Supporting Information

Parsons et al. 10.1073/pnas.1109208108

SI Materials and Methods

Materials. [1-^{14}C]Acetate (specific activity, 55 mCi/mmol) and [1-^{14}C]oleate (specific activity, 55 mCi/mmol) were purchased from American Radiolabeled Chemicals. Cerulenin and human serum were purchased from Sigma-Aldrich. Platensimycin was purchased from BioAustralis. Andrimid was extracted from *Pantoea agglomerans* EH335 culture supernatants and purified by HPLC-MS as described (1). Affinity-purified anti-rabbit acyl carrier protein (ACP) antibody was described previously (2).

Bacterial Strains, Plasmids, and Growth Conditions. *Streptococcus pneumonia* strain R6 and *Staphylococcus aureus* strains Mu50, SA113, and multidrug-resistant *S. aureus* (*S. aureus* strain MRSA) were purchased from ATCC. The restriction-deficient *S. aureus* strain RN4220 (3), *S. pneumonia* strain D39, and its strain LYJ4 (Δ*fabT*) (4) were described previously, as were *P. agglomerans* strain EH335 (1) and plasmid pCL15 (5). Strains CS34 (Δ*fapR*), PS01 (Δ*accD*), and JP102 (Δ*accD* Δ*fabI*) were constructed using the TargeTron Gene Knockout System (Sigma-Aldrich): A 0.9-kb intron was inserted into position 183 bp, 164 bp, and 169 bp of the *fapR*, *accD*, and *fabI* genes, respectively. *S. aureus w*ild-type and mutant *fabI* genes were amplified using primers 5′-TTAAAACTGGGATTAGATATTC and 5′-TTTGCTCACATATATAATAAC. The PCR products were ligated into the XbaI and KpnI restriction sites of pCL15. *S. aureus accDA* genes were amplified using primers 5′-ATTGCTAGCATGGTTAAAGATTTTTTTAATCGAAC and 5′-GTGCGGCCGCTTCTATATAAGAACCGATA. The PCR product was ligated into the XbaI and SmaI sites of pCL15. Growth medium for *S. aureus* was 1% tryptone broth (TB), or TB plus the indicated fatty acid and 0.1% Brij-58 or 10 mg/mL fatty acid free bovine serum albumin (BSA). Growth medium for *S. pneumoniae* was C+Y medium (CY) (6) containing 10 mg/mL BSA and the indicated amount of fatty acid. Acyl-ACPs were synthesized using *Escherichia coli* acyl-ACP synthetase (7) with *S. aureus* ACP.

Fatty Acid Composition. Cultures (10 mL) of *S. aureus* RN4220 were grown to midlog phase in TB/BSA medium containing the specified fatty acid. The lipids were extracted (8), and methyl esters were prepared and quantified using a Hewlett-Packard 5890 gas chromatograph as described previously (9).

Mass Spectrometry. Ten-milliliter cultures of *S. aureus* RN4220 were grown to midlog phase in TB. The cells were pelleted and suspended in 300 μL of medium. These cells were used to inoculate medium containing 10 mg/mL BSA or medium containing 10 mg/mL BSA and 800 μM oleate and were incubated for 4 h. Cells were harvested by centrifugation and washed two times with TB and two times with PBS. The cell pellet was suspended in 1 mL water, and lipids were extracted by using the Bligh and Dyer method (8). Lipids were brought up in 50:50 chloroform:methanol (vol/vol). Mass spectrometry of phosphatidylgylcerol (PtdGro) was performed using a Finnigan TSQ Quantum (Thermo Electron) triple-quadrupole mass spectrometer. The instrument was operated in the negative ion mode using parent ion scanning corresponding to the loss of phosphoryl glycerol-H_2O. Ion source parameters were as follows: spray voltage: 2,000 V; capillary temperature, 270 °C; capillary offset, −35 V; tube lens offset was set by infusion of the polytyrosine tuning and calibration solution (Thermo Electron) in electrospray mode. MS acquisition parameters for parent ion scanning were as follows: scan range, 600–900 *m/z*; scan time, 0.4 s; product mass, 153.0 *m/z*; collision energy, 45 V; peak width, Q1 and Q3 0.7 FWHM; and Q2 collision-induced dissociation (CID) gas. 0.5 mTorr. Instrument control and data acquisition were performed with the Finnigan Xcalibur (version 1.4 SR1) software (Thermo Electron).

