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Deletion of the β-Acetoacetyl Synthase FabY in *Pseudomonas aeruginosa* Induces Hypoacylation of Lipopolysaccharide and Increases Antimicrobial Susceptibility

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The β-acetoacetyl-acyl carrier protein synthase FabY is a key enzyme in the initiation of fatty acid biosynthesis in *Pseudomonas aeruginosa*. Deletion of *fabY* results in an increased susceptibility of *P. aeruginosa in vitro* to a number of antibiotics, including vancomycin and cephalosporins. Because antibiotic susceptibility can be influenced by changes in membrane lipid composition, we determined the total fatty acid profile of the Δ*fabY* mutant, which suggested alterations in the lipid A region of the lipopolysaccharide. The majority of lipid A species in the Δ*fabY* mutant lacked a single secondary lauroyl group, resulting in hypoacylated lipid A. Adding exogenous fatty acids to the growth media restored the wild-type antibiotic susceptibility profile and the wild-type lipid A fatty acid profile. We suggest that incorporation of hypoacylated lipid A species into the outer membrane contributes to the shift in the antibiotic susceptibility profile of the Δ*fabY* mutant.

Fatty acid biosynthesis (FAS) is a central metabolic pathway, producing acyl intermediates destined for incorporation into phospholipids, and hence is an essential pathway for membrane biogenesis among members in all three domains of life. Although common to all three domains, the majority of bacterial species analyzed to date utilize a distinct FAS II type pathway for *de novo* FAS (1–3). The constituent FAS II-type pathway enzymes are dissociated enzymes, in comparison to the modular type I FAS complex of eukaryotes (4), suggesting a potential for selective chemical inhibition of prokaryotic FAS II without affecting host FAS. As such, antibacterial discovery strategies focusing on essential bacterial FAS enzymes have emerged in the pursuit of selective antibacterial agents (5, 6).

The initiating condensation in type II FAS between acetyl coenzyme A (acetyl-CoA) and malonyl acyl carrier protein (malonyl-ACP) is catalyzed by β-acetoacetyl-ACP synthase (FabH) in *Escherichia coli* (7). Our previous study showed that in *Pseudomonas aeruginosa* this reaction is not catalyzed by a FabH-type KASIII (ketoacyl synthase) enzyme as in *E. coli* and other bacterial species studied to date but rather by the highly diverged KASI/II family β-ketoacyl-ACP synthase FabY (formerly annotated as PA5174) (8). Disruption of *fabY* imparts a fitness cost to *P. aeruginosa* PAO1 due to reduced FAS pathway flux, resulting in generation times that are 3-fold longer than that observed for the isogenic parent. The Δ*fabY* phenotype is characterized by muted quorum sensing and diminished siderophore secretion (8), a finding consistent with the need for FAS intermediates in the synthesis of the three major acylated quorum-sensing signal molecules [2-heptyl-3-hydroxy-4-quinolone (PQS), *N*-(3-oxododecanoyl)-L-homoserine lactone, and *N*-butanoyl-L-homoserine lactone] (9) and in siderophore assembly (10). Residual FAS initiation in the absence of *fabY* is thought to be due in part to the β-ketoacyl-ACP synthase PA3286, a KASIII family condensing enzyme with low catalytic activity when using short-chain acetyl-CoA as an initiating substrate (11). The main cellular role for PA3286 in wild-type *P. aeruginosa*, however, is in the shunting of exogenous fatty acid-CoA degradation intermediates from the catabolic β-oxidation pathway into FAS at the octanoyl-CoA (C8-CoA) substrate chain length through condensation with malonyl-ACP to form decanoyl-ACP (11). In addition, there may be yet a third uncharacterized mechanism for FabY-independent fatty acid initiation akin to that observed in *E. coli* lacking FabH (12). Nevertheless, the pleiotropic effects of *fabY* disruption suggest inhibitors of FabY alone may suppress signaling pathways and critical virulence factor production in addition to directly impeding growth through FAS inhibition.

Screens of transposon insertion libraries of *P. aeruginosa* have associated *fabY* (formerly PA5174) disruption mutants with increased susceptibility to a number of antibiotics, including certain β-lactams (13, 14). In this report we confirm that the depletion of FabY leads to increased susceptibility to some antibiotics, and we show that the depletion of FabY induces hypoacylation of lipopolysaccharide (LPS), providing a hypothesis for the molecular mechanism responsible for the observed antibiotic potentiation. We also show that exogenous fatty acids restore the wild-type fatty acid profile and reverse the antibiotic hypersusceptibility of the *fabY* deletion mutant, revealing an intrinsic resistance mechanism that bypasses FAS initiation via the PA3286 fatty acid shunt in *P. aeruginosa*.

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MATERIALS AND METHODS

Strains and growth conditions. All *P. aeruginosa* strains were derived from the prototrophic strain K767 (PAO1) (15). The construction and phenotypic characterization of the Δ*fabY* strain TMT39 (*fabY*::*aacC1*; Gm^r) and the plasmid-complemented strain TMT41 [TMT39(pZEN-PA5174); Gm^r Cb^r] have been described (8). Strains were grown in lysogeny broth (LB)–Miller or cation-adjusted Mueller-Hinton broth (MHB) at 37°C. Where appropriate, strains harboring antibiotic resistance markers were maintained by selection with gentamicin (100 μg/ml) or carbenicillin (150 μg/ml).

FAME composition analysis. Bacteria were streaked onto LB agar plates and incubated overnight at 37°C. The Δ*fabY* strain TMT39 grew 3-fold slower than the parent or plasmid-complemented strains. Biomass was scraped from confluent lawns, suspended in phosphate-buffered saline (PBS), and washed three times with the same buffer. Lipids were saponified, methylated, extracted, and washed according to the recommended protocol for the Sherlock microbial identification system (technical note 101 by M. Sasser, revised February 2001; Microbial ID, Inc., Newark, DE). Fatty acid methyl ester (FAME) composition was determined by gas chromatography with flame ionization detection (GC-FID; Microbial ID). Structural assignments were made by comparison of retention times to fatty acid standards (Microbial ID). For each strain, the average FAME composition was determined from three experimental replicates (using lawns of bacteria grown on different days), with >95% of the total peak area in each spectrum able to be assigned to a given fatty acid.

