Discovery of Bacterial Fatty Acid Synthase Type II Inhibitors Using a Novel Cellular Bioluminescent Reporter Assay

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Novel, cellular, gain-of-signal, bioluminescent reporter assays for fatty acid synthase type II (FASII) inhibitors were constructed in an efflux-deficient strain of *Pseudomonas aeruginosa* and based on the discovery that FASII genes in *P. aeruginosa* are coordinately upregulated in response to pathway disruption. A screen of 115,000 compounds identified a series of sulfonamidobenzamide (SABA) analogs, which generated strong luminescent signals in two FASII reporter strains but not in four control reporter strains designed to respond to inhibitors of pathways other than FASII. The SABA analogs selectively inhibited lipid biosynthesis in *P. aeruginosa* and exhibited minimal cytotoxicity to mammalian cells (50% cytotoxic concentration [CC₅₀] ≥ 80 μM). The most potent SABA analogs had MICs of 0.5 to 7.0 μM (0.2 to 3.0 μg/ml) against an efflux-deficient *Escherichia coli* (Δ*tolC*) strain but had no detectable MIC against efflux-proficient *E. coli* or against *P. aeruginosa* (efflux deficient or proficient). Genetic, molecular genetic, and biochemical studies revealed that SABA analogs target the enzyme (AccC) catalyzing the biotin carboxylase half-reaction of the acetyl coenzyme A (acetyl-CoA) carboxylase step in the initiation phase of FASII in *E. coli* and *P. aeruginosa*. These results validate the capability and the sensitivity of this novel bioluminescent reporter screen to identify inhibitors of *E. coli* and *P. aeruginosa* FASII.

Pseudomonas aeruginosa is a highly virulent, persistent human pathogen with both acquired and intrinsic drug resistances. It is the most common cause of nosocomial pneumonia, causing 15% to 20% of hospital-acquired pneumonias (1), and up to 75% of patients in intensive care units are colonized with this pathogen (2). *P. aeruginosa* is also becoming a major cause of community-acquired pneumonia in severely ill patients (3). An astounding 30% of clinical isolates from critically ill patients are resistant to three or more drugs, which leads to treatment failure (4). The discovery and development of new classes of antibiotics, which are not subject to existing target-based resistance mechanisms, is an important strategy in combating drug resistance, and targeting unexploited or underexploited essential bacterial pathways has been a successful strategy for discovering new compound classes (5, 6).

This study focused on the fatty acid synthase type II (FASII) pathway in *P. aeruginosa*. While most bacteria utilize exogenous fatty acids for phospholipid synthesis (7), the FASII pathway is absolutely essential for bacterial membrane biogenesis in Gram-negative bacteria because the hydroxyl-fatty acid constituents of LPS cannot be obtained from an extracellular source (8). FASII inhibitors also block the production of Gram-negative signaling molecules, including homoserine lactones and hydroxyquinolines, which are important to establish and maintain *P. aeruginosa* virulence (9). This FASII macromolecular synthesis pathway is conserved and essential in Gram-negative bacteria but is absent from the mammalian cytoplasm, which utilizes a distantly related type I FAS enzyme complex (10). While mammalian mitochondria do contain a FASII system (11), its relevance in the treatment of acute infections by bacterial FASII inhibitors is unclear (12, 13). Significantly, a FabI inhibitor is about to enter phase III studies in humans for *Staphylococcus aureus* infections (14), and isoniazid, a FASII inhibitor, is currently used clinically to treat tuberculosis (15), indicating that these inhibitors are highly selective for bacterial FASII or that inhibition of mitochondrial FASII is not toxic

during treatment of acute-phase infections. Clearly, the absence of significant cytotoxicity by a FASII inhibitor is an important criterion for further development of such a potential drug. Although there are no anti-pseudomonal agents in development or approved for clinical use that act on the FASII pathway, existing drugs targeting FASII in other bacterial species indicate the feasibility of targeting this pathway with antibiotics.

The high rate of failure in recent target-based biochemical screens for antibacterials (16, 17) prompted us to design novel cellular reporter screens with the potential to detect inhibitors of any rate-determining step in the entire FASII pathway. To construct such screening strains, we identified FASII promoters that were induced in response to decreased flux through the FAS pathway. These FASII depletion-responsive promoters were fused to the *Photobacterium luminescens* luxCDABE operon to provide a gain-of-signal bioluminescent response upon reduction in the pathway flow. The resulting cellular screens of this study proved to be more sensitive to FASII inhibition than are growth assays, and they select for inhibitors that can penetrate the *P.*

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TABLE 1 Strains and plasmids

