMMFIILTLIIAVAAFNLVSSLVMAVTEKQADIAILRTLGLSPGGVMKIFMVQGA  325
H.pylori LolF      259  WQQNGNFFSAMELEKRALFIVLMLIILMASLNIISSLLMVVMNRRKEIALLFSMGSSQKEIQKTFFYLGN  328
G.sulfur LolF      264  MQMNKNILFALKTEKMVMFIILTLIVLVAAFGIASTLFMVVMEKTKDIAILKSMGATGRSIMKIFVLEGL  333

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Consensus ss:           hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh    hhhhhhhhhhhh

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E.coli MacB        578  LVCLVGGALGITLSLLIAFTLQLFLP-------------------GWEIGFSPLALLLAFLCSTVTGILF  628
V.cholerae MacB    582  LVCLCGGAIGIGVAYLIGGLFATLGS-------------------SFSMIYSTTSIISAFLCSTLIGVLF  632
N.gonor MacB       574  LICIIGGLVGVGLSAAVSLVFNHFVT-------------------DFPMDISAASVIGAVACSTGIGIAF  624
C.jejuni  MacB     571  MICTIGAILGVILSIFVIFAFNTLST-------------------DFPMILNAYSVLLGLLSSMFIGVVF  621
P.aeruginosa PvdT  595  MLSVVGGLAGIALALCIGGVLLLGQ--------------------VAVAFSLSAIVGAFSCALVTGLVF  643

E.coli LolC        320  SAGIIGAILGAALGALLASQLNNLMPIIGVLL----------DGAALPVAIEPLQVIVIALVAMAIALLS  379
S.enterica LolC    357  SAGIIGALLGAALGALLASQLNNLMPIIGAFL----------DGAALPVAIEPLQVIVIALVAMAIALLS  416
V.cholerae LolC    325  SSGVIGALVGGLLGVLLAANLNSLMEALGVALFS--------VGGSLPVAIDPLQIVLVIVLAIVLSLLA  386
Y.pestis LolC      321  TAGVIGALLGAGLGVLLASQLNTLIPILGVLI----------DGATLPVEIDPLQVTVIALLAMVIALLS  380
H.influenzae LolC  316  LVGFVGTLLGAILGVLATLNLTDIVSAVNP------------QGVFLPTELSFVQMIFVIGFSLLLSLLS  373
P.aeruginosa LolC  327  VIGVIGTLVGGVLGVVAALNVSAWISALEKLLGHQFLASDVYFIDYLPSQLMLDDVVLVCGAALVLSFFA  396

E.coli LolE        325  LAGLFGSLCGVIIGVVVSLQLTPIIEWIEKLIGHQFLSSDIYFIDFLPSELHWLDVFYVLVTALLLS-LA  393
S.enterica LolE    325  LAGLLGSLIGVAIGVVVSLQLTAIINGIEKAIGHQFLSGDIYFIDFLPSELHWLDVVYVLVTALLLSLLA  394
V.cholerae LolE    325  FSGVLGSVVGSVLGMVVAFNLTPLIKGLEHLIGHQFLSGDIYFVDFLPSQVEWADVVLVSGTAIVLSLLA  394
Y.pestis LolE      327  LAGLIGSISGAVIGVIVSLQLTTIIRGLEKMVGHQFLSSDIYFIDFLPSELRWFDVACVLATALVLSLIA  396
H.influenzae LolE  327  QAGMKGCLIGIVLGIILALNLTTFIQGIEWVIGKKLLSGDVYFVDFLPSELHWLDVLMVLVAALALSLMA  396
P.aeruginosa LolE  325  VIGVIGTVIGGVLGVFAALNITGMIDRIERLVGHKVFSSDVYFINYLPSDLQVLDVVLICSAALLMSFLA  394

A.baumannii LolF   294  VIGVIGTVAGTVLGVILALTISDIISWFNNVLGLNLF--DAYFVHYLPSYLRWQDVTIIVIVSLLLSFLA  361
C.burnetii LolF    325  IVGIVGTLIGVIGGVILAVNATAIVNGIQQIFHVQFLKSSIYFVNFLPSRLQWLDVLNVSLIAFALSLIA  394
L.pneumo LolF      326  MVGLVGTILGLLGGLVLANNATEIVNALQSFFQVKVLSSSIYFVDYLPSKIMFRDLWQVCAMALLMSFAA  395
F.tularensis LolF  331  IIGLIGTVIGVLLGILLSTYATEIVNFIQNLTGKQFLSASVYLINYIPSELMWSDVIKVTLVSMFLSFLA  400
B.pseudo LolF      328  TIGFVGTATGVALGCLIAWSIPWLIPMIEHAFGVQFLPPSVYFISELPSELVAGDVIKIGVIAFALSALA  397
N.menin LolF       326  FSGFFGTLAGVVCGVLLGWNVGRVVAFFENLLGVHLINSQVYFIDYLPSDVDMGDVALIACISLGLSFVA  395
H.pylori LolF      329  IIGLGGVA----LGVVLAF----LSMYLLSVFPIISLPADVYGINTLPLNLSLMDFTLTLIGSVIIVGLS  390
G.sulfur LolF      334  IIGISGTAIGVIGGLLVALNLEPIVGVIQRVTGFELFSKDVYYLDHFPSQVVPSDVLLISVTAVIISLVA  403

Consensus aa:           hhthhGshhGhhlthlltl..hs.h...l..............s.hs..lph.plh.hhh.thhlthlh
Consensus ss:           hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh                ee hhhhhhhhhhhhhhhh

Conservation:             5 597  76   75 68
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A.actino MacB      594  GYMPAKKAAELNPITALAQE--  644
E.coli MacB        629  GWLPARNAARLDPVDALARE--  648
V.cholerae. MacB   633  GYLPAKNAAQLNPIDALARE--  652
N.gonor MacB       625  GFMPANKAAKLNPIDALAQD--  644
C.jejuni  MacB     622  GFFPARNAANLNPISALSKE--  641
P.aeruginosa PvdT  644  GFMPARKAAQLDPVAALASQ--  663

E.coli LolC        380  TLYPSWRAAATQPAEALRYE--  399
S.enterica LolC    417  TLYPSWRAAATQPAEALRYE--  436
V.cholerae LolC    387  TLFPAYRASSVQPAEALRYE--  406
Y.pestis LolC      381  TLYPSWRAAAAQPAEALRYE--  400
H.influenzae LolC  374  TLYPAYRAAKVEPAAALRYE--  393
P.aeruginosa LolC  397  TLYPAWRAARTQPAEALRYE--  416

E.coli LolE        394  SWYPARRASNIDPARVLSGQ--  413
S.enterica LolE    395  SWYPARRASNIDPARVLSGQ--  414
V.cholerae LolE    395  TWYPARRASRLNPAQVLSSK--  414
Y.pestis LolE      397  SWYPARRASRIDPARVLSGQ--  416
H.influenzae LolE  397  SLYPASRAAKLQPAQVLSSH--  416
P.aeruginosa LolE  395  TLYPSWRAARTQPAESLRYE--  414

A.baumannii LolF   362  TIYPALRAAKVQPAEALRYE--  381
C.burnetii LolF    395  TIYPAFIAFRTEPAEALRYE--  414
L.pneumo LolF      396  TIYPAWRASKTVIAEALHYE--  415
F.tularensis LolF  401  TLYPAWSASKVQPVEALRYE--  420
B.pseudo LolF      398  TLYPSWRGAKVRPAEALRYE--  417
N.menin LolF       396  TLYPSRRASKTQPAEALRYE--  415
H.pylori LolF      391  SYYPSKKASTIDALSVLRNE--  410
G.sulfur LolF      404  TLYPSWQASRLPPAEALRYE--  423

Consensus aa:           shhPt.pAtphpPhphL..c..