LPS SDS-PAGE. Suspensions of PBS-washed cells prepared as described above for FAME analysis were normalized to an optical density of 1.0 measured using a 600-nm wavelength of light (OD_{600}). Cells were pelleted by centrifugation (10,000 × *g*, 10 min), and the supernatant was decanted. Pellets were resuspended in 50 μl of lysing buffer (1 M Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 4% [vol/vol] β-mercaptoethanol, and bromophenol blue) and boiled for 10 min to lyse cells before being treated with proteinase K (1 mg/ml, 50°C, 1 h) (16). Extracts were separated by electrophoresis through 16.5% polyacrylamide Tricine gels (17). LPS bands were visualized by either silver staining (16) or by transfer to polyvinylidene difluoride (PVDF) membranes, followed by immunoblotting with a primary monoclonal monoclonal antibody (1:1,000 [vol/vol]) specific for the O-antigen O5 serotype in LPS B-band (Rougier Bio-Tech, Ltd., Montreal, Quebec, Canada) (18), a secondary goat anti-mouse polyvalent immunoglobulin-alkaline phosphatase conjugate (1:3,000 [vol/vol]; Sigma, catalog no. A0162), and chromogenic substrate (Sigma*FAST* BCIP/NBT; Sigma, catalog no. B5655).

Isolation of lipid A and ESI-MS analysis. Lipid A was isolated from *P. aeruginosa* cultures grown to mid-exponential growth phase (OD_{600} = 0.8) in LB media at 37°C with shaking at 220 rpm. The procedures for LPS extraction, hydrolysis, and purification of lipid A have been described (12). Briefly, cell pellets were delipidated by suspension in PBS to which chloroform and methanol were added to form a single-phase Bligh-Dyer solution of chloroform-methanol-water (19). After stirring for 1 h at room temperature, the cell pellet containing LPS was collected by centrifugation at 10,000 × *g* for 30 min. The pellet was resuspended in 1% acetic acid and incubated at 100°C for 1 h to hydrolyze the oligosaccharide moiety and liberate free lipid A. The solution was mixed in an acidified two-phase Bligh/Dyer mixture (19) to partition lipid A into the chloroform layer, which was isolated, dried, and resuspended in 2:1 chloroform-methanol (vol/vol) to generate lipid A samples for analysis by electrospray ionization mass spectrometry (ESI-MS). Lipid A mass spectra were acquired on a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer in the negative-ion mode, equipped with a Turbo V ion source (Applied Biosystems, Foster City, CA) using a TurboIonSpray probe for ESI as described previously (20).

Determination of antibacterial activity. The MIC of each antibiotic was determined in cation-adjusted MHB (21). Growth was assessed by visual inspection after incubation for 18 h at 37°C. Each MIC was measured in three separate experiments. Where indicated, MHB was supplemented with sodium decanoate (Sigma, 0.01 to 100 μg/ml) to determine the effect of exogenous fatty acids on antibiotic potency.

TABLE 1 Antibiotic susceptibility of *P. aeruginosa* Δ*fabY* mutant and wild-type strains

Antibiotic	MIC (μg/ml)[a]		Fold change
	Wild type	Δ*fabY* mutant	
Vancomycin	>1,024	32 (>1,024)	>32
Cefotaxime	32	1	32
Ceftriaxone	16	0.5	32
Ceftazidime	1	0.06 (2)	16
Carbenicillin	64	4	16
Ticarcillin	32	2	16
Imipenem	1	0.125	8
Meropenem	0.5	0.125	4
Rifampin	32	4	8
Tetracycline	32	8	4
Minocycline	16	4	4
Erythromycin	512	128	4
Azithromycin	256	64	4
Chloramphenicol	32	16	2
Ofloxacin	1	0.25	4
Ciprofloxacin	0.25	0.12 (0.12)	2
Amikacin	4	2	2
Tobramycin	0.5	0.25	2
Sulfamethoxazole	256	128	2
Trimethoprim	128	256 (128)	0.5
Polymyxin B	1	0.5	2
Colistin	1	1	1
Chlorhexidine	16	16	1

[a] MIC measured in MHB broth. MICs in parentheses correspond to the plasmid-complemented Δ*fabY* (p*fabY*) strain TMT41.

RESULTS

Antibiotic hypersusceptibility of the Δ*fabY* strain. Disruption of *fabY* (PA5174) results in increased susceptibility of *P. aeruginosa* to certain antibiotics (13, 14). To further characterize the enhanced susceptibility phenotype, we measured the MIC of a panel of antibiotics (Table 1). Vancomycin had the largest shift in MIC of all of the compounds tested, with a >32-fold increase in potency against the Δ*fabY* mutant compared to wild-type *P. aeruginosa*. The β-lactams also had increased activity against the Δ*fabY* strain, particularly for the third-generation cephalosporins and carboxypenicillins, whose MICs decreased 16- to 32-fold. The MICs of relatively large and hydrophobic antibiotics, including rifampin, tetracyclines, and macrolides, all were 4- to 8-fold lower. However, the Δ*fabY* strain was not uniformly hypersusceptible to all antibiotics because the susceptibility to membrane-disrupting agents, such as chlorhexidine, a bisbiguanide antiseptic, and the cationic lipopeptide polymyxins (polymyxin B and colistin), was unaffected. In addition, there was no difference in the susceptibilities to fluoroquinolones, trimethoprim, and aminoglycosides (Table 1).