Positional Distribution of Fatty Acids. PtdGro extracted from RN4220 grown in TB containing 10 mg/mL BSA with or without 800 μM oleate was used to determine the positional distribution of fatty acids. Lipids were spotted on a silica gel H thin-layer plate (activated at 90 °C for 1 h) and developed in chloroform:methanol:acetic acid (55:20:5, vol/vol/vol). Phosphatidylglycerol (PtdGro) was isolated from the plate and added to 50 mM Tris (pH 8.5), and 1 mM $CaCl_2$. Phospholipase A_2 (Sigma-Aldrich) was added to a concentration of 250 μg/mL to cleave off the fatty acid in the second position of the phospholipid. The fatty acid and the corresponding lyso-phospholipid were separated on a silica gel H thin-layer plate (activated at 90 °C for 1 h) and developed in chloroform:methanol:acetic acid (55:20:5, vol/vol/vol). The fatty acid and lyso-phospholipid were converted to fatty acid methyl esters with anhydrous methanol/acetyl chloride and analyzed by gas chromatography.

Malonyl-CoA Measurements. Cultures of *S. aureus* strain RN4220 in TB or TB/18:1Δ9 medium or *S. pneumoniae* strain R6 in CY/BSA or CY/BSA/18:1Δ9 medium were grown to an A_{600} = 0.8. A 100-mL aliquot of culture was treated with either AFN-1252 (100 ng/mL) or platensimycin (8 μg/mL) for strain RN4220 or cerulenin (100 μg/mL) for strain R6 and incubated for 1 h. Cells were harvested at room temperature and extracted using the Bligh and Dyer method (8), and 250 pmol of [$^{13}C_3$]malonyl-CoA (Stable Isotope Products; Isotec) was added. The aqueous phase was applied to a 100-mg 2-(2-pyridyl)ethyl functionalized silica gel column (Supelco) equilibrated with 2% acetic acid in methanol/water (1:1) (10). The column was washed two times with 1 mL of equilibration buffer. CoAs were eluted with 1 mL of 50% acetonitrile containing 15 mM ammonium hydroxide. Mass spectrometry of acyl-CoA was performed using a Finnigan TSQ Quantum (Thermo Electron) triple-quadrupole mass spectrometer (11). The instrument was operated in positive mode using single ion monitoring (SIM) neutral loss scanning corresponding to the loss of the phosphoadenosine diphosphate from malonyl-CoA. The ion source parameters were as follows: spray voltage, 4,000 V; capillary temperature, 250 °C; capillary offset, −35 V; sheath gas pressure, 20; auxiliary gas pressure, 10; and tube lens offset was set by infusion of the polytyrosine tuning and calibration in electrospray mode. The MS acquisition parameters were as follows: scan time, 0.5 s; collision energy, 30 V; peak width Q1 and Q3, 0.7 FWHM; Q2 CID gas, 0.5 mTorr; source CID, 10 V; neutral loss, 507.0 *m/z*; and SIM mass of 855 *m/z* with a scan width of 10 *m/z* to capture the signals from both light and heavy malonyl-CoA.

Radioactive Labeling of Lipids. A 10-mL culture of *S. aureus* strain RN4220 or *S. pneumoniae* strain R6 was grown to midlog phase in TB or CY medium. The culture was split, and 70 μCi [1-^{14}C]acetate or 15 μCi [1-^{14}C]oleate was added. After incubating for 2 h, cells were harvested and washed twice with PBS. The lipids were extracted, and incorporation was quantified by scintillation counting. The lipids were separated by loading equivalent amounts of radioactivity onto a silica gel H thin-layer plate and were developed in chloroform:methanol:acetic acid (55:20:5, vol/vol/vol). R6 lipids were separated on silica gel 60A (Partisil LK6D; Whatman) and developed in chloroform:methanol:acetic acid:water (80:10:14:3, vol/vol/vol/vol). The radiolabeled lipids were visualized using a Bioscan Imaging detector.