Consensus ss:           hhhhhhhhh   hhhhh
```

Figure S4. Protein alignment of members of the MacB superfamily. Sequence alignment was generated with Promals3D excluding the nucleotide-binding domain of MacB and PvdT. Sequences corresponding to predicted helices are highlighted in red, β-sheets in blue. Abbreviations are as follows E.coli, *Escherichia coli*; A.actino, Aggregatibacter actinomycetemcomitans; V.cholerae, *Vibrio cholerae*; N.gonor, *Neisseria gonorrhoeae* ; C.jejuni, *Campylobacter jejuni*; P.aeruginosa, *Pseudomonas aeruginosa*; S.enterica, *Salmonella enterica* serovar Typhimurium; Y.pestis, *Yersinia pestis*; H.influenzae, *Haemophilus influenzae*; A.baumannii, *Acinetobacter baumannii*; C.burnetii, *Coxiella burnetii*; L.pneumo, *Legionella pneumophila*; F.tularensis, *Francisella tularensis*; B.pseudo, *Burkholderia pseudomallei*; N.menin, *Neisseria meningitidis*; H.pylori, *Helicobacter pylori*; G.sulfur, *Geobacter sulfurreducens*.

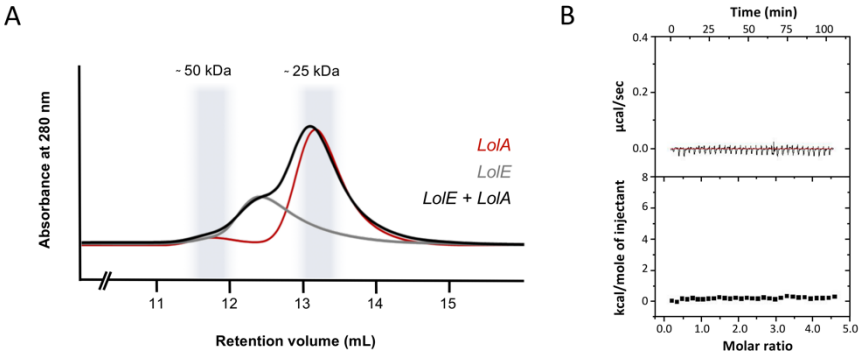

Figure S5. LolA does not bind to the LolE periplasmic domain. (A) Size-exclusion chromatography profiles for LolA, LolE periplasmic domain and a mixture of the two proteins. (B) Isothermal titration calorimetry using LolE and LolA. Both experiments were performed under conditions where LolC and LolA interact with high affinity.

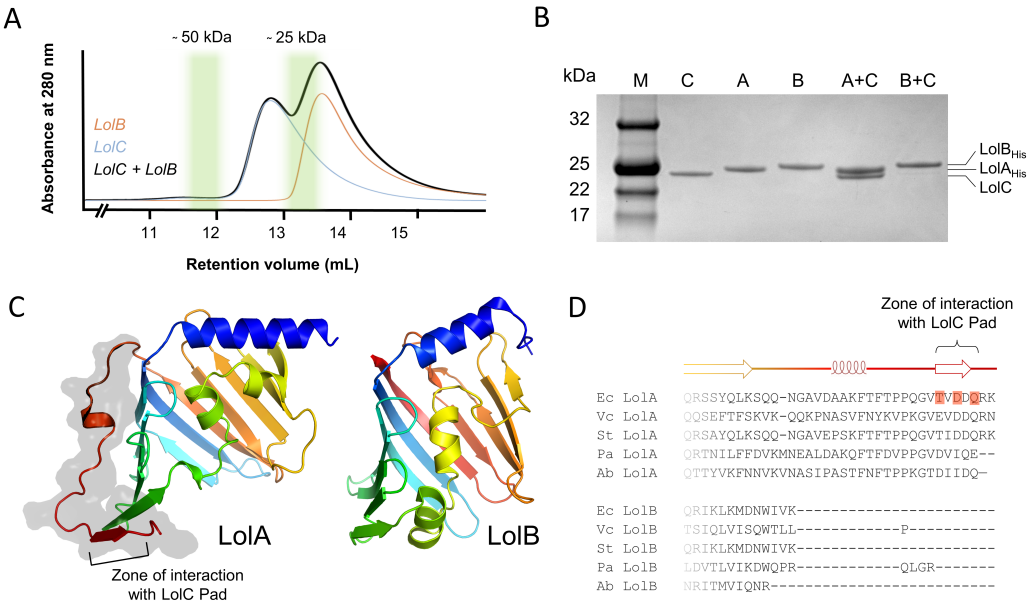

Figure S6. LolB does not interact with LolC. (A) Size-exclusion chromatography experiment for LolB, LolC periplasmic domain and a mixture of the two proteins. (B) Assessment of the in vitro interaction of LolC with LolA or LolB. Untagged LolC periplasmic domain was added to His-tagged LolA (A+C) or LolB (B+C) immobilized on IMAC resin. After washing, bound proteins were eluted with imidazole and analyzed on SDS-PAGE. Purified LolC periplasmic domain, C; LolA, A; and LolB, B are loaded as a reference. Molecular masses of protein standards (M) are indicated. (C) Comparison of LolA (6F3Z) and LolB (1IWM) showing the presence of an extra loop in LolA (dark surface). (D) Sequence alignment of LolA and LolB proteins showing the C-terminal region. Secondary structural elements of LolA are indicated above the sequence alignment. Residues in *E. coli* LolA that interact with the LolC Pad are highlighted in red. Abbreviations are as follows Ec, *Escherichia coli*; Vc, *Vibrio cholerae*; St, *Salmonella enterica* serovar Typhi; Pa, *Pseudomonas aeruginosa*; Ab, *Acinetobacter baumannii*.