LPS specific fatty acid content is decreased in the Δ*fabY* mutant. The pattern of increased antibiotic susceptibility to compounds normally excluded by the outer membrane (OM) in the Δ*fabY* strain was not consistent with those reported upon loss of specific efflux systems (22–24) but rather suggested a change in OM permeability. Alterations in the ratio of unsaturated to saturated fatty acids, in the average fatty acid chain length, or in the

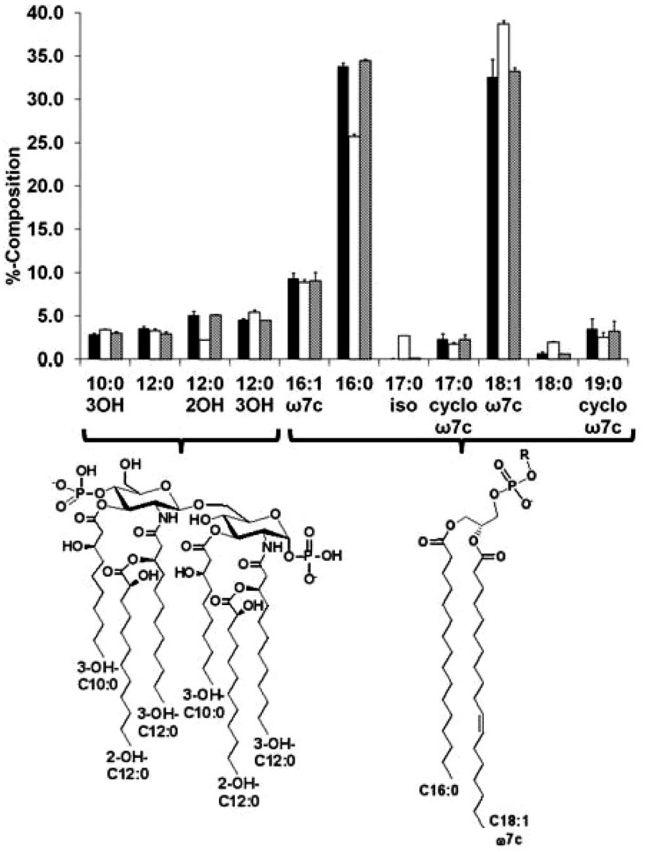

FIG 1 Fatty acid composition of *P. aeruginosa*. The fatty acid content of each *P. aeruginosa* strain was determined by GC-FID analysis of the corresponding methyl ester. For each analysis, >95% of the summed peak areas on each chromatogram could be assigned. Values represent the averages of three independent samples isolated from cells grown on LB agar at 37°C for 18 to 24 h. Bars: ■, wild type; □, Δ*fabY* mutant; ▨, p*fabY* complemented mutant. (Bottom left) Hexa-acylated lipid A structure in *P. aeruginosa* with primary (3-OH-$C_{10:0}$ and 3-OH-$C_{12:0}$) and secondary (2-OH-$C_{12:0}$) acylation; (bottom rght) *P. aeruginosa* glycerophospholipid substituted with the most common fatty acyl chains ($C_{16:0}$ and $C_{18:1\ \omega7c}$). Minor fatty acyl chains detected include acyl chains 16-carbons or longer that are saturated (i.e., $C_{18:0}$), unsaturated (i.e., $C_{16:1\ \omega7c}$), with a cyclopropane (i.e., $C_{17:0\ cyclo\ \omega7c}$), or that are branched (i.e., $C_{17:0\ iso}$). The different phospholipid headgroups vary at position R.

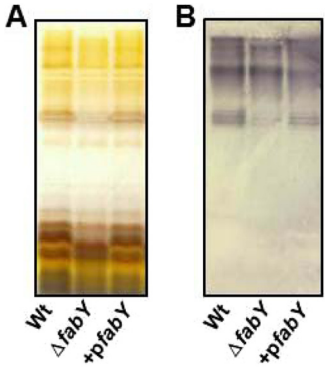

FIG 2 SDS-PAGE and immunoblot analysis of LPS from *P. aeruginosa*. (A) LPS samples were extracted from biomass obtained by scraping cells off LB agar that had been incubated at 37°C overnight. Samples were treated with proteinase K (4) and separated by SDS-PAGE using a Tris-Tricine running buffer system (5). (B) LPS samples were transferred to a PVDF membrane and probed with mouse primary antibody specific for the O5 O-antigen serotype (B-band). Blots were visualized by using an antibody-alkaline phosphatase conjugate.

acylation of LPS could all potentially impact antibiotic susceptibility through alterations in the permeability barrier (25, 26). Because the deletion of *fabY* is known to reduce FAS flux (8), we initially suspected that alterations in membrane fatty acid composition might be responsible for increasing cellular permeability and hence the antibiotic susceptibility. Fatty acid composition analysis of the parent, Δ*fabY* mutant, and *fabY*-complemented mutant was performed (Fig. 1). The fatty acids captured in the FAME analysis include those from phospholipids, lipid A, acyl-ACP, and other minor cellular pools. FAME analysis revealed only minor changes in the levels of phospholipid-specific fatty acids (C_{16} and longer) (Fig. 1). In comparison to the isogenic wild-type parent, the most notable shift was an ~10% increase in the relative abundance of $C_{18:1\ \omega7c}$ content at the expense of the saturated $C_{16:0}$ relative abundance. In addition, the branched-chain $C_{17:0\ iso}$ fatty acid, which is normally not present in planktonic *P. aeruginosa* (27), was readily detected at ~3% of total fatty acids in the Δ*fabY* strain. In Gram-positive bacteria, *iso* branched-chain fatty acids originate from condensation of the leucine degradation intermediate 3-methylbutanoyl-CoA with malonyl-ACP to make β-keto 5-methyl hexanoyl-ACP by FabH type KASIII enzymes (28). Assuming a similar origin in Gram-negative bacteria, the $C_{17:0\ iso}$ fatty acid in the *fabY*-null mutant likely reflects the increased ratio of β-keto 5-methyl hexanoyl-ACP to the *fabY* product β-acetoacetyl-ACP, the normally preferred elongation substrate of the FAS machinery.