Strain ^a or plasmid	Relevant genotype ^b	Reference
<i>P. aeruginosa</i> strains		
PAO1	Wild-type laboratory strain	20
PAO1-LAC	PAO1 with <i>lacI^q lacZ'</i> inserted at <i>attB</i> in chromosome via mini-CTX- <i>lacM15</i>	37
PAO397	PAO1 $\Delta(\text{mexAB-oprM}) \text{ nfxB } \Delta(\text{mexCD-oprJ}) \Delta(\text{mexJKL}) \Delta(\text{mexXY}) \Delta(\text{mexEF-oprN}) \text{ opmH}^{+}$	28
MDM1505	PAO397 pGSV3-Sp ^r - <i>Paer</i> -PA1365- <i>luxCDABE</i> (<i>secA</i> reporter)	19
MDM1519	PAO397 pGSV3-Sp ^r - <i>Paer</i> -PA0614- <i>luxCDABE</i> (<i>gyrA</i> reporter)	19
MDM1520	PAO397 pGSV3-Sp ^r - <i>Paer</i> -PA2030- <i>luxCDABE</i> (<i>coaD</i> reporter)	19
MDM1522	PAO397 pGSV3-Sp ^r - <i>Paer</i> -PA1494- <i>luxCDABE</i> (<i>glmU</i> reporter)	19
MDM1284	PAO1-LAC $\Delta\text{fabG}::\text{tet}/\text{pUCP24GW-lacPO-Paer-fabG}$	This study
MDM1325	PAO1-LAC $\Delta\text{accD}::\text{tet}/\text{pUCP24GW-lacI}^{\text{q}}\text{lacPO-Paer-accD}$	This study
MDM1460	PAO397 mini-Tn7-P-PA2968(<i>fabD</i>)- <i>luxCDABE</i>	This study
MDM1471	PAO397 mini-Tn7-P-PA3645(<i>fabZ</i>)- <i>luxCDABE</i>	This study
MDM1472	PAO397 mini-Tn7-P-PA0500(<i>bioB</i>)- <i>luxCDABE</i>	This study
MDM1473	PAO397 mini-Tn7-P-PA3112(<i>accD</i>)- <i>luxCDABE</i>	This study
MDM1480	PAO397 pGSV3-Sp ^r -P-PA4848(<i>accC</i>)- <i>luxCDABE</i>	This study
MDM1439	PAO1-LAC mini-Tn7-P-PA3645(<i>fabZ</i>)- <i>luxCDABE</i> $\Delta\text{accD}/\text{pUCP24GW-lacI}^{\text{q}}\text{lacPO-accD}$	This study
MDM1420	PAO1-LAC mini-Tn7-P-PA3645(<i>fabZ</i>)- <i>luxCDABE</i> /pUCP24GW- <i>lacI}^{\text{q}}\text{lacPO}</i> (empty vector)	This study
MDM1440	PAO1-LAC mini-Tn7-P-PA2968(<i>fabD</i>)- <i>luxCDABE</i> $\Delta\text{accD}/\text{pUCP24GW-lacI}^{\text{q}}\text{lacPO-accD}$	This study
MDM1421	PAO1-LAC mini-Tn7-P-PA2968(<i>fabD</i>)- <i>luxCDABE</i> /pUCP24- <i>lacI}^{\text{q}}\text{lacPO}</i> (empty vector)	This study
MDM1441	PAO1-LAC mini-Tn7-P-PA0500(<i>bioB</i>)- <i>luxCDABE</i> $\Delta\text{accD}/\text{pUCP24GW-lacI}^{\text{q}}\text{lacPO-accD}$	This study
MDM1422	PAO1-LAC mini-Tn7-P-PA0500(<i>bioB</i>)- <i>luxCDABE</i> /pUCP24- <i>lacI}^{\text{q}}\text{lacPO}</i> (empty vector)	This study
MDM2363	PAO397 mini-Tn7-P-PA2968(<i>fabD</i>)- <i>luxCDABE</i> $\Delta\text{accC}/\text{pUCP24GW-lacI}^{\text{q}}\text{lacPO-E. coli-accC(WT)}$	This study
MDM2365	PAO397 mini-Tn7-P-PA2968(<i>fabD</i>)- <i>luxCDABE</i> $\Delta\text{accC}/\text{pUCP24GW-lacI}^{\text{q}}\text{PO-E. coli-accC(F193V)}$	This study
MDM2402	PAO397 mini-Tn7-P-PA2968(<i>fabD</i>)- <i>luxCDABE</i> $\Delta\text{accC}/\text{pCUP24GW-lacI}^{\text{q}}\text{lacPO-Pae-accC(WT)}$	This study
<i>E. coli</i> strains		
K-12	Wild type	51
KLE701	K-12 <i>tolC::tet</i>	51
MDM2250	K-12 <i>tolC::tet accC(F193V)</i>	This study
MDM2251	K-12 <i>tolC::tet accC(L61Q)</i>	This study
MDM2252	K-12 <i>tolC::tet accC(L61R)</i>	This study
MDM2254	K-12 <i>tolC::tet accC(R167H)</i>	This study
MDM2298	K-12 <i>tolC::tet accC(F193V)</i> , derived by P1 transduction of the <i>accC</i> SABA-resistant allele from MDM2250 into <i>E. coli</i> K-12 <i>tolC::tet</i>	This study
MDM2295	MDM2298/pUCP24GW- <i>lacI}^{\text{q}}\text{lacPO-E. coli accC(WT)}</i>	This study
MDM2296	K-12 <i>tolC::tet/pUCP24GW-lacI}^{\text{q}}\text{lacPO-E. coli accC(F193V)}</i>	This study
MDM2430	K-12 <i>tolC::tet/pUCP24GW-lacI}^{\text{q}}\text{lacPO-E. coli accC(L61R)}</i>	This study
MDM2431	K-12 <i>tolC::tet/pUCP24GW-lacI}^{\text{q}}\text{lacPO-E. coli accC(R167H)}</i>	This study
Plasmids		
pEX18ApGW	pEX18Ap modified with Invitrogen Gateway vector conversion kit	52
pUCP24GW	pUCP24 modified with Invitrogen Gateway vector conversion kit	18
pUCP24GW- <i>lacI}^{\text{q}}</i>	pUCP24GW modified by addition of <i>lacI}^{\text{q}}</i> at BspHI site	18
mini-Tn7-GW-lux	Mini-Tn7-GW modified to accept DraIII-cut DNA fragments proximal to the <i>P. luminescens luxCDABE</i> operon	18
pGSV3-lux-Sp ^r	pGSV3-lux modified by replacement of Gm ^r with Sp ^r element at SacI sites	27

^a Commonly used *E. coli* hosts and cloning vectors are described and referenced in Materials and Methods.

^b Ap^r, Sp^r, Gm^r, and Tc^r indicate ampicillin, spectinomycin, gentamicin, and tetracycline resistance, respectively.

aeruginosa cell. In this study, we optimized and applied one of the FASII screens to over 100,000 diverse compounds and identified a novel series of compounds that generate highly significant luminescent responses in several FASII reporter strains but not in reporter strains designed to respond to inhibition of other targets (18, 19). To verify the ability of these new reporter screens to identify novel FASII inhibitors, we used molecular genetic tools and biochemical assays to identify the molecular target of the most potent hit compound series. These studies demonstrate that these screening hits target AccC, which carries out the biotin carboxylase half-reaction of the acetyl coenzyme A (acetyl-CoA) carboxylase step in the initiation phase of the FASII pathway.

MATERIALS AND METHODS

Strains, plasmids, and growth media. Bacterial strains and plasmids used for assays are described in Table 1. All *P. aeruginosa* strains were derivatives of PAO1 (20). *Escherichia coli* TOP10 (Invitrogen), *E. coli* DB3.1 (host strain for Gateway vectors from Invitrogen, Inc.), *E. coli* SM10 (21), and *E. coli* S17-1 (ATCC 47055) were used as hosts for molecular cloning. Vogel-Bonner minimal medium (VBMM) was prepared as described previously (21). Luria-Bertani (LB) medium (Lennox version; liquid and agar) was purchased from Difco. LB was supplemented with gentamicin (LBG) and/or isopropyl-β-D-thiogalactopyranoside (IPTG), as noted for each experiment.