[1-^{14}C]Oleate and [1-^{14}C]Acetate Incorporation in AFN-1252- and Andrimid-Treated Cells. *S. aureus* RN4220 and *S. pneumonia* R6 were grown in medium containing 10 mg/mL BSA to an A_{600} = 0.8. Five-milliliter aliquots were labeled with 5 μCi of [1-^{14}C]oleate or 20 μCi of [1-^{14}C]acetate and treated with the indicated concentrations of AFN-1252 or andrimid with vigorous shaking. Incorporation into solvent-treated cells represented 100% incorporation. The cells were harvested by centrifugation and washed two times with medium and two times with PBS. The cell pellet was suspended in 100 μL of water, and lipids were extracted with 360 μL of chloroform:methanol:hydrochloric acid (1:2:0.02). Phases were separated following the addition of 120 μL of chloroform and 120 μL of 2 M potassium chloride. Radiolabeled lipids were quantified by scintillation counting.

Minimal Inhibitory Concentration. The minimal inhibitory concentrations (MICs) for AFN-1252, andrimid, cerulenin, and platensimycin against *S. aureus* and *S. pneumoniae* were determined using a broth microdilution method. The medium was TB plus 10 mg/mL BSA for strain RN4220 or CY plus 10 mg/mL BSA for strain R6. *S. aureus* and *S. pneumoniae* were grown to A_{600} = 1.0 and diluted 30,000-fold in medium. A 10-μL aliquot of diluted cells was added to each well of a U-bottomed, 96-well plate containing 100 μL of medium with the appropriate concentration of drug. The MIC was tested in medium containing 800 μM oleate, 800 μM *S. aureus* fatty acids (a15:0 and a17:0 fatty acids in equal amounts), and 800 μM human serum lipids or TB supplemented with 50% human serum. The plate was incubated at 37 °C for 20 h and read using a Fusion plate reader at 600 nm. Cells grown in medium containing solvent were used as 100% growth.

Gel Electrophoresis and Immunoblotting of ACP. *S. aureus* strain RN4220 was grown to an A_{600} = 0.4 in TB and TB/18:1. A 10-mL aliquot of culture was treated with 80 ng/mL AFN-1252, 75 μg/mL andrimid, 1 mg/mL cerulenin, or an equal amount of DMSO as control and was incubated for 15 min. Cells were lysed using lysostaphin and Triton X-100 and spun at 80, 000 × *g* to remove cell debris. The lysates with and without treatment with 200 mM DTT were run a 13% acrylamide gel containing 1 M urea. The separated proteins were transferred to polyvinylidene difluoride membrane by electroblotting. ACP was detected using the ECF detection kit (GE Healthcare). The primary ACP-specific antibody was used at 1:500 dilution followed by the secondary anti-rabbit IgG conjugated with alkaline phosphatase at a 1:5,000 dilution. The blot was developed using the ECF substrate, and the fluorescent signal was recorded on the Typhoon 9200.

Glycerol-Phosphate Acyltransferase Assays. *S. aureus* strain RN4220 was grown to an A_{600} of 1.0, and the cells were harvested and lysed with lysostaphin at a final concentration of 5 μg/mL The membrane fraction was isolated, and glycerol-phosphate acyltransferase assays were performed as described previously (12). A 30-μL assay mixture contained 0.1 M Tris-HCl (pH 7.4), 150 mM NaCl, 100 μM [1-^{14}C]glycerol-3P, 1 mg/mL BSA, 5 mM Na_3VO_4, and 4 μg purified membrane protein. Reactions were started by the addition of the acyl donors (50 μM palmitoyl-PO_4, 50 μM palmitoyl-ACP, or 50 μM palmitoyl-CoA) and were incubated at 37 °C for 15 min. The products were extracted with 60 μL chloroform-methanol–concentrated HCl (1:2:0.02) followed by 30 μL of 2 M KCl containing 5 mM EDTA. The products in the organic phase were separated by thin-layer chromatography (TLC) on silica gel H layers developed with chloroform:methanol:ammonium hydroxide:water:0.25 M EDTA (45:35:1.5:8.34:0.16). Products were detected with a Typhoon 9200 PhosphorImager and quantified using ImageQuant software v. 5.2.