Although the changes in the relative abundances and types of phospholipid-related fatty acids were minor overall, the relative abundance of 2-hydroxy-laurate (2-OH-$C_{12:0}$) was decreased >50% in the Δ*fabY* strain (Fig. 1). The 2-OH-$C_{12:0}$ fatty acid is only found esterified in the secondary acyl chains of *P. aeruginosa* lipid A (Fig. 1), where they are symmetrically distributed in acyloxyacyl linkages to the 3-OH of each primary amide-linked acyl group on GlcNI and GlcNII (29). The 2-OH-$C_{12:0}$ fatty acid is formed by nonstoichiometric oxidation of $C_{12:0}$ fatty acid in the lipid A, catalyzed by *P. aeruginosa* LpxO orthologs, and presumably occurs after incorporation into lipid A, as has been reported in *Salmonella enterica* serovar Typhimurium (30). Because the relative abundance of $C_{12:0}$ did not increase in the Δ*fabY* strain, the decreased relative abundance of 2-OH-$C_{12:0}$ in the Δ*fabY* strain was unlikely to be due to inactivation or downregulation of LpxO activity. The relative abundance of 3-hydroxylated fatty acids (3-OH-$C_{10:0}$ and 3-OH-$C_{12:0}$), primary lipid A fatty acids directly attached to the GlcN disaccharide of lipid A, also did not appreciably change upon deletion of *fabY*. The FAME analysis may not quantitatively reflect lipid A acylation states because there may be differences in methyl ester conversion efficiency and the possibility of contamination by rhamnolipids and/or polyhydroxyalkanoates, both of which contain 3-OH-$C_{10:0}$ (31). Nevertheless, the FAME data suggested that lipid A was hypoacylated in the Δ*fabY* strain.

Lipid A remains glycosylated upon deletion of *fabY*. To determine whether the LPS was altered in the Δ*fabY* strain, we first analyzed the LPS profile by SDS-PAGE (Fig. 2). The LPS separated into two regions, characteristic of LPS from many Gram-negative bacteria; the high-molecular-mass ladder pattern at the top of gel

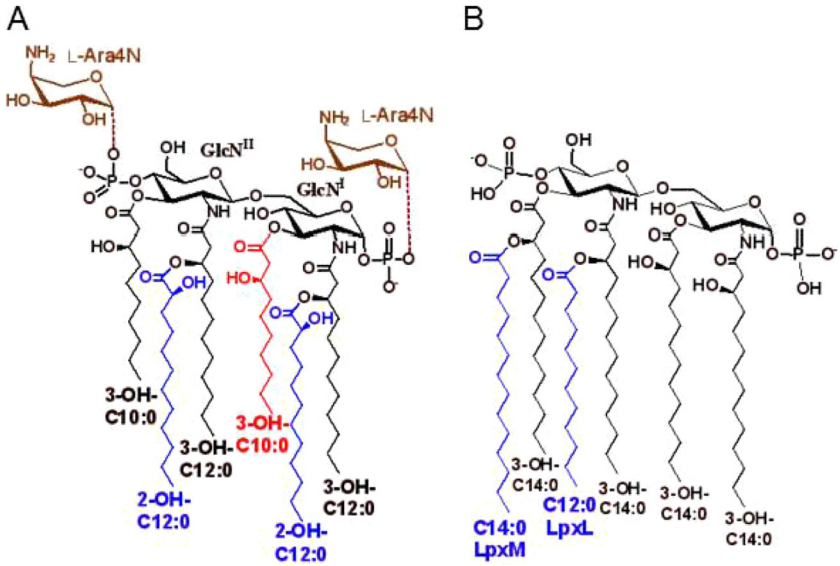

FIG 3 Lipid A structure. (A) *P. aeruginosa* lipid A with potential modification that include: cleavage of the 3-O-acyl chain (3-OH-$C_{10:0}$, red), addition of 1 or 2 L-Ara4N moieties (brown), and 2-hydroxylation of one or both of the secondary acyl chains (blue). (B) Typical *E. coli* lipid A. The secondary acyl chains (blue) are added by LpxL ($C_{12:0}$) and LpxM ($C_{14:0}$) as indicated. The loss of either or both of these acyl chains results in antibiotic hypersusceptibility phenotypes.

from the O antigen containing fraction, along with the low-molecular-mass fraction at the bottom gel (Fig. 2A). Immunoblotting with an antibody specific for the O5 serotype of *P. aeruginosa* B-band PAO1 O antigen confirmed extensive decoration with polysaccharide in the Δ*fabY* strain, with retention of both the overall quantity and laddering seen in the wild type (Fig. 2B). The *fabY* deletion did not prevent maturation of the oligosaccharide core and ligation of the O-antigen polymer to lipid A. Within the uncapped LPS region, however, changes in the banding pattern were apparent (Fig. 2A). LPS species in aggregate shifted to faster-migrating bands in the Δ*fabY* strain. The wild-type profile could be restored by complementation with plasmid-encoded *fabY* (Fig. 2A, lane 2 versus lane 3, respectively).