PCR and primers. Synthetic oligonucleotide primers (Operon, Inc.) were designed using the published genome sequence for *P. aeruginosa*

(22) or *Escherichia coli* (23) and web-based PRIMER3 (Whitehead Institute) (see Table S1 in the supplemental material). Primers were used at 10 μ M in PCR amplifications with PhusionGC polymerase (New England BioLabs) for *P. aeruginosa* chromosomal DNA templates or with AccuPrime Taq DNA high-fidelity polymerase (Invitrogen, Inc.) with *E. coli* chromosomal DNA templates. Primers used for plasmid construction are noted below; those used for sequencing the *E. coli* *accA*, *accB*, *accC*, and *accD* genes following PCR amplification from sulfonamidobenzamide (SABA)-resistant mutants are shown as numbers 37 to 54 in Table S1 in the supplemental material.

Construction of *P. aeruginosa* complemented deletions. Plasmid constructs to generate knockouts of *accC*, *accD*, and *fabG* in *P. aeruginosa* were built in the vector pEX18ApGW by means of SOE (splicing by overlap extension) PCR and Gateway technology using primers 1 to 26 (see Table S1 in the supplemental material) as described previously (24). For deletion of the *accD* and *fabG* genes, the pEX18ApGW vectors carried a tetracycline resistance (*Tet*^r) element in place of the genes and about 1 kb of homology on both sides of the locus to be deleted. For the *accC* gene, the pEX18ApGW vector carried a markerless, in-frame deletion that was flanked by about 1 kb of homologous DNA on each side. These constructs were used successfully to create merodiploids in *P. aeruginosa* strains PAO-LAC and PAO397, which were then resolved in the presence of a complementing copy of the appropriate wild-type or mutant gene on the extrachromosomally replicating plasmid pUCP24GW to create *lac*-regulated complemented deletions as described previously (18). The pUCP24 vector carried the *lacI*^q gene for the *accC* and *accD* deletions but not for the *fabG* deletion, because complementation appeared to be inadequate to support growth in the presence of multiple copies of *lacI*^q. Complemented *fabG* deletions were made in *P. aeruginosa* PAO-LAC carrying a single integrated copy of *lacI*^q. Putative deletions were confirmed by PCR with flanking primers (primers out-F and out-R primers in Table S1 in the supplemental material) outside the regions cloned into pEX18ApGW.

Determination of mammalian-cell cytotoxicity. The 50% cytotoxic concentration (CC₅₀) of compounds for cultured mammalian cells (HeLa, ATCC CCL-2; American Type Culture Collection, Manassas, VA) was determined as the concentration of a compound that inhibits 50% of the conversion of MTS to formazan (25) using VP-SFM medium without serum, as previously described (26).

RNA preparation, labeling, and array hybridization. Cultures of the *lac*-regulated strains MDM1284 (PAO-LAC Δ *fabG*/pUCP24-*lacPO*-*fabG*) and MDM1325 (PAO-LAC Δ *accD*/pUCP24-*lacI*^q-*lacPO*-*accD*) were grown in VBMM with either limiting IPTG (0.06 mM and 0.02 mM, respectively) or excess IPTG (1 mM) for about 120 min after detectable effects on growth were observed in the limiting IPTG cultures. The cultures were treated with a 2:1 mixture of RNA Protect bacterial reagent (Qiagen, Inc.) according to the manufacturer's instructions. Total RNA was prepared from each culture using an RNeasy kit (Qiagen, Inc.). Experimental replicates were generated by duplicating the entire growth, harvesting, and RNA preparation for each strain. The duplicate RNA preparations were sent to NimbleGen Systems, Inc. (Madison, WI), for labeling and hybridization to microarrays representing the *P. aeruginosa* PAO1 genome (22).

Construction of bioluminescent reporter strains. The upstream regulatory regions of several target-responsive genes (i.e., *fabD*, *fabZ*, *bioB*, *accC*, and *accD*) were amplified by PCR (primers 27 to 36 in Table S1 in the supplemental material), cut with the restriction endonuclease DraIII or EcoRI (New England BioLabs, Inc.) as indicated by the primer name, gel purified, ligated to the *Photobacterium luminescens* *luxCDABE* operon in the mini-Tn7 (18) or pGSV3 (27) vector, and integrated into an efflux-competent *P. aeruginosa* PAO1 strain (20) or into its efflux-deficient derivative PAO397 (28).

Compound screening libraries and analogs. A high-throughput screen for FASII inhibitors (see below) was applied to 63,785 compounds in the Microbix, Inc., library and to 52,746 compounds in the collection at the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) at Har-

vard Medical School (Boston, MA). Analogs of the SABA scaffold were purchased from ChemBridge, Inc. (San Diego, CA). Liquid chromatography-mass spectrometry (LC-MS) analyses established that the analogs were $\geq$ 98% pure and of the correct molecular mass. Nuclear magnetic resonance (NMR) spectra were consistent with the assigned structures. Control compounds, including a benzoyl-amino-benzoate (BAB; compound 24c of reference 29) inhibitor of *Enterococcus faecalis* and *Streptococcus pyogenes* FabH, a pyridopyrimidine (PYP; compound 1 of reference 30) inhibitor of AccC, and a phenoxycetamide (PhAA; compound MBX 2359 of reference 31) inhibitor of the *P. aeruginosa* type III secretion system (T3SS), were synthesized as described previously (29–31); thiolactomycin and ciprofloxacin were purchased (Sigma).

High-throughput screen for FASII inhibitors. A cellular high-throughput screen (HTS) utilizing strain MDM1460 was carried out as follows. Overnight cultures of MDM1460 were inoculated from frozen stock into LB containing 10 μ g/ml gentamicin. In the morning, MDM1460 was subcultured at an optical density at 600 nm (OD₆₀₀) of 0.005 and grown to an OD₆₀₀ of $\sim$ 0.1. Then, 100 μ l of cells was added to each well of white, opaque 384-well plates containing 2 μ l of screening compound per well (50 μ M final concentration). The negative control was dimethyl sulfoxide (DMSO), and the positive control was the published acetyl-CoA carboxylase (ACC) inhibitor PYP (30) at 2 μ M. Plates were sealed with a gas-permeable seal and incubated at 30°C overnight. In the morning, relative luminescence unit (RLU) values were measured in an Envision Multilabel multiplate reader (PerkinElmer) at an elapsed time of 18 to 25 h. Optimization studies demonstrated that the cellular assay RLU values were sensitive to DMSO, but the Z' factor and signal/background ratio were suitable for HTS at 2% DMSO (see Fig. S1A in the supplemental material). A pilot screen of known bioactive compounds (Spectrum library; MicroSource Discovery Systems, Inc.) exhibited excellent screening parameters and identified a hit known to inhibit FASII (see Fig. S1B in the supplemental material). A Z score was calculated for each screened compound as the number of multiples of the DMSO negative-control standard deviation that the sample signal exhibited above the DMSO signal. A hit was defined as a compound that exhibited a Z score of $\geq$ 5.