Acyl-ACP Synthetase Assay. *S. aureus* strain RN4220 or *S. pneumoniae* strain R6 was harvested by centrifugation and resuspended in lysis buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA] containing 0.5 mg/mL lysostaphin and 0.01 mg/mL DNase I and was incubated at room temperature for 2 h. Reaction mixtures contained 100 mM Tris-HCl (pH 7.5), 10 mM ATP, 20 mM $MgCl_2$, 2% Triton X-100, 2 mM DTT, 14 μM ACP, 18 μM [1-^{14}C] oleate, and 75 μg strain RN4220 cell lysate in a volume of 50 μL. Assays were incubated at 37 °C for 20 min before transfer to a 2.3-cm Whatman 3-mm paper disk. The disk was washed with chloroform:methanol:acetic acid to remove unreacted fatty acid, and the bound [1-^{14}C]acyl-ACP was quantified by liquid scintillation counting.

Selection of Mutants. AFN-1252 mutants were selected on plates containing 40 ng/mL AFN-1252. An average of 9.4 ± 0.5 mutants arose per 2×10^{10} cells plated for a frequency of 1 per 2×10^{9} cells, although AFN-1252 does not stop cell growth for two or three divisions (Fig. 3*B*). AFN-1252–resistant mutants also were isolated by plating the same number of cells on TB plates containing 40 ng/mL AFN-1252 plus a 2:1 mixture of a15:0 and a17:0 fatty acid (500 μM total) in 0.1% Brij-58. Two types of colonies were isolated: AFN-1252–resisitant prototrophs and 93% were fatty acid auxotrophs. Individual colonies were screened on fatty acid and AFN-1252 plates for positive identification of fatty acid auxotrophs and AFN-1252–resistant mutants.

1. Jin M, Fischbach MA, Clardy J (2006) A biosynthetic gene cluster for the acetyl-CoA carboxylase inhibitor andrimid. *J Am Chem Soc* 128:10660–10661.
2. Jackowski S, Rock CO (1983) Ratio of active to inactive forms of acyl carrier protein in *Escherichia coli*. *J Biol Chem* 258:15186–15191.
3. Kreiswirth BN, et al. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 305:709–712.
4. Lu Y-J, Rock CO (2006) Transcriptional regulation of fatty acid biosynthesis in *Streptococcus pneumoniae*. *Mol Microbiol* 59:551–566.
5. Luong TT, Lee CY (2006) The *arl* locus positively regulates *Staphylococcus aureus* type 5 capsule via an *mgrA*-dependent pathway. *Microbiology* 152:3123–3131.
6. Lacks S, Hotchkiss RD (1960) A study of the genetic material determining an enzyme in Pneumococcus. *Biochim Biophys Acta* 39:508–518.
7. Rock CO, Garwin JL (1979) Preparative enzymatic synthesis and hydrophobic chromatography of acyl-acyl carrier protein. *J Biol Chem* 254:7123–7128.
8. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917.
9. Zhang Y-M, Marrakchi H, Rock CO (2002) The FabR (YijC) transcription factor regulates unsaturated fatty acid biosynthesis in *Escherichia coli*. *J Biol Chem* 277: 15558–15565.
10. Minkler PE, Kerner J, Ingalls ST, Hoppel CL (2008) Novel isolation procedure for short-, medium-, and long-chain acyl-coenzyme A esters from tissue. *Anal Biochem* 376: 275–276.
11. Haynes CA, et al. (2008) Quantitation of fatty acyl-coenzyme As in mammalian cells by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Lipid Res* 49:1113–1125.
12. Lu Y-J, et al. (2006) Acyl-phosphates initiate membrane phospholipid synthesis in Gram-positive pathogens. *Mol Cell* 23:765–772.
13. Band L, Yansura DG, Henner DJ (1983) Construction of a vector for cloning promoters in *Bacillus subtilis*. *Gene* 26:313–315.