Lipid A is hypoacylated and highly substituted with L-Ara4N in the Δ*fabY* strain. In order to confirm the lipid A hypoacylation in the *fabY* mutant, we isolated the complete lipid A from each strain for analysis by ESI-MS. The structure of *P. aeruginosa* lipid A with reported lipid A modifications (20) is shown in Fig. 3A. Figure 4 shows the entire window of peaks for the lipid A species, which were detected as $[M-2H]^{2-}$ ions. The spectra from the parental and *fabY*-complemented Δ*fabY* strains were nearly identical (Fig. 4A and C; peak assignments listed in Table 2). Two peaks had values consistent with those previously observed for the major hexa-acylated lipid A species (A-1-0 at 807.5 *m/z* and A-2-0 at 815.5 *m/z*) (20). The 8-*m/z* unit difference between A-1-0 and A-2-0 is consistent with hydroxylation of one or both secondary $C_{12:0}$ lipid A acyl groups, denoted by the first number in the peak designations (e.g., A-1-0). Prominent peaks were also identified that are consistent with penta-acylated lipid A (B-1-0 at 722.5 *m/z* and B-2-0 at 730.5 *m/z*). The penta-acylated lipid A species (labeled "B") were produced from hexa-acylated lipid A (labeled "A") by the activity of PagL, a 3-*O*-deacylase that removes the primary 3-OH-$C_{10:0}$ acyl group from lipid A (32). This penta-acylated lipid A still retains both secondary acyl groups ($C_{12:0}$ and/or 2-OH-$C_{12:0}$). A fraction of lipid A was modified with a single 4-deoxy-L-aminoarabinose (L-Ara4N) residue, catalyzed by ArnT (33), giving rise to peaks shifted by 65.53 *m/z* for both hexa-acylated (A-1-1 at 873.1 *m/z* and A-2-1 881.1 *m/z*) and penta-acylated (B-1-1 at 788.0 *m/z* and B-2-1 796.0 *m/z*) lipid A congeners. The second number in the peak designation (e.g., A-1-0) indicates the number of L-Ara4N moieties.

In contrast to the lipid A composition in wild-type and plasmid-complemented strains, where most lipid A species were unmodified by L-Ara4N, nearly every lipid A species detected in the Δ*fabY* strain contained one or two L-Ara4N moieties (Fig. 4B with peak assignments in Table 2). The additional complexity of the lipid A spectrum from the Δ*fabY* strain arises from multiple peaks consistent with the absence of a single secondary acyl chain ($C_{12:0}$ or 2-OH-$C_{12:0}$). These hypoacylated lipid A species include penta-acylated variants (e.g., a-1-1 at 782.0 *m/z*) that contain four primary and one secondary acyl chain as well as tetra-acylated variants that have only three primary and one secondary acyl chain (e.g., b-1-1 at 696.9 *m/z*). The penta-acylated lipid A species (labeled "a") give rise to the tetra-acylated lipid A species (labeled "b") by the action of PagL. There is no MS evidence for lipid A species lacking both secondary acyl chains.

Exogenous fatty acids reverse the antibiotic hypersusceptibility of the Δ*fabY* strain. We next investigated whether exogenous fatty acids could restore the secondary acylation of lipid A and reverse the antibiotic hypersusceptibility phenotype observed in the Δ*fabY* strain. As expected based on the PA3286 fatty acid shunt (11), secondary lipid A acylation was partially restored when the Δ*fabY* strain grown on agar supplemented with 100 μg of decanoate/ml (estimated by FAME analysis [data not shown]). Beginning with decanoate supplementation at 1 μg/ml, the susceptibility of the *P. aeruginosa* Δ*fabY* strain to ceftazidime and vancomycin decreased, with complete restoration at 100 μg of decanoate/ml (Fig. 5). The decanoate supplementation did not change the MICs against the parent strain, which suggested the rescue mechanism is not an artifact of drug sequestration by free