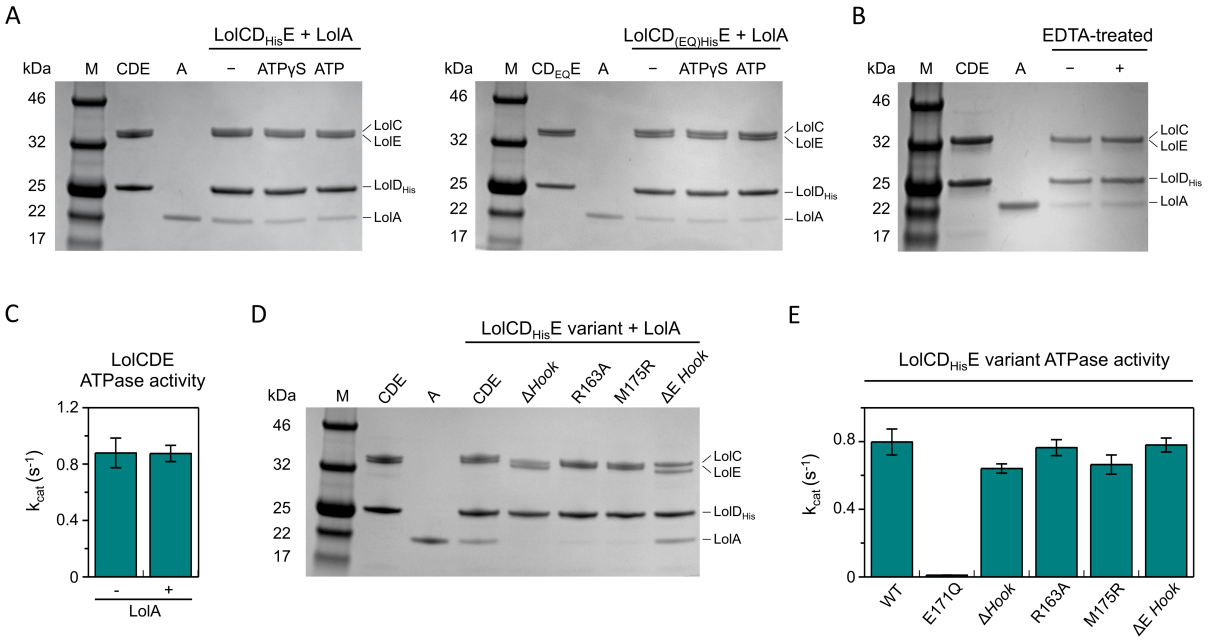

Figure S7. LolA binding and ATPase assays for wild-type and variant LolCDE complexes. (A) In vitro interaction of LolA with wild-type LolCDE (*left*) or LolCD(E171Q)E variant (*right*) in the presence and absence of ATP or ATPγS. Wild-type LolCDE or E171Q variant bearing a His-tag on LolD were incubated with no nucleotide (-), 1 mM ATP or ATPγS as indicated, and immobilized on IMAC resin. Untagged LolA was then added, the resin washed, and bound proteins eluted with imidazole and analyzed on SDS-PAGE. Purified proteins loaded as references are LolCDE, CDE; LolCD(E171Q)E, $CD_{EQ}E$ and LolA, A. Molecular masses of protein standards (M) are indicated. (B) In vitro interaction of LolA with wild-type LolCDE untreated or treated with 5 mM EDTA. (C) ATPase assays for wild-type LolCDE in the absence and presence of 5 μM LolA. Results correspond to the mean ± standard deviation for triplicate determinations. (D) Assessment of the in vitro interaction of LolA with wild-type or variant LolCDE. LolA binding assay in the absence of nucleotide for wild-type LolCDE and indicated variants: ΔHook, R163A, M175R, correspond to mutations in LolC component of LolCDE; ΔE Hook corresponds to LolCDE with the Hook removed from LolE. (E) ATPase assays for wild-type and variant LolCDE complexes. Results correspond to the mean ± standard deviation for triplicate determinations.

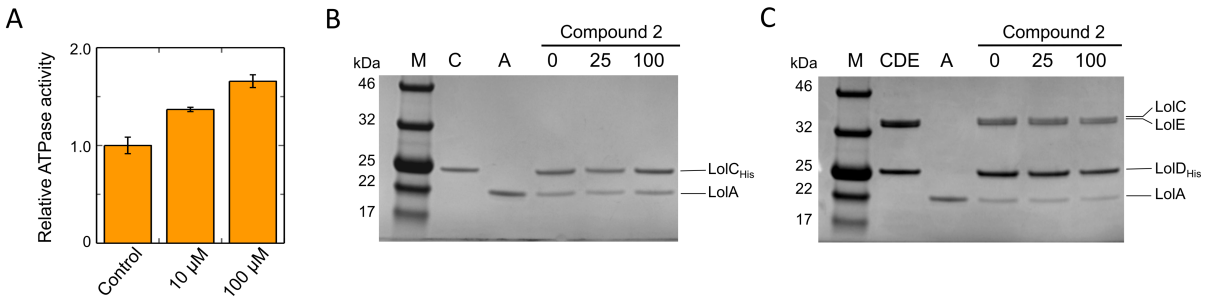

Figure S8. LolCDE inhibitor Compound 2 stimulates ATPase activity but does not interfere with LolA binding. (A) ATPase assay for wild-type LolCDE in the presence of 0, 10 or 100 μM Compound 2. ATP hydrolysis rates correspond to the mean ± standard deviation for triplicate determinations. (B) Effect of Compound 2 on the in vitro interaction of LolC and LolA. His-tagged LolC periplasmic domain was incubated with the indicated concentration (μM) of Compound 2 and immobilized on IMAC resin prior to the addition of untagged LolA. After washing, bound proteins were eluted with imidazole and analyzed on SDS-PAGE. Purified LolC periplasmic domain, C; and LolA, A are loaded as a reference. Molecular masses of protein standards (M) are indicated. (C) Effect of Compound 2 on the in vitro interaction of His-tagged LolCDE and LolA.

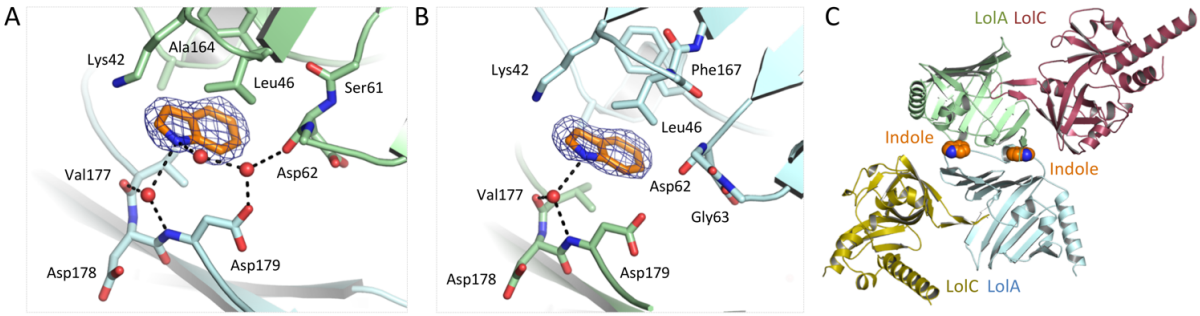

Figure S9. Additional electron density in the LolA-LolC structure suggestive of indole. (A, B) Sites within the asymmetric unit with additional difference map electron density suggestive of indole. The difference electron density map is shown as a blue mesh contoured at 3 σ. (C) Locations of putative indole sites within the context of the asymmetric unit. The presence of indole was biochemically confirmed for the *E. coli* culture used to express these proteins, but not for the protein solution. We therefore chose to omit indole from the deposited coordinates while highlighting its possible presence here.

Supplemental Tables

Table S1. X-ray data and refinement statistics.