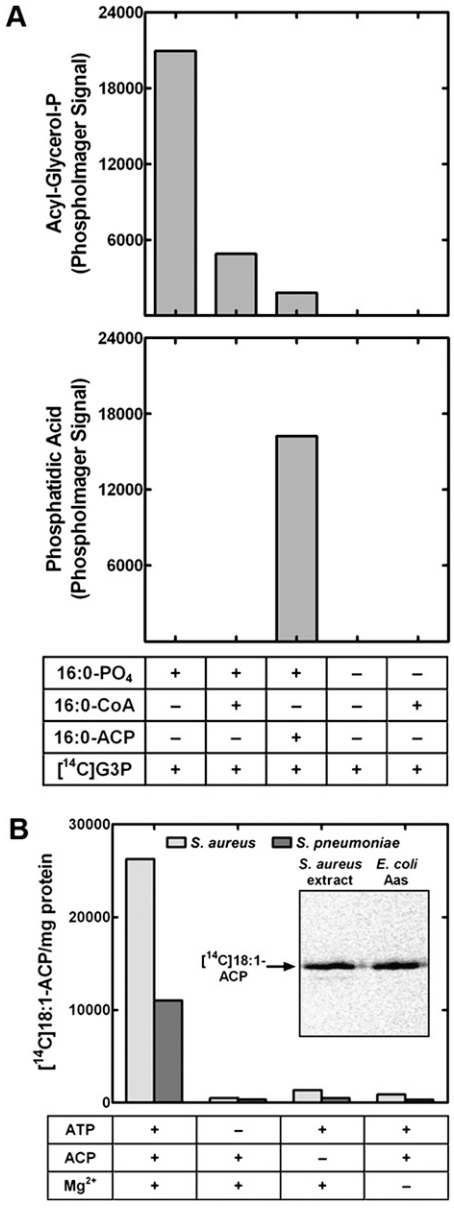

Fig. S1. Enzymes for the acylation and uptake of exogenous fatty acids. (*A*) Membranes were isolated from *S. aureus* strain RN4220 and assayed for acyltransferase activities as described in *Materials and Methods*. Palmitoyl-phosphate (16:0-PO_4) was the only acyl donor used for acylglycerolphosphate synthesis by PlsY, and palmitoyl-ACP was only the acyl donor for phosphatidic acid formation via PlsC. (*B*) Soluble cell extracts of *S. aureus* strain RN4220 or *S. pneumoniae* strain R6 were assayed for acyl-ACP synthetase activity with [1-^{14}C]18:1Δ9 in the presence or absence of the indicated cofactors using the filter disk assay described in *Materials and Methods*. *Inset* shows an autoradiogram of an assay using a *S. aureus* extract analyzed by urea gel electrophoresis. The standard was prepared using the *E. coli* acyl-ACP synthetase to generate [^{14}C]18:1Δ9-ACP. The ACP used in these experiments was *S. aureus* ACP.

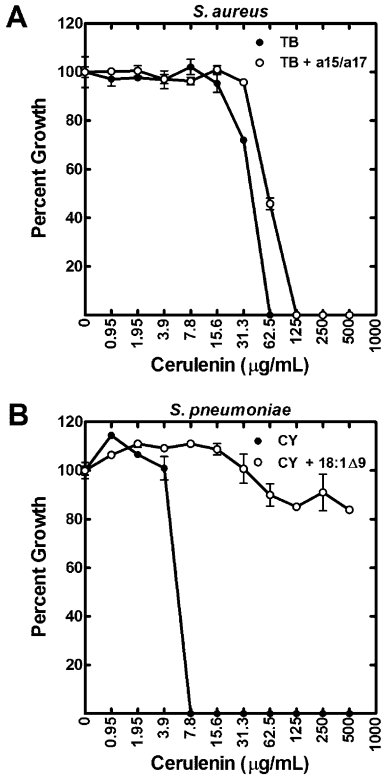

Fig. S2. MIC determinations in the presence and absence of exogenous fatty acids. (*A*) MIC for cerulenin against *S. aureus* strain RN4220 in the presence or absence of exogenous a15:0/a17:0 (500 μM). (*B*) MIC for cerulenin against *S. pneumoniae* strain R6 in the presence or absence of 18:1Δ9.