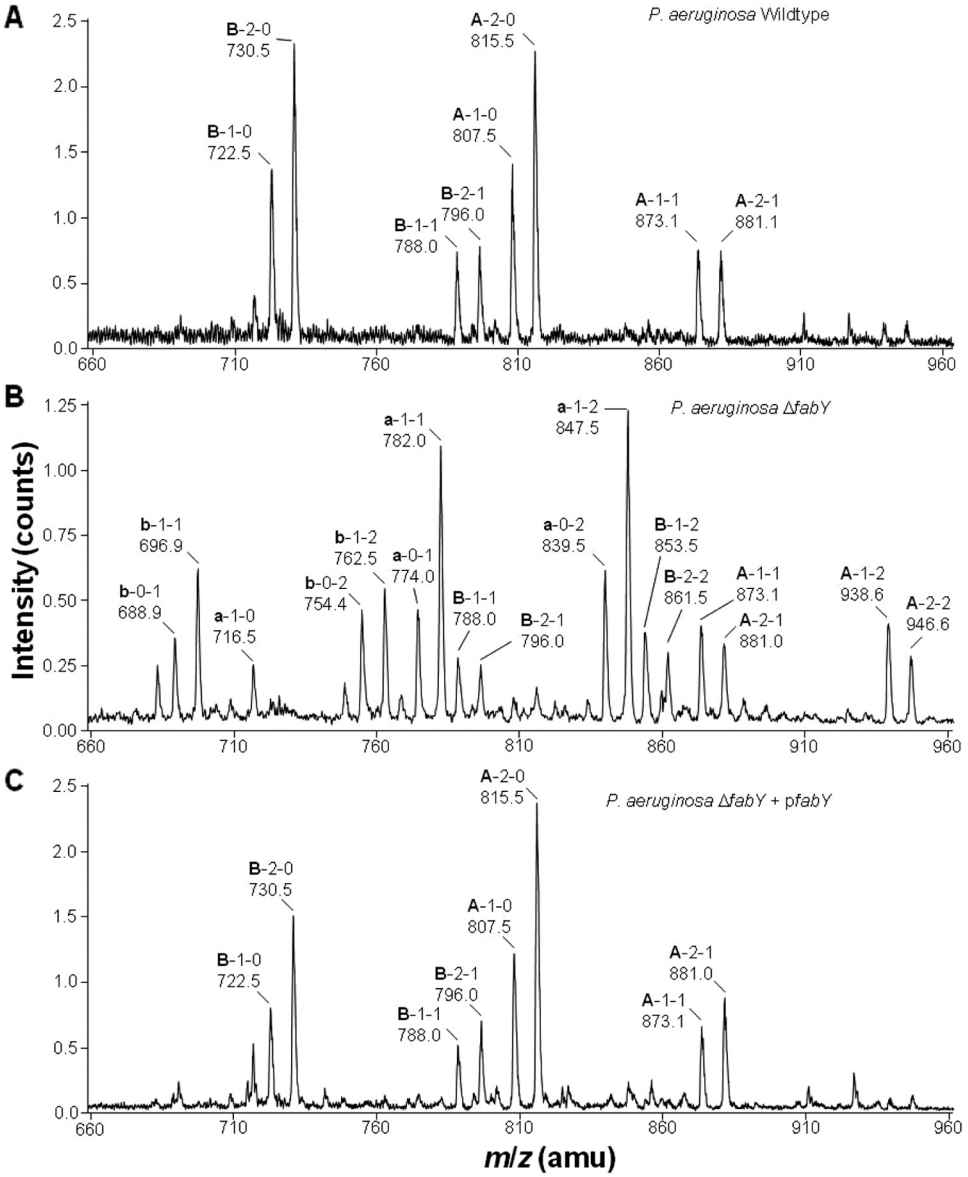

FIG 4 Mass spectra of the lipid A fraction obtained from *P. aeruginosa*. Lipid A was isolated and subjected to ESI-MS as previously described (3). (A) Wild type; (B) Δ*fabY* mutant; (C) p*fabY* complemented Δ*fabY* mutant. Peaks: A, four primary and two secondary; B, three primary and two secondary; a, four primary and one secondary; b, three primary and one secondary acyl chains. The first number indicates 2-OH modifications (0, 1, or 2). The second number indicates L-Ara4N modifications (0, 1, or 2).

fatty acid. In comparison to ceftazidime and vancomycin, the MICs of ciprofloxacin and trimethoprim against either strain were only modestly affected by addition of exogenous fatty acid.

DISCUSSION

We found that the *P. aeruginosa* Δ*fabY* strain is hypersusceptible to certain antibiotics (Table 1), a finding consistent with previous reports (13, 14). We initially hypothesized that upon deletion of *fabY*, alterations in the lipid composition could potentially increase the permeability of the membrane and induce antibiotic susceptibility. In order to determine the cellular mechanism of the antibiotic hypersusceptibility, we first determined the fatty acid profile of the Δ*fabY* strain. Similar to *fabH* mutants in *E. coli* (34, 35), fatty acid composition analysis did not reveal striking changes in phospholipid-associated fatty acids outside an ~10% shift to unsaturated fatty acid content (Fig. 1). The most striking difference in the fatty acid profile between the wild type and the Δ*fabY* strain was a 50% decrease in an LPS-specific fatty acid, 2-OH-$C_{12:0}$ (Fig. 1). Using SDS-PAGE analysis (Fig. 2) and ESI-MS (Fig. 4), we were able to confirm alterations in LPS and determine that the majority of the lipid A species in the Δ*fabY* strain were lacking a single secondary acyl group ($C_{12:0}$) and hence propose a mechanism for the observed antibiotic hypersensitization.

Lipid A is the membrane-embedded anchor of LPS, a glycosaccharolipid located in the external leaflet of the OM of *P. aeruginosa* (29). As in most Gram-negative bacteria (36), LPS is composed of a serotype-specific polysaccharide connected to a highly conserved core oligosaccharide unit which in turn is connected to

TABLE 2 Assignment of lipid A species present in *P. aeruginosa*

Strain[a]	Total no. of acyl chains	No. of specific acyl chains		No. of 2-OH modifications[d]	No. of L-Ara4N modifications	Mass (*m/z*)		
		Primary[b]	Secondary[c]			Calculated (−1)[e]	Calculated (−2)[f]	Observed[g]
Wild type								
A-1-0	6	4	2	1	0	1,615.99	807.49	807.5
A-2-0	6	4	2	2	0	1,631.98	815.49	815.5
A-1-1	6	4	2	1	1	1,747.05	873.02	873.1
A-2-1	6	4	2	2	1	1,763.04	881.02	881.1
B-1-0	5	3	2	1	0	1,445.86	722.42	722.5
B-2-0	5	3	2	2	0	1,461.85	730.42	730.5
B-1-1	5	3	2	1	1	1,576.92	787.96	788.0
B-2-1	5	3	2	2	1	1,592.91	795.96	796.0
Δ*fabY* mutant								
A-1-1	6	4	2	1	1	1,747.05	873.02	873.1
A-2-1	6	4	2	2	1	1,763.04	881.02	881.0
A-1-2	6	4	2	1	2	1,878.10	938.55	938.6
A-2-2	6	4	2	2	2	1,894.10	946.55	946.6
B-1-1	5	3	2	1	1	1,576.92	787.96	788.0
B-2-1	5	3	2	2	1	1,592.91	795.96	796.0
B-1-2	5	3	2	1	2	1,707.97	853.48	853.5
B-2-2	5	3	2	2	2	1,723.97	861.48	861.5
a-1-0	5	4	1	1	0	1,433.82	716.41	716.5
a-0-1	5	4	1	0	1	1,548.88	773.94	774.0
a-1-1	5	4	1	1	1	1,564.88	781.94	782.0
a-0-2	5	4	1	0	2	1,679.94	839.47	839.5
a-1-2	5	4	1	1	2	1,695.94	847.47	847.5
b-0-1	4	3	1	0	1	1,378.75	688.88	688.9
b-1-1	4	3	1	1	1	1,394.75	696.88	696.9
b-0-2	4	3	1	0	2	1,509.81	754.40	754.4
b-1-2	4	3	1	1	2	1,525.81	762.40	762.5
Δ*fabY* (p*fabY*) mutant								
A-1-0	6	4	2	1	0	1,615.99	807.49	807.5
A-2-0	6	4	2	2	0	1,631.98	815.49	815.5
A-1-1	6	4	2	1	1	1,747.05	873.02	873.1
A-2-1	6	4	2	2	1	1,763.04	881.02	881.0
B-1-0	5	3	2	1	0	1,445.86	722.42	722.5
B-2-0	5	3	2	2	0	1,461.85	730.42	730.5
B-1-1	5	3	2	1	1	1,576.92	787.96	788.0
B-2-1	5	3	2	2	1	1,592.91	795.96	796.0