	LolA bound to LolC periplasmic domain	LolC ΔHook	LolA F47E
PDB code	6F3Z	6F49	6FHM
Data Collection			
Beamline	ESRF 30B	ESRF 30B	Diamond I03
Wavelength (Å)	0.9763	0.9763	0.9763
Crystal Parameters			
Space Group	$P2_1\ 2_1\ 2$	$P2_1\ 2_1\ 2_1$	$P2\ 2_1\ 2_1$
Unit Cell Dimensions (Å)	146.0, 68.2, 94.8	75.3, 108.5, 109.5	61.0, 77.6, 103.6
Unit Cell Angles (°)	90, 90, 90	90, 90, 90	90, 90, 90
Mosaic Spread (°)	0.58	0.58	0.44
Reflection Data			
Resolution Range (Å)	73.01-2.00 (2.05-2.00)	62.04-2.02 (2.07-2.02)	62.11-2.39 (2.48-2.39)
Unique Reflections	61745 (4379)	58914 (4321)	20165 (2087)
R_{sym}	0.103 (0.844)	0.166 (0.684)	0.196 (1.167)
I/σ(I)	10.6 (2.2)	6.7 (2.0)	6.7 (2.1)
CC½	0.997 (0.883)	0.990 (0.704)	0.984 (0.893)
Completeness (%)	95.8 (97.0)	99.1 (99.5)	100.0 (100.0)
Multiplicity	10.2 (9.9)	5.0 (5.2)	11.4 (11.8)
Wilson B ($Å^2$)	33.5	14.1	47.2
Refinement			
Resolution (Å)	73.01 (2.00)	62.04 (2.02)	62.11 (2.39)
Number of Reflections	58544	55872	19110
R_{work}	0.2022	0.1884	0.2147
R_{free}	0.2492	0.2328	0.2693
Rms (Bond Lengths) (Å)	0.019	0.015	0.015
Rms (Bond Angles) (°)	1.85	1.70	1.67
Model Composition			
Protein atoms	6536	6639	2987
Waters	152	452	48
Other	0	84	18
Model B-factors			
Protein atoms ($Å^2$)	47.1	22.9	61.4
Waters ($Å^2$)	42.6	26.9	54.1
Other	-	39.1	68.6
Ramachandran Statistics			
Favoured (%)	97.0	99.0	95.5
Allowed (%)	3.0	1.0	4.5
Outliers (%)	0.0	0.0	0.0

Values in parentheses indicate the outer resolution bin.
Reflection data is as reported by Aimless (1).
Refinement statistics as reported by Refmac (2).
Ramachandran statistics from Rampage (3).

Table S2. ITC data for LolA binding by LolC periplasmic domain variants.

	K_D (μM)	*N*	*ΔG*	*ΔH*	*-TΔS*
LolC	0.3, 0.4, 0.4, 0.5 **0.4 ± 0.1**	1.02, 0.92, 0.91, 1.21 **1.01 ± 0.14**	-8.9, -8.7, -8.7, -8.6 **-8.7 ± 0.1**	7.0, 7.7, 8.3, 6.2 **7.3 ± 0.9**	-15.9, -16.4, -17.0, -14.8 **-16.0 ± 0.9**
LolC ΔHook	No binding	-	-	-	-
LolC R163A	No binding	-	-	-	-
LolC Q171A	0.4, 0.5 **0.4 ± 0.1**	0.97, 0.99 **0.98 ± 0.01**	-8.7, -8.6 **-8.7 ± 0.1**	7.1, 7.0 **7.0 ± 0.1**	-15.8, -15.6 **-15.7 ± 0.1**
LolC F172A	4.0, 3.6 **3.8 ± 0.3**	1.02, 1.04 **1.03 ± 0.01**	-7.4, -7.4 **-7.4 ± 0.0**	8.5, 7.6 **8.0 ± 0.6**	-15.8, -15.0 **-15.4 ± 0.6**
LolC T173A	62.5, 74.3, 71.9 **69.6 ± 6**	**1.00 ***	-5.7, -5.6, -5.6 **-5.7 ± 0.1**	8.5, 8.0, 6.4 **7.6 ± 1.1**	-14.2, -13.6, -12.1 **-13.3 ±1.1**
LolC M175A	4.9, 4.0 **4.5 ± 0.6**	1.03, 1.03 **1.03 ± 0.00**	-7.2, -7.4 **-7.3 ± 0.1**	11.9, 9.8 **10.8 ± 1.5**	-19.2, -17.1 **-18.1 ± 1.4**
LolC R177A	2.5, 1.7 **2.1 ± 0.5**	0.87, 0.92 **0.90 ± 0.04**	-7.6, -7.8 **-7.7 ± 0.2**	-7.2, -7.4 **7.3 ±0.1**	-15.4, -14.8 **-15.1 ± 0.4**
LolC I178A	12.7, 10.0 **11.4 ± 1.9**	0.93, 1.11 **1.02 ± 0.13**	-6.7, -6.8 **-6.7 ± 0.1**	6.1, 6.1 **6.1 ± 0.0**	-12.8, -12.9 **-12.9 ± 0.1**
LolC Q181A	1.6, 1.4 **1.5 ± 0.1**	1.06, 1.09 **1.07 ± 0.02**	-7.9, -8.0 **7.1 ± 0.0**	7.3, 6.9 **7.1 ± 0.3**	15.2, -14.9 **-15.1 ± 0.3**
LolC R182A	26.3, 36.2 **31.2 ± 7.0**	**1.00 ***	-6.2, -6.1 **3.6 ± 0.1**	3.4, 3.8 **3.6 ± 0.3**	-9.7, -9.9 **-9.8 ± 0.1**
LolC F172R	8.4, 3.7 **6.1 ± 3.3**	1.07, 1.08 **1.08 ± 0.01**	-6.9, -7.4 **6.8 ± 0.3**	7.7, 5.8 **6.8 ± 1.4**	14.7, -13.2 **-13.9 ± 1.0**
LolC M175R	No binding	-	-	-	-

Mean ± standard deviation in bold. *ΔG*, *ΔH* and *TΔS* reported in kcal mol^{-1}. *T*=25 °C.
* Stoichiometry was fixed at 1:1 for LolA binding by the T173A and R182A LolC variants.
Fits and thermograms in **Figure S2**.

Table S3. Correlation between in vivo crosslinking data and the LolA-LolC crystal structure.

Photo-crosslinker substitution in LolA	Crosslink to LolC?	Nearest LolC* residue	Distance (Å)
F20		P174	6.50
V24	+	**F172**	**4.06**
Q33	+	**F172**	**2.65**
W40		P174	9.70
R43			-
D55	+	**R213**	**4.82**
L59	+	**T173**	**3.60**
K64		M175	9.26
F72	+	**R210**	**3.11**
S99			-
V114			-
K118			-
F127			-
R133			-
E144	+	**F172**	**5.19**
R149		F172	7.57
K155			-
A165			-
Q173		R182	9.72
T176	+	**R182**	**3.23**
Q180		Q181	3.01

*Nearest neighbour located more than 10 Å away are not reported.
Distance measurements are for chains A and B in the LolA-LolC crystal structure (6F3Z).
Columns 1 and 2 from Tokuda (4), columns 3 and 4 this work.

Table S4. List of primers for PCR amplification.