MIC. MICs were determined using the broth microdilution method described in the CLSI (formerly NCCLS) guidelines (32) and are expressed in micromolar units to facilitate convenient comparisons with the activities of the compounds in other assays. The upper limit of concentrations evaluated was 100 μ M, since this is the aqueous solubility limit of the particular SABA analogs described here.

Selection for SABA-resistant mutants. Approximately 10⁹ cells from concentrated frozen stocks of *E. coli* *tolC::tet* in LB were spread on LB agar plates containing 12.5 μ g/ml tetracycline and 2.6 μ g/ml SABA-1 (about 8-fold MIC) and incubated overnight at 37°C. Colonies were picked and retested in MIC assays to identify clones which had resistance to SABA-1 and -2 but which exhibited unaltered sensitivity to three unrelated antibiotics. Genomic DNA was prepared from seven independent colonies with specific resistance to SABA-1 and -2. The *accA*, *accB*, *accC*, and *accD* genes were sequenced from all seven clones using the primers indicated in Table S1 in the supplemental material.

MMS evaluations. Macromolecular synthesis (MMS) assays for lipid, protein, RNA, and DNA pathway labeling were performed essentially as described previously (33). Briefly, bacterial cells were grown to an OD₆₀₀ of 0.5 before being split into 10-ml aliquots and incubated with a given inhibitor for 15 min. Then, either [¹⁴C]acetate (10 μ Ci) to determine fatty acid synthesis, a [³H]-labeled amino acid mix (10 μ Ci) to determine protein synthesis, [³H]uracil (10 μ Ci) for stable RNA synthesis, or [³H]thymidine (10 μ Ci) for DNA synthesis was added to the cultures for 30 min. The labeled cells were then collected via vacuum filtration, and the radioactivity was counted and normalized to the OD₆₀₀ of the culture. The amount of label incorporated by the untreated cell population was set at 100%. Averages and standard errors were obtained from triplicate samples derived from biological duplicate experiments.

***E. coli* ACC assay.** The radiochemical *E. coli* ACC assay used the reconstituted AccABCD multiprotein complex to determine the 50% inhibitory concentrations (IC₅₀s) for SABA analogs. The assay was modeled after the work of Alves et al. (34); however, instead of using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect malonyl-CoA, we monitored the rate of [¹⁴C]CO₂ fixation onto acetyl-CoA. Briefly, AccAD were coexpressed and purified as a tetramer. AccC was expressed and purified as a dimer. Apo-AccB was purified and converted to AccB using purified BirA to biotinylate the carrier protein. Complete biotinylation was checked by intact protein mass spectrometry. The calculated mass of AccB is 18,850, and our observed mass was 18,719; the difference is accounted for by the presence of a histidine tag and the removal of the N-terminal methionine in the cells. The observed mass of the holo-AccB was 18,945, consistent with the addition of a biotin group with a mass of 226. The three protein preparations were mixed together (AccAD-AccC-AccB; 0.125/0.125/0.25 μM) and preincubated for 20 min at room temperature. The assay mixture contained 0.5 mM ATP, 5 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, and 0.1 M Tris-HCl (pH 8.0) in a total volume of 50 μL. NaH¹⁴CO₂ (1 mM; 55 mCi/mmol) was added to start the reaction, and the mixture was incubated at 37°C for 5 min. The reaction was stopped by adding 12.5 μL of 6 N HCl, and the mixture was applied to a 2.3-cm Whatman filter disc and dried prior to scintillation counting. The assay was linear and reproducible, and unlike the spectrophotometric assays for the half-reactions, the fluorescent and UV absorbing properties of compounds did not interfere.

Array data accession number. The gene expression profiles based on the NimbleGen, Inc., array data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/), which is a public functional genomics data repository supporting MIAME-compliant data submissions, under accession number GSE66045.

RESULTS

FASII genes are coordinately upregulated when fatty acid synthesis is impeded. To detect FASII inhibitors that are active against *P. aeruginosa* cells, we undertook the construction of screening strains that would report inhibition of the FASII pathway as an increased luminescent signal. This strategy combined the simplicity and speed of a homogenous assay with the low cost of autoluminescence and the enhanced specificity of a gain-of-signal assay. We used expression profiling by microarray hybridization to identify genes that are induced in response to reduced FASII pathway flow. Our strategy was to fuse FASII depletion-responsive promoters to the *P. luminescens luxCDABE* operon and create reporter strains for screening. To simulate reduced FASII pathway flow, we constructed *P. aeruginosa* strains in which the expression of FASII genes could be reduced to levels that inhibit normal growth. Since most genes encoding the FASII pathway are essential (35), these strains were constructed by deleting the essential gene in the presence of a P_{lac}-regulated complementing copy carried on the replicating plasmid pUCP24GW (18, 19), enabling the expression of the FASII genes to be regulated by IPTG. Accordingly, we generated *P. aeruginosa* strains with regulated expression of *accD*, which encodes a subunit of the FASII initiation activity acetyl-CoA carboxylase, and *fabG*, which encodes β-ketoacyl-acyl carrier protein (ACP) reductase, the first reduction step in the FASII elongation cycle. As expected, both of these strains, MDM1284 (*fabG*) and MDM1325 (*accD*), failed to grow in the absence of IPTG. MDM1284 and MDM1325 were subjected to IPTG limitation during growth and harvested for RNA preparation when detectable effects on the growth rates were observed (~1 to 2 h later). The resulting RNA was used to measure changes in gene expression by microarray hybridization (see Materials and Methods).