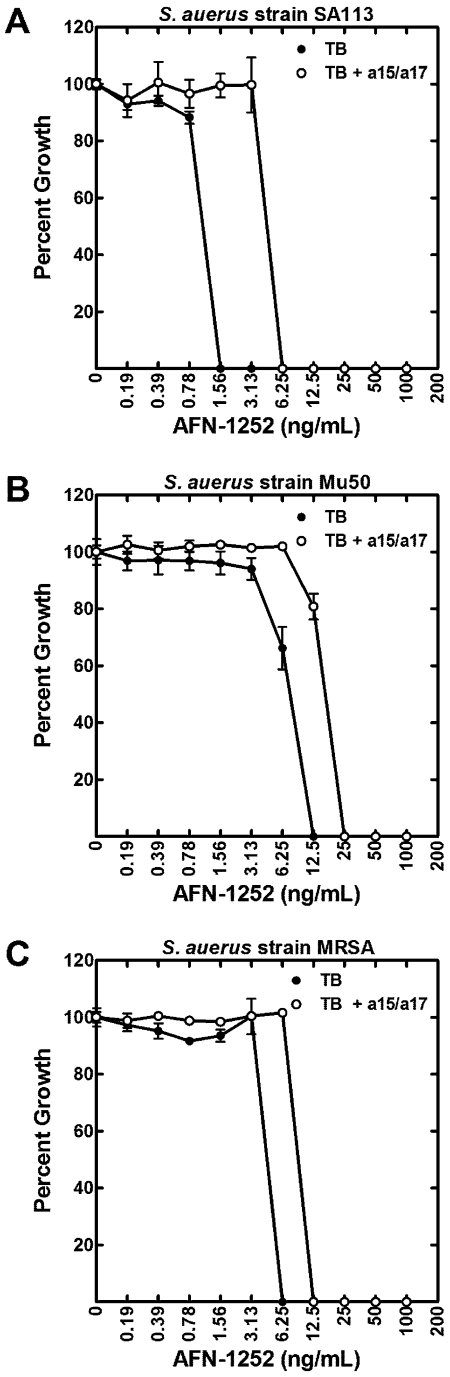

Fig. S3. MIC determinations with three *S. aureus* strains. MIC determinations were carried out in TB plus Brij-58 or TB/Brij-58 supplemented with 500 μM a15:0/a17:0. (*A*) MIC for AFN-1252 against wild-type strain SA113. (*B*) MIC for AFN-1252 against *S. aureus* strain Mu50. (*C*) MIC for AFN-1252 against *S. aureus* strain MRSA.