Primers	Description	Sequence (5' to 3')
P1	LolA_NheI_F	GCGCGCTAGCATGGATGCCGCAAGCGATCTGAAAAGC
P2	LolA_BamHI_R	GCGCGGATCCTTACTACTTACGTTGATCATCTACCGTGACGCC
P3	Soluble LolB_NdeI_F	GCGCCATATGTCCGTTACCACGCCCAAAGGTCCTG
P4	Soluble LolB_BamHI_R	GCGCGGATCCTTATTTCACTATCCAGTTATCCATTTTTAAC
P5	periLolC_NdeI_F	GCGCCATATGAACGGCTTTGAGCGCGAGCTG
P6	periLolC_BamHI_R	GCGCGGATCCTTACATATTTTTTTCCATGCGTACGGCCTG
P7	periLolC ΔHook_R	GAACAGGCGCTGGCTCGCACCTACCATCACGCGGATTTGATC
P8	periLolC_NotI_R	GCGCGCGGCCGCCATATTTTTTTCCATGCGTACGGCCTG
P9	periLolC ΔHook_F	GATCAAATCCGCGTGATGGTAGGTGCGAGCCAGCGCCTGTTC
P10	periLolE_NdeI_F	GCGCCATATGCATGGTGAAATCGAGGCG
P11	periLolE_XhoI_R	GCGCCTCGAGCCAGCTTTTAATATAAACATAGCTGTTGGTCAC
P12	periLolE ΔHook_R	GCAAACGCACACGTTTCGCACCGATCATAATCGACACCC
P13	periLolE ΔHook_F	GGGTGTCGATTATGATCGGTGCGAAACGTGTGCGTTTGC
P14	periLolC_BspHI_F	GCGCTCATGAAATACCTGCTGCCGACCGCTGC
P15	T7_F	TAATACGACTCACTATAGGG
P16	His-tag_HindIII_R	GCGCAAGCTTTTAGTGGTGGTGGTGGTGGTGCTCG
P17	LolCD_PciI_F	GCGCACATGTACCAACCTGTCGCTCTATTTATTGGCCTGCGTTACATG
P18	LolCD_NotI_R	GCGCGCGGCCGCTTAGTGGTGGTGGTGGTGGTGAGAACCCTCCGCCC CCATCAGGCTCAGTTCCGC
P19	LolE_NdeI_F	GCGCCATATGGCGATGCCTTTATCGTTATTAATTGGCCTG
P20	LolE_AvrII_R	GCGCCCTAGGTTACTGGCCGCTAAGGACTCGCGCAGG
P21	LolA F74E_F	CTGTGGGTGAAACGTCCAAACTTAGAGAACTGGCATATGACACAAC
P22	LolA F74E_R	GTTGTGTCATATGCCAGTTCTCTAAGTTTGGACGTTTCACCCACAG
P23	periLolC R163A_F	CGTTAATCGCGGTGATCAAATCGCGGTGATGGTACCATCTGCC
P24	periLolC R163A_R	GGCAGATGGTACCATCACCGCGATTTGATCACCGCGATTAACG
P25	periLolC Q171A_F	CGTGATGGTACCATCTGCCAGCGCGTTCACGCCGATGGGGCG
P26	periLolC Q171A_R	CGCCCCATCGGCGTGAACGCGCTGGCAGATGGTACCATCACG
P27	periLolC F172A_F	GGTACCATCTGCCAGCCAGGCGACGCCGATGGGGCGTATTCC
P28	periLolC F172A_R	GGAATACGCCCCATCGGCGTCGCCTGGCTGGCAGATGGTACC
P29	periLolC F172R_F	GGTACCATCTGCCAGCCAGCGCACGCCGATGGGGCGTATTCC
P30	periLolC F172R_R	GGAATACGCCCCATCGGCGTGCGCTGGCTGGCAGATGGTACC
P31	periLolC T173A_F	CCATCTGCCAGCCAGTTCGCGCCGATGGGGCGTATTCC
P32	periLolC T173A_R	GGAATACGCCCCATCGGCGCGAACTGGCTGGCAGATGG
P33	periLolC M175A_F	GCCAGCCAGTTCACGCCGGCGGGGCGTATTCCAAGCCAGC
P34	periLolC M175A_R	GCTGGCTTGGAATACGCCCCGCCGGCGTGAACTGGCTGGC
P35	periLolC M175R_F	GCCAGCCAGTTCACGCCGCGCGGGCGTATTCCAAGCCAGC
P36	periLolC M175R_R	GCTGGCTTGGAATACGCCCGCGCGGCGTGAACTGGCTGGC
P37	periLolC R177A_F	GCCAGTTCACGCCGATGGGGGCGATTCCAAGCCAGCGCCTG
P38	periLolC R177A_R	CAGGCGCTGGCTTGGAATCGCCCCCATCGGCGTGAACTGGC
P39	periLolC I178A_F	GTTCACGCCGATGGGGCGTGCGCCAAGCCAGCGCCTGTTC
P40	periLolC I178A_R	GAACAGGCGCTGGCTTGGCGCACGCCCCATCGGCGTGAAC
P41	periLolC Q181A_F	CGATGGGGCGTATTCCAAGCGCGCGCCTGTTCAATGTGATTGG
P42	periLolC Q181A_R	CCAATCACATTGAACAGGCGCGCGCTTGGAATACGCCCCATCG
P43	periLolC R182A_F	GGGCGTATTCCAAGCCAGGCGCTGTTCAATGTGATTGGC
P44	periLolC R182A_R	GCCAATCACATTGAACAGCGCCTGGCTTGGAATACGCCC
P45	LolD E171Q_F	CCTGGTACTGGCGGATCAGCCTACCGGTAACC
P46	LolD E171Q_R	GGTTACCGGTAGGCTGATCCGCCAGTACCAGG

Table S5. List of plasmid constructs used in this study.

Name	Description	Reference
pET28a	Expression vector	Novagen
pET24a	Expression vector	Novagen
pET26b	Vector encoding pelB signal sequence	Novagen
pETDuet-1	Expression vector	Novagen
pET28-LolA	Expresses LolA (residues 22-203) with an N-terminal His-tag	This study
pET24-LolA	Expresses LolA (residues 22-203) with a C-terminal His-tag	This study
pET28-mLolB	Expresses mLolB (residues 23-207) with an N-terminal His-tag	This study
pET28-LolA(F47E)	Expresses LolA F47E (residues 22-203) with an N-terminal His-tag	This study
pET24-periLolC	Expresses LolC (residues 48-266) with a C-terminal His-tag	(5)
pET28-periLolC	Expresses LolC (residues 48-266) with an N-terminal His-tag	This study
pET24-periLolC(ΔHook)	Expresses LolC (residues 48-266) with a C-terminal His-tag. Residues 167-179 replaced by a GA linker	This study
pET24-periLolC(XnY)	Expresses LolC (residues 48-266) with a C-terminal His-tag, residue X at position n mutated to residue Y	This study
pET24-periLolE	Expresses LolE (residues 65-254) with a C-terminal His-tag	This study
pET24-periLolE(ΔHook)	Expresses LolE (residues 65-254) with a C-terminal His-tag. Residues 171-182 replaced by a GA linker	This study
pETDuet-LolCDE	*lolCD* cloned in the first MCS of pETDuet-1 with a C-terminal His-tag on *lolD, lolE* cloned in the 2nd MCS	This study
pETDuet-LolC(R163A)DE	Expresses LolCDE with an R163A variant of LolC	This study
pETDuet-LolC(M175R)DE	Expresses LolCDE with an M175R variant of LolC	This study
pETDuet-LolCD(E171Q)E	Expresses LolCDE with an E171Q variant of LolD	This study
pETDuet-LolC(ΔHook)DE	Expresses LolCDE with residues 167-179 of LolC replaced by a GA linker	This study
pETDuet-LolCDE(ΔHook)	Expresses LolCDE with residues 171-182 of LolE replaced by a GA linker	This study
pBAD18-pelBperiLolC	Expresses LolC (residues 48-266) with an N-terminal PelB signal peptide and a C-terminal His-tag	This study
pBAD18-pelBperiLolC(ΔHook)	Expresses LolC (residues 48-266) with an N-terminal PelB signal peptide and a C-terminal His-tag. Residues 167-179 replaced by a GA linker	This study
pBAD18-pelBperiLolC(XnY)	Expresses LolC (residues 48-266) with an N-terminal PelB signal peptide and a C-terminal His-tag. Residue X at position n mutated to residue Y	This study

Supplemental Movies

Movie 1. Roving camera tour of the LolA-LolC structure showing representative electron density. A weighted $2|F_o|-|F_c|$ electron density map, calculated with model phases, is shown as blue mesh contoured at 1 σ.