To identify target-responsive genes, we searched the resulting normalized gene expression levels (GEO accession number GSE66045) for genes that are upregulated in response to downregulation of each FASII gene. A total of 147 genes were upregulated ≥4-fold (≥2-log₂ change) in response to downregulation of *accD* in the Δ*accD* strain (MDM1325). These responsive genes fell into a variety of functional categories based on clusters of orthologous groups (COGs) (36); however, 12.6% and 7.5% of the upregulated genes are involved in translation and lipid metabolism, respectively (Fig. 1A). A total of 23 genes exhibit an upregulated expression response of a ≥8-fold change (≥3 log₂), 13 of which encode proteins involved in lipid metabolism or biotin cofactor synthesis (Fig. 1A and B). Similarly, 28 genes were upregulated ≥4-fold (≥2-log₂ change) in the complemented Δ*fabG* strain in response to downregulation of *fabG*, including 10 of the 13 genes involved in lipid metabolism or biotin cofactor synthesis in the ≥3-log₂ set from the Δ*accD* strain. The three missing genes in the lipid metabolism COG category are *fabG* itself, which was downregulated in this engineered strain, *fabD*, whose expression may have been affected by polarity due to its linkage to *fabG*, and *fabB*, which was upregulated but only 2.6-fold (1.4-log₂ change). The lower levels of upregulation in general from the complemented Δ*fabG* strain were probably due to the accumulation of mutations in the single *lacI*^q copy integrated into the chromosome of the PAO-LAC strain (37) (see Materials and Methods). All but two of the 13 highly upregulated lipid metabolism and biotin cofactor genes identified in the Δ*accD* strain play known roles in the FASII pathway (Fig. 1B). The two exceptions are PA3644 (*lpxA*), which is likely upregulated because of its linkage in an apparent operon with *fabZ*, and PA3267, encoding a hypothetical protein of unknown function that clusters with several phospholipid/glycerol acyltransferases. Conversely, the only putative FASII genes that are not highly upregulated in the IPTG-limited growth of the Δ*accD* strain are *fabH1* (PA0999), *fabH2* (PA3333), and *fabF2* (PA1373), which have been shown previously to play no known role in FASII (38), and *fabI* (PA1806), whose encoded function is duplicated in *P. aeruginosa* by a triclosan-resistant enoyl-ACP reductase encoded by *fabV* (PA2950) (39). The *accD* gene was not highly upregulated because it was engineered in this strain; however, it exhibited a 5-fold (2.3-log₂ change) during IPTG-limited growth of the Δ*fabG* strain (see GSE66045).

FASII-sensitive promoter-*lux* fusions respond to downregulated FASII genes. To confirm the FASII depletion response of the genes identified in the expression profiling experiments, the upstream regulatory regions of several upregulated genes (*accC*, *accD*, *fabD*, *fabZ*, and *bioB*) were fused to the *P. luminescens luxCDABE* operon and transferred to the complemented Δ*accD* deletion strains by using either mini-Tn7 integrations (18) or single-crossover insertions with pGSV3 (27). Cells with regulated complemented deletions or wild-type cells carrying the same reporter constructs were grown overnight in various inducer (i.e., IPTG) concentrations and were then subcultured in the morning without inducer. The resulting cultures were examined for their luminescent response to downregulation of *accD*. For example, reduction in *accD* expression in the P_{*fabD*}-*lux* strain MDM1440 decreased the growth rate and induced luminescence (Fig. 2A). As expected, a wild-type strain carrying the same reporter construct, MDM1421, grew to a higher density than the IPTG-limited deletion strain but exhibited the lowest level of luminescence throughout its growth. The more rapid increase in RLU production by the

FIG 1 Summary of expression profiling of *P. aeruginosa* Δ accD cells complemented by *lac*-promoted *accD* in limited versus excess IPTG inducer levels. (A) Abundance of genes upregulated ≥ 4 -fold (gray bars) and ≥ 8 -fold (black bars) as a percentage of genes in each COG category. (B) Log₂ fold upregulation of FASII-related genes from the lipid metabolism COG category. Error bars represent standard deviations ($n = 5$).

complemented deletion versus the wild-type strain produced an increasingly larger separation between the luminescent responses of the two strains. The luminescence values (RLU) for three wild-type and complemented Δ accD strain pairs containing three different FASII gene promoter fusions to *luxCDABE* (i.e., *fabD*, *fabZ*, and *bioB*), which were normalized to cell density (OD₆₀₀), revealed a consistent luminescent response of cells to FASII pathway depletion by lowered IPTG at 270 min (Fig. 2B). These results were consistent with the expected responses of the FASII promoters to impedance of the FASII pathway, as determined by expression profiling.

FASII-sensitive promoter-*lux* fusions respond to FASII inhibitors. In preparation for high-throughput screening, we evaluated the responses of several reporter strains to known FASII inhibitors. Because most existing FASII inhibitors are subject to efflux by *P. aeruginosa* (35, 40), we integrated the P_{FASII} -*luxCDABE* reporter constructs into the genome of PAO397, a derivative of PAO1 in which five RND-family efflux pump genes have been deleted (28), to generate FASII reporter strains with increased sensitivity to FASII inhibitors. We investigated the sensitivity, kinetics, and specificity of the luminescent response of the resulting strains to treatment with the FabB inhibitor thiolactomycin (TLM) (41) and a pyridopyrimidine (PYP) AccC inhibitor (30). As controls, the reporter strains were treated with inhibitors of targets unrelated to *P. aeruginosa* FASII, including the gyrase inhibitor ciprofloxacin (CIP), a phenoxyacetamide (PhAA) inhibitor of *P. aeruginosa* type

III secretion (31), and a benzoylamino-benzoate (BAB) targeting *E. faecalis* and *S. pyogenes* FabH (29). The P_{fabD} -*lux* reporter strain MDM1460 produced highly significant luminescence in response to doses of TLM and PYP 20- to 30-fold below their MICs (Fig. 3A and Table 2) but did not respond to CIP, PhAA, or BAB.

The time course of the luminescent response was examined with TLM at a concentration that was 8-fold below its MIC, using two different FASII reporter strains, P_{fabD} -*lux* and P_{accC} -*lux*. The differences in the luminescent responses of the reporter cells to TLM versus the diluent DMSO at 270 min (Fig. 3B) were not as significant as those observed for reporter strains carrying wild-type and downregulated FASII genes (Fig. 2). However, cultures treated with TLM continued to produce luminescence after 21 h, and the luminescent responses were significantly elevated in the presence of the sub-MIC of TLM compared to the diluent DMSO. The reporter luminescent signal declined slightly from 21 h to 28 h but remained significantly selective for FASII inhibitor TLM throughout the observation period during the second day (>5 -fold for P_{fabD} -*lux*; >3 -fold for P_{accC} -*lux*), providing a durable response to TLM and a choice of time points for an endpoint reading in a screen.

Finally, to demonstrate that the reporter strain responses are highly selective for FASII inhibitors and promoters, we evaluated two FASII inhibitor-sensitive reporter strains and four reporter strains designed to respond to inhibitors of other targets (18, 19, 27) for sensitivity to five inhibitors of FASII and non-FASII targets

FIG 2 Luminescence reports from wild-type and regulated complemented $\Delta accD$ strains. (A) Kinetics of growth (OD_{600} [open symbols, dotted lines]) and luminescence (RLU [closed symbols, solid lines]) for the $\Delta accD/pUCP24-lac^q lacPO-accD$ strain MDM1440 and the wild-type PAO1 strain MDM1421, both carrying mini-Tn7- P_{fabD} -luxCDABE reporter constructs (circles, WT; triangles, $\Delta accD$ strain in 0.05 mM IPTG; diamonds, $\Delta accD$ strain in 0.025 mM IPTG). (B) Luminescence normalized to cell number (RLU/ OD_{600}) at 270 min of growth in LB for the complemented $\Delta accD$ strains (black) MDM1439, MDM1440, and MDM1441 in 0.025 mM IPTG and wild-type strains (gray) MDM1420, MDM1421, and MDM1422 carrying three different FASII depletion reporters as indicated. Error bars represent standard deviations ($n = 3$).