[a] That is, the source of lipid A. Peak designations: A, four primary and two secondary; B, three primary and two secondary; a, four primary and one secondary; b, three primary and one secondary acyl chains. The first number indicates 2-OH modifications (0, 1, or 2), and the second number indicates the number of L-Ara4N modifications (0, 1, or 2).
[b] 3-OH-$C_{10:0}$ and 3-OH-$C_{12:0}$.
[c] $C_{12:0}$ and 2-OH-$C_{12:0}$.
[d] Hydroxylation on $C_{12:0}$.
[e] These *m/z* values are for the $[M\text{-}H]^-$ ions.
[f] These *m/z* values are for the $[M\text{-}2H]^{2-}$ ions.
[g] Enhanced resolution MS was used to identify the *m/z* value of the first peak in the isotope window and to confirm the charge state of the ions.

lipid A (Fig. 3). The 1,4′-bisphosphorylated D-glucosamine (GlcN) backbone of lipid A is acylated with primary 3-OH acyl chains (via amide and ester linkages to GlcNI and GlcNII), which are subsequently acylated with secondary acyl groups. The mature hexa-acylated lipid A structures of *P. aeruginosa* and *E. coli* (Fig. 3A versus 3B) each contain two secondary acyl chains, which are transferred by homologous secondary acyltransferases to species-specific positions in the lipid A (29, 36). In *E. coli*, impaired function of the secondary acyltransferases (LpxL and LpxM) increases susceptibility to many antibiotics, including vancomycin, rifampin, novobiocin, and erythromycin (37–40). The density of lipid A acyl chains directly impacts the permeability barrier, since the tight packing of acyl groups enhances lateral interactions and the barrier function within the lipid A monolayer via hydrophobic van der Waals interactions (25). The *P. aeruginosa* secondary lipid A acyltransferases have been assigned to PA0011 and PA3242 based on sequence similarity to LpxL (29) and a preliminary report of targeted deletions (34). Three separate studies in two different strain backgrounds (*P. aeruginosa* PAO1 and PA14) have associated deletion of PA0011 with hypersusceptibility to antibiotics, including ceftazidime (13), carbenicillin (14, 41), and ciprofloxacin, tetracycline, chloramphenicol, rifampin, and erythromycin (41). These findings are consistent with the assignment of PA0011 as a lipid A lauroyltransferase. Thus, lipid A alterations

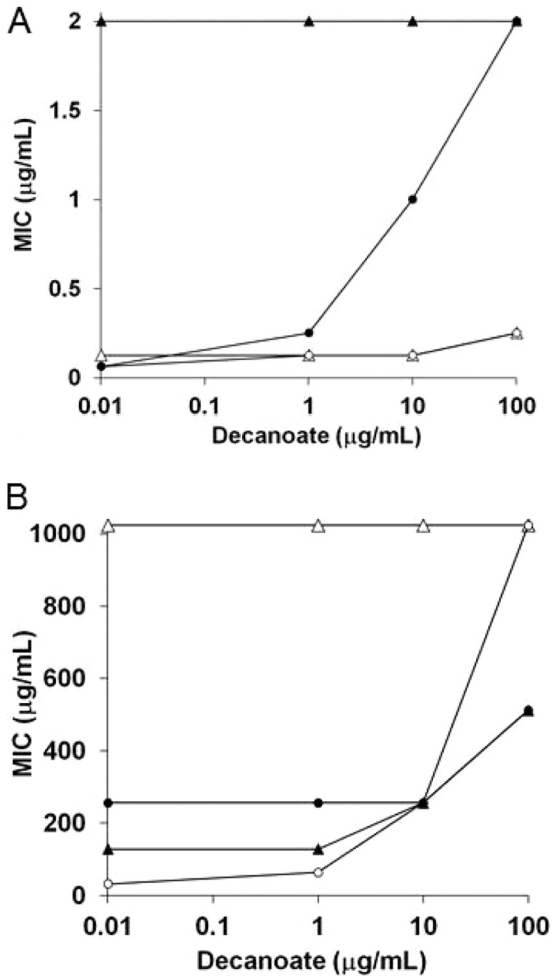

FIG 5 MIC as a function of exogenous fatty acid concentration. (A) The MIC for ciprofloxacin (open symbols) and ceftazidime (closed symbols) was measured for the *P. aeruginosa* wild type (triangles) and Δ*fabY* mutant (circles) in MHB broth supplemented with various concentrations of decanoate at 37°C. (B) The MIC for vancomycin (open symbols) and trimethoprim (closed symbols) was measured for the *P. aeruginosa* wild type (triangles) and Δ*fabY* mutant (circles) in MHB broth supplemented with various concentrations of decanoate at 37°C.

achieved through *fabY* deletion in *P. aeruginosa* phenocopies that of secondary acyl chain transferase deletion mutants in causing lipid A hypoacylation, increasing OM permeability, and imparting antibiotic hypersusceptibility.