Movie 2. Molecular morph showing conformational changes in LolA due to LolC binding. *Left*, cartoon structure of LolA alternating between its conformation in isolation (1IWL) and within the LolA-LolC complex (6F3Z). *Right*, the same morph using a surface representation of LolA (yellow) with the LolC Hook (teal). Orientations differ by a quarter turn about the horizontal axis; on the left hand side, the mouth of LolA is located at the bottom of the frame, on the right hand side, it is viewed face-on.

Movie 3. Electron density for the LolA F47E variant. One monomer is coloured in red, one in blue to demonstrate the strand exchange between the two monomers, within the domain-swapped dimer. The glutamate residues at position 47 are shown in yellow. A weighted $2|F_o|-|F_c|$ electron density map, calculated with model phases, is shown as blue mesh contoured at 1 σ.

Supplemental Methods

Construction of strains and plasmids

Details of the primer sequences and constructs used in this study appear in **Tables S4** and **S5** respectively. For cytoplasmic expression of LolA, the mature domain of LolA (residues 22-203) lacking the N-terminal secretion signal was amplified from *E. coli* M1655 genomic DNA using primers P1/P2, digested NheI-BamHI, and inserted into pET28a (Novagen) digested with the same enzymes. The resultant vector, pET28-LolA, encodes N-terminal His-tagged mature LolA. Similarly, a plasmid expressing the mature domain of LolB (residues 23-207) with an N-terminal His-tag was amplified with primers P3/P4, digested NdeI-BamHI and ligated into pET28a resulting in pET28-mLolB. pET28-periLolC encoding LolC periplasmic domain (residues 48-266) with an N-terminal thrombin-cleavable His-tag was created by amplification with P5/P6, digestion with NdeI/BamHI and ligation into pET28a digested with the same enzymes. pET24-periLolC encoding the C-terminally His-tagged periplasmic domain of LolC was previously described (5). Residues 167-179 inclusive were replaced by a Gly-Ala linker by two-step PCR using primers P5/P7 and P8/P9. A mixture of these reactions served as a template for a final reaction with P5/P8. Digestion of this product with NdeI-NotI and introduction into NdeI-NotI digested pET24a resulted in pET24-periLolC(ΔHook). The extent of the periplasmic region of LolE (residues 65-254) was determined using the periplasmic LolC structure (5NAA) as a guide and amplified from MG1655 *E. coli* genomic DNA using the primers P10/P11. After digestion by NdeI and XhoI, PCR products were ligated into pET24a digested with the same enzymes, resulting in pET24-periLolE. The LolE Hook was removed in a similar manner to that described for LolC using two stages of PCR P10/P12 and P11/P13 and then an amplification of a mixture of the products with P10/P11. The resultant fragment was digested and ligated into pET24. The resultant plasmid, pET24-periLolE(ΔHook) encodes the LolE periplasmic domain with residues 171-182 inclusive replaced by a Gly-Ala linker. Point mutations in LolA or LolC were created by Quikchange site-directed mutagenesis from pET28-LolA or pET24-periLolC respectively using the primers listed in **Table S4**.

To target the periplasmic domain of LolC (wild-type or variant) to the periplasm, the region corresponding to residues 48-266 was amplified with primers P8/P14, digested BspHI-NotI and cloned into NcoI-NotI digested pET26b (Novagen). The entire region comprising the LolC periplasmic domain with an N-terminal pelB secretion signal and C-terminal His-tag was then amplified with primers P15/P16, digested Xba-HindIII and introduced into pBAD18 (6) resulting in plasmid pBAD18-pelBperiLolC or indicated variant.

To express *E. coli* LolCDE with a His-tag on the C-terminus of LolD, the *lolCD* contiguous region was amplified with primers P17/P18 digested with PciI and NotI, and cloned into the first MCS (Multiple Cloning Site) of pETDuet digested with the same enzymes. *lolE* was amplified with primers P19 and

P20, digested NdeI-AvrII and introduced into the second MCS of the resulting plasmid to create pETDuet-LolCDE. Variants in LolCDE were created by a two-step PCR using mutagenic internal primers and P17/P18 or P19/P20 with pETDuet-LolCDE as template. After restriction enzyme digest, the variant *lolCD* or *lolE* PCR products were ligated into pETDuet-LolCDE from which the wild-type copies of *lolCD* or *lolE* had been excised. All clones were verified by DNA sequencing (Source BioScience).

Protein purification

Purification of wild-type and variant E. coli LolCDE

E. coli C43 (DE3) (7) carrying pETDuet-LolCDE or variants: LolC(R163A)DE, LolC(M175R)DE, LolC(E171Q)DE, LolC(ΔHook)DE, LolCDE(ΔHook) were grown in 2YT media supplemented with 100 μg/mL carbenicillin for 16h at 30 °C. Cells were pelleted at 3500 *g* for 15 min, resuspended in fresh media and protein expression induced with 1 mM IPTG. After 2.5 hours of induction at 30 °C, cells were harvested by centrifugation at 6000 *g* for 6 min and pellets frozen at -80 °C. Bacterial pellets were thawed at room temperature and resuspended in buffer composed of 50 mM Tris pH 7.5, 150 mM NaCl and 10 % (vol/vol) glycerol. Cells were then lysed by passage through a Constant Systems cell disruptor at 30200 psi. Unbroken cells and debris were removed by centrifugation at 10000 *g* for 10 min. Membranes were recovered from the supernatant by centrifugation at 115000 *g* at 5 °C for 2h and resuspended in the same buffer containing 1 % (wt/vol) DDM (dodecyl maltopyranoside) for solubilisation. After 1h, the soluble fraction was recovered by centrifugation (1h at 115000 *g*, 5 °C), supplemented with 40 mM imidazole and loaded on IMAC resin (Biorad Profinity) for 1h. The resin was washed with 50 mM Tris pH 7.5, 150 mM NaCl, 10 % (vol/vol) glycerol, 0.03 % DDM and 40 mM imidazole and the protein eluted with the same buffer containing 500 mM imidazole. Eluted LolCDE complex was buffer exchanged into 20 mM HEPES pH 7.5, 150 mM NaCl, 0.03 % DDM using either PD10 columns (GE Healthcare) or Amicon Ultra 100 kDa cut-off centrifugal concentrators and concentrated using the same device to 5-10 mg/mL before flash freezing and storage at -80 °C.