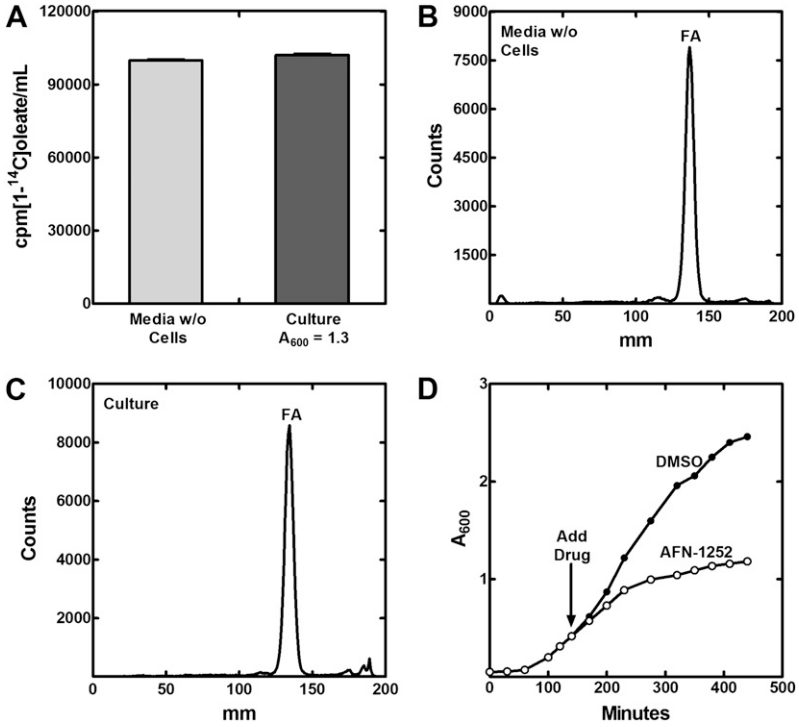

Fig. S4. Fatty acid degradation does not occur in *S. aureus*. TB was prepared with 40 μM [1-^{14}C]18:1Δ9 (2.2 μCi/mL). Triplicate aliquots of medium alone or medium inoculated with *S. aureus* strain RN4220 were incubated with shaking at 37 °C until the cell density reached 1.3 A_{600}. The samples (medium or medium plus cells) were treated with KOH and heated at 100 °C for 3 h. The mixtures were acidified, and the saponified fatty acids were extracted. (*A*) The amount of radioactivity present was quantified by liquid scintillation counting, and the cpm/mL of initial culture was calculated. Samples of the saponified lipids were analyzed by TLC on silica gel G layers developed in chloroform:methanol:acetic acid (98/2/1). Fatty acid (FA) was the only species recovered either in the absence (*B*) or the presence (*C*) of cells. Radioactivity distribution was determined using a Bioscan Imaging Detector. Quantifying the amount of label remaining in the medium after cell growth showed that 31% of the label was incorporated into the cells. (*D*) *S. aureus* strain RN4220 was adapted to 18:1Δ9 by 3 d of repetitive growth in TB/Brij-58/18:1Δ9 and remained sensitive to AFN-1252 (80 ng/mL). An inoculum from this culture was grown to the indicated time in TB/Brij-58/18:1Δ9, the culture was split, and AFN-1252 (80 ng/mL) was added.

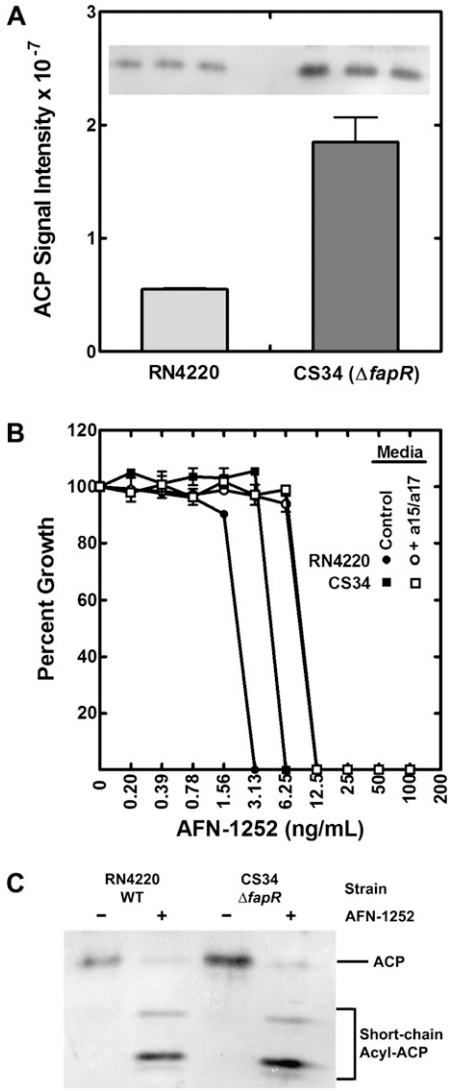

Fig. S5. ACP content and sensitivity of *S. aureus* to AFN-1252 in the presence and absence of exogenous fatty acid. (*A*) Wild-type strain RN4220 and its Δ*fapR* derivative, strain CS34, were grown to midlog phase. Then the cells were harvested and subjected to urea gel electrophoresis. ACP was detected by immunoblotting with anti-ACP, and the bands were quantified using the PhosphorImager. Triplicate samples were processed. ACP content was 3.6-fold higher in strain CS34. (*B*) MIC for AFN-1252 for *S. aureus* strains RN4220 and CS34 grown in TB/Brij-58 compared with the MIC for AFN-1252 for the strains grown in TB/Brij-58 plus 500 μM a15:0/a17:0. (*C*) Strains RN4220 and CS34 (Δ*fapR*) were grown to an A_{600} of 0.5. Then each culture was split into two flasks containing 80 ng/mL AFN-1252 or an equal volume of DMSO. Cultures were incubated for an additional 30 min. Cells were lysed, equal numbers of cells were loaded onto the urea gel, and ACP was detected by immunoblotting with anti-ACP IgG.