(Fig. 3C). The FASII inhibitor-sensitive reporter strains carrying P_{fabD} -lux and P_{accD} -lux responded to the FASII inhibitors TLM and PYP but not to CIP, PhAA, or the BAB inhibitor of FASII, found in Gram-positive strains. The response of the P_{PA2030} -lux reporter strain to incubation with BAB was unexpected and may indicate a degree of nonspecificity for BAB. Conversely, the non-FASII reporters did not respond to TLM or PYP, but the reporter for gyrase inhibitors (P_{PA0614} -lux) did respond to ciprofloxacin as described previously (18). In summary, the luminescent signals generated by the FASII reporter strains were selectively induced in response to FASII inhibitors that target the initiation stage (AccC) or the elongation cycle (FabB) of the FASII pathway. In addition, the reporters were sensitive to inhibitors at levels as low as 1/30 the MIC, confirming the potential utility of these reporter strains for high-throughput screening to identify inhibitors of the FASII pathway.

Implementation of a high-throughput cellular bioluminescent reporter screen for inhibitors of the FASII pathway. To develop a high-throughput screen (HTS) for FASII inhibitors, we selected the P_{fabD} -luxCDABE reporter strain MDM1460 because it exhibited the strongest and most reproducible luminescent response to the known FASII inhibitors TLM and PYP. The cellular screening parameters, including culture volume, cell density, compound concentration, endpoint time, and DMSO concentration tolerance, were adjusted to optimize the luminescent response in 384-well microplates, as measured by Z' factor (42),

using 2 μ M PYP and the diluent DMSO as positive and negative controls, respectively (see Materials and Methods). The optimized screen produced signal-to-background ratios (S/B) and Z' factors in each 384-well screening plate typical of those shown in Fig. S1 in the supplemental material (S/B = 8.3; Z' factor = 0.68). We applied the HTS to over 115,000 discrete small molecules, selected potential inhibitors with Z scores of ≥ 5 , confirmed them by re-assay in the primary screen, and validated them based on the following features: (i) specificity for FASII (i.e., highly significant Z scores of ≥ 5 versus P_{fabD} -lux and P_{accD} -lux reporter strains but much reduced and mostly insignificant Z scores versus non-FASII reporter strains); (ii) minimal cytotoxicity versus HeLa cells ($CC_{50} \geq 80 \mu$ M), as measured in serum-free medium to ensure that serum binding did not mask cytotoxicity; and (iii) selectivity for inhibition of lipid synthesis versus inhibition of DNA synthesis in preliminary macromolecular synthesis assays (see Materials and Methods). Five compounds representing three distinct chemical scaffolds met these stringent criteria (data not shown). Here, we focus on two of these compounds, which share a core sulfonamidobenzamide (SABA) scaffold, SABA-1 and SABA-2, together with the structurally related but inactive SABA-3 (Table 3 and Fig. 3A and C).

SABA compounds target the biotin carboxylase (AccC) of acetyl-CoA carboxylase. To determine the molecular target of the SABA analogs, we first evaluated them for inhibition of the growth of efflux-competent and -deficient strains of *P. aeruginosa* and *E.*

FIG 3 Sensitivity, kinetics, and specificity of luminescent response of reporter strains to known FASII inhibitors. (A) Concentration dependence of RLU response of the efflux-deficient FASII reporter strain MDM1460 (*P_{fabD}-lux*) at 20 h to two FASII inhibitors: a pyridopyrimidine AccC inhibitor (PYP) and a thiolactomycin FabB inhibitor (TLM). Also included are several non-FASII inhibitor negative controls: a phenoxyacetamide inhibitor of *P. aeruginosa* type III secretion (PhAA), the gyrase inhibitor ciprofloxacin (CIP), and a benzoyl aminobenzoate (BAB) inhibitor of *E. faecalis* and *S. pyogenes* FabH. Two active analogs of the SABA screening hit series, SABA-1 and SABA-2, and one inactive analog are shown. (B) RLU values versus time of incubation in the presence of sub-MICs of FabB inhibitor TLM (1.25 μM, closed symbols) or DMSO (open symbols) for efflux-deficient *P. aeruginosa* strains carrying *luxCDABE* driven by the *accC* (blue squares) (MDM1480) or *fabD* (red circles) (MDM1460) promoter. (C) Z score of RLU values after 20 h of incubation of efflux-deficient reporter strains in the presence of PYP (2 μM), TLM (1.25 μM), BAB (20 μM), a PhAA T3SS inhibitor (20 μM), and ciprofloxacin (CIP) (2 ng/ml) compared to DMSO. Promoter elements driving *luxCDABE* and their inhibitory response pathways are given in the key and were integrated into PAO397 to generate strains MDM1460, MDM1473, MDM1505, MDM1419, MDM1520, and MDM1522. Error bars represent standard deviations (*n* = 3).

coli (Table 2). The two active SABA analogs in the *P. aeruginosa* reporter assay, SABA-1 and SABA-2, failed to exhibit antibacterial activity against wild-type *E. coli* or *P. aeruginosa* at concentrations up to 100 μM (45 μg/ml). However, SABA-1 and SABA-2 did exhibit MICs in the range of 0.45 to 9 μM (0.2 to 0.4 μg/ml) and 3.5 to 7 μM (1.5 to 3.0 μg/ml), respectively, when tested against an efflux-deficient strain of *E. coli* (Δ tolC) (Table 2), indicating that efflux likely rescued *E. coli* cells from growth inhibition. Furthermore, the compounds reduced the growth rate (increased the exponential-phase doubling time) of the efflux-deficient *P. aeruginosa* strain PAO397, but not the wild-type strain PAO1, by 30 to 40% when added at 50 μM (24 μg/ml) (Table 3; also, see Fig. S2 in the supplemental material), and they exhibited statistically significant increases in RLU values in the corresponding efflux-deficient reporter strain (MDM1460) at compound concentrations as

low as 6 to 10 μM (3 to 5 μg/ml) (Fig. 3A). By comparison, the structurally related SABA-3 failed to generate a luminescent report in the screening strain and did not produce a significant effect in the growth or reporter assays (Fig. 3A and C and Table 2; also, see Fig. S2 in the supplemental material). These results demonstrated that SABA-1 and -2 are subject to efflux from *E. coli* and *P. aeruginosa* but are capable of inhibiting growth of *E. coli* and the growth rate of *P. aeruginosa* when efflux is reduced.