Purification of wild-type and variant E. coli LolC periplasmic domain

E. coli BL21 (DE3) cells bearing plasmid pET24-periLolC or pET24-periLolC(XnY) variant were grown in 1L of 2YT medium supplemented with 50 μg/mL kanamycin at 30 ºC. When the culture achieved an OD_{600} of 0.8 the temperature was reduced to 18 ºC and protein expression induced with 0.1 mM IPTG. After 16h further growth, cells were harvested by centrifugation at 4000 *g* and the pellet resuspended in 50 mL of 50 mM HEPES pH 7.5, 300 mM NaCl, supplemented with protease inhibitor cocktail (Roche), lysozyme and DNase. Bacteria were lysed by cell disruption (Constant Systems) at 30200 psi before removal of bacterial debris by ultracentrifugation (1h, 115000 *g* at 5 ºC). The soluble

fraction was supplemented with 20 mM imidazole and loaded on to a 5 mL HisTrap FF column using an ÄKTAxpress FPLC (GE Healthcare). Bound proteins were washed with 15 column volumes (CV) of the same buffer, before elution with 250 mM imidazole. Peak fractions were analyzed on SDS-PAGE and pooled according to purity in a 10 kDa exclusion size centricon filter (Amicon). Proteins were buffer exchanged into 20 mM HEPES pH 7.5, 150 mM NaCl using a 10 kDa cut-off centricon device (Amicon) and concentrated to 20-30 mg/mL, before flash freezing and storage at -80 °C. When required the C-terminal His-tag was removed using the Thrombin CleanCleave Kit (Sigma) according to the manufacturer's instructions.

Purification of E. coli wild-type and variant LolE periplasmic domain

The periplasmic domain of LolE and equivalent ΔHook variant were produced and purified as described for LolC with a purification buffer composed of 50 mM Tris pH 8.0, 300 mM NaCl and 10 % (vol/vol) glycerol and a desalting buffer comprising 20 mM HEPES pH 7.5, 150 mM NaCl and 5 % (vol/vol) glycerol. Proteins were stored at -80 °C at 15 mg/mL.

Purification of E. coli wild-type LolA and LolA F47E

Wild-type and LolA F47E proteins were produced in *E. coli* BL21 (DE3) bearing pET28-LolA or pET28-LolA(F47E). Cells were grown at 37 °C in 1L of 2YT medium supplemented with 50 μg/mL kanamycin. Cultures were induced with 0.1 mM IPTG when an OD_{600} of 0.8 was reached and temperature was reduced to 18 °C. After 16h, bacteria were harvested by centrifugation at 4000 *g* and resuspended in a buffer composed of 50 mM Tris, pH 8.0, 300 mM NaCl before lysis in a cell disruptor (Constant Systems) at 30200 psi in the presence of lysozyme and DNase. Cell debris were removed by ultracentrifugation (1h, 115000 *g* at 5 °C). The soluble fraction was supplemented with 20 mM imidazole and loaded onto a 5 mL HisTrap FF column using an ÄKTAxpress system (GE Healthcare). Bound proteins were washed with 15 CV of the same buffer, before elution with 250 mM imidazole. Peak fractions were analyzed on SDS-PAGE and pooled in a 10 kDa cut-off centrifugal concentrator (Amicon). Proteins were then buffer exchanged into 20 mM HEPES at pH 8.0 and 200 mM NaCl and concentrated to 25 mg/mL. When required, the N-terminal His-tag was removed using the Thrombin CleanCleave Kit (Sigma) according to the manufacturer's instructions. After cleavage, the protein was re-purified using Ni-IMAC to remove free His-tags and uncleaved His-tagged protein.

Purification of E. coli wild-type soluble LolB

Soluble LolB was produced in *E. coli* BL21 transformed with pET28-mLolB and purified as described for wild-type LolA with a buffer composed of 20 mM Tris pH 7.4, 150 mM NaCl and 0.25 mM TCEP. The protein was desalted with the same buffer containing no TCEP. Proteins were stored at -80 °C at 30 mg/mL.

Size-exclusion chromatography analysis

Size-Exclusion Chromatography (SEC) was performed on a Superdex 75, 10/300 GL column run at 0.8 mL/min using an ÄKTA Pure FPLC system (GE Healthcare) equipped with a 100 µL injection loop. The running buffer was composed of 20 mM HEPES at pH 7.5, 150 mM NaCl. For analysis of individual proteins, 0.5 mg of protein was loaded onto the column. To assess the interaction of two proteins, 0.5 mg of each protein was mixed and incubated for 5 minutes prior to injection.

Isothermal titration calorimetry (ITC)

ITC experiments were carried out at 25 °C in a VP-ITC calorimeter (MicroCal, GE Healthcare) by injecting 300 or 450 µM of wild-type or variant LolC periplasmic domain into 25 µM LolA. ITC buffer was composed of 20 mM HEPES pH 7.5, 200 mM NaCl. Initially, 5 µL was injected over 10 s followed by injections of 10 µL over 20 s until the syringe was empty. Injections occurred every 200 s and the cell stirring speed was 300 rpm. To characterise the interaction of LolA and LolE, LolA (450 µM) was injected into the cell containing 25 µM periplasmic LolE while LolA F47E (200 µM) was injected into 25 µM LolC periplasmic domain. For each titration, a control run with injectant and buffer alone in the cell was performed. The resulting signal was subtracted as a linear fit from protein-protein data. Binding affinity, stoichiometry and thermodynamic parameters were obtained by nonlinear least-squares fitting of experimental data using a single-site binding model from the Origin software package.

Crystallization and structure determination

All crystals were grown at 15 °C by the sitting drop vapour diffusion method over a reservoir of 80 µL in MRC 2-drop plates (Molecular Dimensions).

LolA-LolC complex

Individually purified LolC periplasmic domain and LolA were incubated together (both at a final concentration of 6 mg/mL) in 20 mM HEPES pH 7.5, 150 mM NaCl and then mixed with the precipitation solution at a 1:1 ratio in a final volume of 1 µL over a reservoir of 80 µL. Crystals of the LolA-LolC complex were obtained in 100 mM HEPES pH 6.5, 45 % (wt/vol) poly(acrylic acid sodium salt) 2100. Crystals were obtained after two days following seeding with crushed crystals of LolA F47E and LolC periplasmic domain obtained in 13-17 % PEG 8000, 10-20 % (vol/vol) glycerol and 30-60 mM KH_2PO_4. The cryoprotective solution was composed of the reservoir solution supplemented with 20 % ethylene glycol. Data were collected on beamline ID30B at ESRF. The structure was solved by molecular refinement with Phaser (8) using the wild-type LolC periplasmic domain (5NAA) after trimming residues 48-63, 170-179, 252-273 and LolA (1IWL) after removing loops corresponding to amino acids 115-124 and 180-182. Iterative cycles of density modification with Parrot (9) and automated model building with Buccaneer (10) produced a model that was further improved with several rounds of Refmac (2) and manual building in Coot (11). Extra density present at the interface of LolA

monomers was consistent with indole (**Figure S9**). Indole was positively identified in the growing bacterial culture using Kovac's reagent but not in the protein solution, possibly due to insensitivity of the test. Consequently, indole was not included in the final coordinate file (PDB 6F3Z).