Table S1. Fatty acid distribution (weight %) of PtdGro from *S. aureus* grown in TB medium

		Position	
Fatty acid	Total	1	2
i15:0	7.2	3.3	12.8
a15:0	32.3	4.2	87.2
16:0	3.7	4.0	—
i17:0	8.4	13.4	—
a17:0	24.4	38.8	—
18:0	7.0	10.0	—
i19:0	4.4	6.9	—
a19:0	7.8	12.9	—
20:0	4.8	6.4	—

Average of duplicate experiments. These data support the mass spectra shown in Fig. 1*A*. —, <0.2%.

Table S2. Fatty acid composition (weight %) of PtdGro isolated from *S. aureus* grown in TB/BSA/18:1Δ9 medium

		Position	
Fatty acid	Total	1	2
i15:0	1.8	2.7	9.6
a15:0	21.0	3.2	90.4
16:0	1.5	2.4	—
i17:0	2.6	—	—
a17:0	3.3	4.0	—
18:0	3.3	4.5	—
18:1Δ9	33.8	41.6	—
i19:0	—	—	—
a19:0	—	—	—
20:0	1.4	2.7	—
20:1Δ11	31.4	38.9	—

Average of duplicate experiments. These data support the mass spectra shown in Fig. 1*B*. —, <0.2%.

Table S3. Fatty acid composition (weight %) of *S. aureus* strains RN4220 (wild type) and CS34 (Δ*fapR*) grown in TB/BSA/18:1Δ9 medium and *S. pneumoniae* strains D39 (wild type) and LYJ4 (Δ*fabT*) grown in CY/BSA/18:1Δ9 medium

	S. aureus		*S. pneumoniae*	
Fatty acid	WT	Δ*fapR*	WT	Δ*fabT*
i15:0	1.5	2.1	—	—
a15:0	24.6	29.4	—	—
16:0	1.2	1.4	1.9	1.9
i17:0	—	—	—	—
a17:0	—	Tr	—	—
18:0	1.7	1.8	1.5	2.0
18:1Δ9	40.8	41.2	96.6	89.2
18:1Δ11	—	—	—	3.9
i19:0	—	—	—	—
a19:0	—	—	—	—
20:0	—	Tr	—	—
20:1Δ11	30.1	22.2	—	—

Averages of duplicate experiments.These data support the experiments in Fig. 2.*D*. Tr, <1.0%; —, <0.2%.

Table S4. Strains and plasmids

Strain	Genotype	Phenotype	Source
RN4220	*S. aureus*	None	(1)
MWF32	*S. aureus fabI*^{M99T}	AFN-1252 resistant	t296c
MWF33	*S. aureus fabI*^{Y147H}	AFN-1252 resistant	t439c
MWF23	*S. aureus* Δ*accD*	Fatty acid auxotroph	g232t STOP at residue 77
MWF26	*S. aureus accC*^{E86V}	Fatty acid auxotroph	a257t
MWF28	*S. aureus* Δ*accC*	Fatty acid auxotroph	Deletion (301 bp) at residue 77
PS01	*S. aureus* Δ*accD*	Fatty acid auxotroph	Intron insert, at 164 bp
JP102	*S. aureus* Δ*accD* Δ*fabI*	Fatty acid auxotroph	Intron insert, at 167 bp
CS34	*S. aureus*	Δ*fapR*	Intron insert, at 186 bp
R6	*S. pneumoniae*	None	(2)
D39	*S. pneumoniae*	None	(2)
LY4	*S. pneumoniae*	Δ*fabT*	(2)
SA113	*S. aureus*	None	ATCC
Mu50	*S. aureus*	None	ATCC
MRSA	*S. aureus*	None	ATCC
Plasmid			
pCL15	*E. coli-S. aureus* shuttle vector		(3)
pCS57	pCL15 expressing *Sa*FabI		This study
pCS58	pCL15 expressing *Sa*FabI[M99T]		This study
pPS7	pCL15 expressing the *Sa*AccDA operon		This study

1. Kreiswirth BN, et al. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 305:709–712.
2. Lu Y-J, et al. (2006) Acyl-phosphates initiate membrane phospholipid synthesis in Gram-positive pathogens. *Mol Cell* 23:765–772.
3. Band L, Yansura DG, Henner DJ (1983) Construction of a vector for cloning promoters in *Bacillus subtilis*. *Gene* 26:313–315.