Next, we verified that SABA-1 and SABA-2 inhibit lipid synthesis selectively in the efflux-deficient *P. aeruginosa* strain PAO397 by examining their effect on cellular assays measuring the synthesis of several key macromolecules, including DNA, RNA, protein, and lipids. SABA-1 and SABA-2 inhibited acetate incorporation into lipids at least 2-4-fold more potently than they inhibited DNA, RNA, or protein synthesis, while SABA-3 did not

TABLE 2 MICs of FASII inhibitors for *E. coli* and *P. aeruginosa* wild-type, mutant, and engineered strains

Strain	Description	MIC (μM)					
		CIP	TLM	PYP	SABA-1	SABA-2	SABA-3
MDM2170	<i>E. coli</i>	0.06	300	40	>100	>100	>100
MDM2171	<i>E. coli</i> <i>tolC::tet</i>	0.03	30–60	0.5–1.0	0.45–0.9	3.5–7.0	>100
MDM2252	<i>E. coli</i> <i>tolC::tet accC</i> (L61R)	0.03	30–60	0.5–1.0	>100	>100	>100
MDM2254	<i>E. coli</i> <i>tolC::tet accC</i> (R167H)	0.03	30–60	0.5–1.0	>100	>100	>100
MDM2250	<i>E. coli</i> <i>tolC::tet accC</i> (F193V)	0.03	30–60	0.5–1.0	>100	>100	>100
MDM2295	<i>E. coli</i> <i>tolC::tet accC</i> (F193V)/pUCP24- <i>lacPO-E. coli accC</i> (WT)	0.03	30–60	0.5–1.0	25–50	25	>100
MDM2296	<i>E. coli</i> <i>tolC::tet/pUCP24-lacPO-E. coli accC</i> (F193V)	0.03	30–60	0.5–1.0	25	25	>100
MDM2430	<i>E. coli</i> <i>tolC::tet/pUCP24-lacPO-E. coli accC</i> (L61R)	0.03	30–60	0.5–1.0	25	25–50	>100
MDM2431	<i>E. coli</i> <i>tolC::tet/pUCP24-lacPO-E. coli accC</i> (R167H)	0.03	30–60	0.5–1.0	25	12–25	>100
MDM1233	<i>P. aeruginosa</i> PAO1	0.18	>100	>160	>100	>100	>100
MDM1370	<i>P. aeruginosa</i> PAO397	0.015	48	25–40	>100	>100	>100
MDM2263	PAO397 $\Delta accC$ /pUCP24- <i>lacPO-E. coli accC</i> (WT)	0.015	14–24	0.8	>100 ^a	>100 ^a	>100
MDM2265	PAO397 $\Delta accC$ /pUCP24- <i>lacPO-E. coli accC</i> (F193V)	0.015	14–24	0.8	>100	>100	>100
MDM2402	PAO397 $\Delta accC$ /pUCP24- <i>lacPO-P. aeruginosa accC</i> (WT)	0.015	14–24	25	>100 ^b	>100 ^b	>100

^a Significant reduction in extent of growth was observed at 25 μM (e.g., see Fig. 6C3), but it was not sufficient to result in a detectable MIC.
^b Significant reduction in extent of growth was observed at 50 μM (e.g., see Fig. 6B3), but it was not sufficient to result in a detectable MIC.

inhibit any of the pathways significantly (Fig. 4A). This reduction in fatty acid synthesis is consistent with the growth rate inhibition observed for these compounds but is insufficient to generate detectable MICs in efflux-deficient *P. aeruginosa*.
The antibacterial activity of SABA-1 and SABA-2 against the efflux-deficient strain of *E. coli* allowed a direct genetic approach to identifying the pathway target. Mutants resistant to SABA-1 at 8-fold its MIC were selected in the *E. coli* $\Delta tolC$ strain and arose at a frequency of about 10^{−9}. Seven independent mutants exhibited resistance to SABA-1 and SABA-2 but not to PYP, TLM, or ciprofloxacin (e.g., strains MDM2250, MDM2252, and MDM2254 in Table 2). Since SABA analogs failed to inhibit the elongation cycle of FASII in a reconstituted *E. coli* fatty acid synthase assay (43) (data not shown), we reasoned that they do not target any of the enzymes comprising the two-carbon addition cycle (e.g., FabH, FabD, FabB, FabG, FabI, and FabZ). Therefore, we focused on acetyl-CoA carboxylase (ACC), which catalyzes the initial step in FASII and is not measured in the elongation cycle assay. Accord-

ingly, we sequenced all four *acc* genes from all seven SABA-resistant mutants. Each of the strains contained a single codon-altering mutation only in *accC*. We found 5 distinct missense mutations that altered one of three amino acid codons: L61R, L61Q, R167G, R167H, and F193V. All three amino acid residues are conserved in *E. coli* and *P. aeruginosa* AccC (see Fig. S3 in the supplemental material), suggesting that the mutations may also affect *P. aeruginosa* susceptibility to SABA-1 and SABA-2. To verify the role of the mutations in *accC* in the resistance phenotype, we transferred the resistance from one of the original spontaneous resistant mutants [*accC*(F193V)] into the wild-type *E. coli* *tolC::tet* strain using P1 phage transduction and selected SABA-resistant transductants. DNA sequencing of SABA-resistant transductants revealed the presence of the F193V mutation in the *accC* locus, indicating that the single *accC* mutation is necessary and sufficient to confer resistance to SABA-1. Next, we expressed the WT, F193V, R167H, and L61R *accC* alleles in *E. coli* and *P. aeruginosa* using the expression vector pUCP24GW-*lacI^qlacPO* (19). In MIC assays, expres-

TABLE 3 Characterization of SABA screening hits and negative control in multiple assays

Structure	Compound	MW	Reporter screen response in HTS (Z score)	HeLa cell CC ₅₀ (μM)	Relative effect on lipid versus DNA synthesis in PAO397 (% of control ratio at 50 μM)	Prolongation of PAO397 doubling time at 50 μM (%)	<i>E. coli</i> ACC inhibition IC ₅₀ (μM)	<i>E. coli</i> $\Delta tolC::tet$ MIC (μM)
SABA-1	459	87	80	2.2	30	4.0	0.45–0.9
SABA-2	473	84	>100	3.5	39	12.5	3.5–7.0
SABA-3	441	0.8	>100	1.0	1	>50	>100