LolA F47E mutant

Crystals of LolA F47E protein were obtained by mixing 0.5 µL of protein at 12 mg/mL in 20 mM HEPES pH 7.5, 150 mM NaCl with the same volume of a precipitant solution composed of 13 % (wt/vol) PEG 8000, 20 % (vol/vol) glycerol. No seeding procedure was used. Crystals appeared after three days and were cryoprotected in the reservoir solution containing glycerol at a final concentration of 36 % (vol/vol) before being frozen in liquid nitrogen. X-ray diffraction data were obtained at Diamond (UK) on beamline I03 equipped with a Pilatus3 6M detector. LolA (1IWL) was used as a search model in Phaser (8) for molecular replacement after trimming residues 1-26, 32-51 and 88-161. After a round of refinement in Refmac (2), a new set of phases was generated by density modification using Parrot (9). The final model was obtained after a round of auto-building with Buccaneer (10), manual manipulation using Coot (11) and refinement with Refmac (2).

LolC ΔHook periplasmic domain

LolC periplasmic domain lacking the Hook (Δ167-179 GA) was crystallized similarly to LolA F47E with protein concentrated to 12 mg/mL and a precipitant solution composed of 30 % (wt/vol) PEG 2000 MME, 150 mM sodium acetate pH 4.6, 200 mM ammonium sulfate. Seeds of wild-type LolC periplasmic domain were used to favour crystallization. Crystals were flash-frozen in liquid nitrogen after a brief immersion in the precipitation solution supplemented with 20 % (vol/vol) glycerol as cryoprotectant. Data were collected under cryogenic conditions on beamline ID30B at ESRF (Grenoble, France) on a Pilatus3 6M detector. Images were integrated with Imosflm (12) and scaled with Aimless from the CCP4 suite (1). Structure was refined by molecular replacement with Phaser (8) using the wild-type LolC periplasmic domain structure (5NAA) as the molecular replacement probe. The atomic model was manually built in Coot (11) and refined with Refmac (2) using NCS restraints.

Structure depositions

Structures were deposited in the Protein DataBank with accession codes **6F3Z** (LolA-LolC complex), **6F49** (LolC ΔHook), and **6FHM** (LolA F47E variant).

Measurement of LolCDE ATPase activity

The ATPase activity of LolCDE proteins was evaluated using the EnzCheck Phosphate Assay Kit (Thermofisher) that couples the release of inorganic phosphate to purine nucleoside phosphorylase (PNP) mediated breakdown of 2-amino-6-mercapto-7-methyl-purine riboside (MESG). One unit of PNP enzyme was added to a reaction mix containing 50 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 0.1 mM azide,

500 μM MgATP (saturating concentration), 200 μM MESG and 0.03 % DDM in a final volume of 350 μL. The mixture was incubated for 3 minutes and the reaction initiated with addition of 1 μM LolCDE (wild-type or variant). The reaction was followed spectrophotometrically at 360 nm using a NanoPhotometer (Implen). Where indicated, the LolCDE inhibitor, Compound 2 (13), was added at 10 or 100 μM in 1 % DMSO (final concentration) and compared to addition of 1 % DMSO alone. To assess the effect of LolA on LolCDE ATPase activity, 5 μM untagged LolA (a five-fold molar excess) was incubated with LolCDE for 3 minutes prior to initiation of the reaction. The rate of hydrolysis was calculated using GraFit 7.0.3 software from the slope of the initial linear phase of the reaction. A calibration curve obtained using known concentrations of phosphate was used to convert absorbance readings to meaningful units.

Periplasmic targeting of LolC domain

Overnight cultures of C43 (DE3) cells bearing plasmid pBAD18-pelBperiLolC or indicated variant were grown overnight at 37 ºC in LB supplemented with 0.5 % (vol/vol) glycerol and 100 μg/ml carbenicillin. Cultures were diluted to an OD_{600} of 0.02 in fresh medium and grown at 37ºC. After 45 minutes, 0.2 % (wt/vol) arabinose (final concentration) was added to induce protein expression and the growth followed by monitoring OD_{600} for a further 4 hours. To assess expression of the LolC constructs in the periplasm, cultures were inoculated as described above and centrifuged at 3000 *g* for 30 minutes at 4 ºC after 60 minutes growth post-induction. Cells were resuspended in 200 mM Tris, 500 mM sucrose, 1 mM EDTA and incubated on ice for 30 mins. Following centrifugation at 16000 *g* for 30 minutes at 4 ºC, the supernatant was taken as the extracytoplasmic fraction. Samples were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-His (Qiagen) and a dye-conjugated Donkey anti-mouse secondary (Licor) antibodies. Immunoblots were revealed using an Odyssey Licor fluorescence imager.

IMAC-based LolA binding assay

His-tagged LolC periplasmic domain (15 μM final concentration) in 20 mM Hepes pH 7.5, 150 mM NaCl, in a final volume of 250 μL was incubated with 100 μL of Ni-IMAC resin (Biorad) for 5 minutes in a microbatch spin column (Generon). Non-tagged LolA (15 μM final concentration) was then added. After a further 5 minutes, the resin was washed three times with 250 μL of buffer before elution of bound proteins with the same volume of buffer containing 250 mM imidazole. Eluted proteins were analyzed on gradient SDS-PAGE gels with purified proteins as references. Interaction of His-tagged LolCDE with LolA was assessed in the same manner except that 0.01 % DDM was added to all buffers. To assess the effect of any endogenously bound nucleotide, LolCDE was incubated with 5 mM EDTA, the sample desalted and the experiment performed as described above. Where specified, 1 mM MgATPγS or MgATP (final concentration) were added during incubation, wash and elution steps. Where indicated, 25 or 100 μM of Compound 2 inhibitor (14), dissolved in 1 % DMSO (final

concentration), was incubated with the His-tagged protein prior to addition of LolA and the effect compared to addition of DMSO alone. To assess interaction between LolB and the LolC periplasmic domain, the binding assay was performed with His-tagged mature LolB and untagged LolC and compared to the interaction of His-tagged LolA with untagged LolC.

Construction of LolCDE homology model

The LolCDE homology model was built with assistance from the PHYRE2 server (15). LolD and the inner membrane helices of LolC and LolE were built using the MacB structures 5LIL and 5NIL as respective models for the closed and open state. The periplasmic domain of LolC comes from the LolA-LolC structure (6F3Z) in which LolC Sabre and Porter subdomains were split and separately aligned to corresponding Sabre and Porter domains of MacB in the open (5NIL) or closed state (5LIL). LolA was positioned according to the coordinates of the LolA-LolC structure (6F3Z) which was superposed onto the homology model Sabre subdomain. The periplasmic domain of LolE was built with PHYRE2 using the structure of LolC periplasmic domain (5NAA) as a template. The Sabre and Porter subdomains of LolE were separated and placed in the same manner described for those of LolC.

Sequence alignments

The multiple and structure alignment server PromalS3D (16) was used to align the amino acid sequences of LolC, LolE, LolF, MacB and PvdT. The nucleotide binding domain of MacB and PvdT proteins were excluded from the alignment.

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