Although we certainly cannot rule out differences in gene regulation of either a secondary lipid A lauroyl transferase, a putative lauroyl deacylase, or another component of the multifaceted Δ*fabY* phenotype to explain the hypoacylation, the most plausible molecular mechanism invokes the decreased FAS pathway flux previously observed in the Δ*fabY* strain (8). As in other Gram-negative bacteria, all of the acyl groups on lipid A originate from acyl-ACP produced by the FAS pathway (42). It is likely that decreased flux of the FAS pathway in the Δ*fabY* strain limits cellular pools of the $C_{12:0}$-ACP donor available for the secondary acyl transferases. The hypoacylated lipid A may then be prematurely transported across the inner membrane and incorporated into the OM, leading to a permeability defect and antibiotic hypersusceptibility. In *E. coli*, the inner-membrane LPS flippase MsbA inefficiently translocates hypoacylated lipid A precursors (36, 43). The presence of hypoacylated lipid A in the Δ*fabY* strain modified with one or two L-Ara4N groups (Fig. 4) confirms that this lipid A species was transported to the periplasmic leaflet of the inner membrane because the L-Ara4 modification is catalyzed there by ArnT (33). Furthermore, the hypoacylated lipid A fraction can be transported to the OM because a fraction was deacylated by PagL, which is active in the external leaflet of the OM (32). Interestingly, some *P. aeruginosa* clinical isolates from patients with sepsis and bronchiectasis naturally contained lipid A possessing a single $C_{12:0}$ acyl chain (44, 45), supporting the ability of LPS transport machinery to utilize hypoacylated lipid A species. We did not observe by ESI-MS any lipid A species lacking both secondary lauroyl groups (Fig. 3 and Table 1). The absence may reflect the substrate specificity of the LPS inner membrane transport machinery, which serves a critical role in OM permeability barrier quality control through exacting lipid A substrate specificity that ensures only mature LPS is exported. A preference for lipid A with at least one secondary lauroyl group by MsbA of *P. aeruginosa* would prevent export of lipid A lacking both secondary acyl chains and the resulting membrane defects.

The Δ*fabY* strain was hypersusceptible to a number of antibiotics (Table 1), a phenotype consistent with lipid A hypoacylation. Of note, the MIC of vancomycin (molecular weight [MW] of 1,450 g/mol) was >32-fold lower in the *fabY* mutant, whereas the MIC values of the third-generation cephalosporins (MW 450 to 550 g/mol) decreased 16- to 32-fold (Table 1). Normally, polar, hydrophilic molecules do not passively diffuse across the OM but rather depend on the water-filled channels of OM porins (OMP) to traverse the bilayer (26). The *P. aeruginosa* OM is highly impermeable, in part due to the low rate of diffusion through nonspecific OMP channels, coupled with a large repertoire of narrow, substrate-specific OMP (35). As a result, passive diffusion through the bilayer can make a relevant contribution to total uptake rates (46) and shift the MIC values of modestly polar compounds such as cephalosporins and other β-lactams in mutants with compromised OM barriers (47, 48).

In comparison to *E. coli*, where the loss of secondary acylation increases vancomycin sensitivity by ~10-fold (38), the loss of a single secondary acylation in the *P. aeruginosa* Δ*fabY* strain decreased the MIC by >32-fold. In enterobacteria, vancomycin is too large to enter through the narrow OMP channels (38, 49). It is thought that vancomycin-sensitive mutants must elaborate a highly compromised OM that transiently ruptures and reseals, allowing vancomycin to cross the hydrophobic interior of the membrane (49). In contrast to *E. coli*, the major nonspecific OMP of *P. aeruginosa* (OprF) is theoretically large enough to accommodate vancomycin (MW exclusion limit of 3,000 versus 500 in *E. coli* [35]). A shift in the open OprF channel population or conformation induced by the hypoacylated lipid A monolayer, or increased expression, could conceivably increase uptake. Another factor that may contribute to the vancomycin susceptibility of the Δ*fabY* is the nature of the hypoacylated lipid A structures from *P. aeruginosa* (Fig. 3). In contrast to *E. coli*, loss of a secondary acyl group in *P. aeruginosa* generates a population of tetra-acylated lipid A species (Fig. 4). The abundant 2-hydroxy modification of secondary acyl groups is also specific to *P. aeruginosa* (29). This modification stabilizes the OM leaflet through increasing potential intermolecular hydrogen bonding capacity (26). The barrier

function of the lipid A monolayer in *P. aeruginosa* may therefore be less tolerant of losing secondary acylations and/or 2-hydroxy groups compared to *E. coli*.

The antibiotic hypersusceptibility phenotype stemming from deletion of *fabY* was not general to all antibiotics tested. Wild-type levels of susceptibility to cationic agents, including to the cyclic lipopeptide polymyxins (polymyxin B and colistin), aminoglycosides, and the membrane-disrupting bisbiguanide antiseptic chlorhexidine, was retained in the Δ*fabY* mutant (Table 1). Modification of the lipid A phosphates with covalently bound L-Ara4N reduces the net negative surface charge, imparting resistance to polymyxins and cationic peptides (50–52). A disconnect between lipid A L-Ara4N content and polymyxin resistance has been noted previously in *P. aeruginosa* (53) and LPS mutants (20), leading to the suggestion that OM permeability defects in some *P. aeruginosa* mutant strains may mask any resistance imparted by L-Ara4N-modification of the LPS (20).

We originally became interested in FAS initiation in *P. aeruginosa* as an antibiotic target based on the pleiotropic phenotype we observed upon deletion of *fabY* (8). In addition to compromised fitness, the production of a number of virulence factors was decreased, and the activity of a panel of antibiotics was enhanced. In theory, a *fabY* small-molecule inhibitor with marginal OM penetration could induce and then exploit this permeability defect, in effect promoting its own uptake via the hydrophobic pathway. However, exogenous fatty acids complemented the antibiotic hypersusceptibility phenotypes in a dose-dependent fashion (Fig. 5). The PA3286 shunt mechanism endows *P. aeruginosa* with a unique metabolic capacity among bacteria for FAS initiation using C8-CoA primer obtained directly from degradation of exogenous fatty acid (11, 54). Deuterated fatty acid labeling studies have shown that all cellular demands for FAS can be met via the PA3286 shunt provided exogenous fatty acids C8 and longer are present in the medium (11), including for acylation of lipid A and hence the reestablishment of wild-type antibiotic susceptibility. There is a rich supply of long-chain fatty acids in human serum (55), thus the PA3286 shunting mechanism could bypass the function of FabY *in vivo* and prevent lipid A from being hypoacylated.

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