FIG 4 Identification of the SABA mechanism of action. (A) Effect of three SABA analogs at 50 μ M on the synthesis of cellular macromolecules in *P. aeruginosa* PAO397 as measured by incorporation of radiolabeled precursors (see Materials and Methods). (B) Effect of three SABA analogs on the activity of purified *E. coli* acetyl-CoA carboxylase (ACC).

sion of the plasmid-encoded copy of the *accC*(F193V), *accC*(R167H), and *accC*(L61R) alleles produced SABA resistance in *E. coli* Δ *tolC* carrying a wild-type chromosomal copy of the *accC* gene. In contrast, the plasmid-encoded wild-type *E. coli* *accC* allele had a minimal effect (≤ 2 -fold) on the MIC when added to the SABA-resistant mutant *E. coli* strains (Table 2; also, see Fig. S4 in the supplemental material). As expected, addition of the SABA-resistant *accC* allele failed to alter the MICs for ciprofloxacin or PYP against *E. coli* Δ *tolC* cells. Taken together, these results indicate that the SABA-resistant allele is dominant in *E. coli*.

The ability of the SABA compounds to inhibit *E. coli* acetyl-CoA carboxylase (ACC) activity was determined using an *in vitro* biochemical assay (34). Acetyl-CoA carboxylase is a biotin-dependent, multifunctional enzyme that catalyzes the first regulated step in fatty acid synthesis. The enzyme complex is composed of a homodimeric biotin carboxylase (AccC₂), biotinylated biotin carboxyl carrier protein (AccB, four copies), and an AccA₂AccD₂ heterotetrameric carboxyltransferase (44) and catalyzes two half-reactions to form malonyl-CoA. SABA-1 and SABA-2 inhibited ACC with IC₅₀s of 4 and 12.5 μ M, respectively (Fig. 4B). In contrast, SABA-3 failed to show any significant inhibition of *E. coli* ACC (Fig. 4B). Additional experiments will be required to deter-

mine the exact biochemical mechanism for SABA inhibition of ACC, but these results are consistent with the genetic locus of the resistance mutations and indicate that AccC is the target of SABA-1 and SABA-2.

The genetic experiments were expanded to confirm that the SABA compounds target AccC in *P. aeruginosa*. The wild-type *P. aeruginosa* *accC* gene was deleted from the FASII reporter strain MDM1460 in the presence of *lac*-regulated complementing copies of each of three different *accC* alleles—the *P. aeruginosa* wild-type allele and the *E. coli* wild-type and F193V resistant alleles. Growth of each of the three resulting *P. aeruginosa* strains with complemented deletions was directly proportional to the IPTG concentration, with little or no growth in the absence of IPTG, and the luminescent response was inversely proportional to the IPTG level except at very low IPTG levels, which inhibited growth very stringently (see Fig. S5 in the supplemental material). The presence of the *E. coli* *accC*(F193V) mutant allele, but not the wild-type *E. coli* *accC* allele, eliminated the bioluminescent response of *P. aeruginosa* PAO397 cells carrying the *P_{fabD}-lux* reporter to SABA-1 but not to TLM (Fig. 5). Furthermore, the final cell density of the parental *P. aeruginosa* strain PAO397 in stationary phase is decreased by thiolactomycin and ciprofloxacin, but not by SABA-1 (Fig. 6A1 to A3). In contrast, the final cell densities of *P. aeruginosa* *accC* deletion strains complemented by a *lac*-regulated wild-type *P. aeruginosa* *accC* allele or by a *lac*-regulated *E. coli* *accC* allele were reduced by SABA-1 in an IPTG-dependent manner (Fig. 6B1 to B3 and C1 to C3), and complementation by the *lac*-regulated *E. coli* resistant *accC*(F193V) allele significantly alleviated the growth sensitivity to SABA-1 (Fig. 6D1 to D3) with no effect on the sensitivity of cells to CIP and PYP. These results confirmed that specific mutations in the *accC* gene confer resistance to SABA compounds in *P. aeruginosa* as well as in *E. coli*.

DISCUSSION

This study describes a new cell-based reporter screen for inhibitors of the FASII pathway and its validation by the identification of a novel chemical series of inhibitors of ACC in the initiation stage of FASII. These new screening strains are based on the cellular transcriptional response to depletion of FASII. The observation that the genes induced strongly in response to FASII depletion in *P. aeruginosa* primarily encode FASII enzymes permits the construction of gain-of-signal reporter strains with promoters mechanistically linked to the targeted pathway. Application of the FASII cellular reporter screens in this study illustrates several important benefits of these types of screens, including the following: (i) simplicity and low cost of use, (ii) gain-of-signal output that reduces the frequency of false positives such as toxic compounds or luciferase inhibitors, (iii) sensitivity that is superior to that of cell growth-based outputs, (iv) the ability to detect inhibitors that cross the poorly permeable *P. aeruginosa* outer membrane, (v) the ability to detect inhibitors of several different steps in the pathway, and (vi) specificity for FASII. There are also limitations. For example, the high degree of sensitivity of the reporter screens to FASII inhibition is both beneficial and potentially problematic for screening. It enables the identification of weak inhibitors, providing an entry point for chemistry for further optimization as drugs. This sensitivity may prove useful in screening fragment libraries to identify small scaffolds but may preclude the identification of FASII inhibitors that are capable of killing cells or FASII inhibitors with cytotoxic off-target activities, since the reporter strain would

FIG 5 Effect of a SABA-resistant *accC* allele on the bioluminescent response of a *P. aeruginosa* FASII reporter strain. PAO397 reporter (P_{fabD} -*luxCDABE*) cells were engineered to carry the *E. coli* wild-type *accC* gene (A) or the *E. coli* SABA-resistant *accC*(F193V) allele (B) on the *lac*-regulated pUCP24GW-*lac*^q*lacPO* plasmid in place of the chromosomal copy of the *accC* gene and incubated with a concentration range of SABA-1, TLM, or diluent (DMSO) as shown. Luminescence (RLU) was measured after 20 h. Error bars represent standard deviations ($n = 3$).

be unable to produce a significant signal. In practice, such a scenario is unlikely, because few compounds in current small-molecule libraries are capable of arresting the growth of *P. aeruginosa*. This may be a more significant limitation for screens using efflux-deficient *P. aeruginosa*, as described in this report, but the efflux-deficient reporter strain improves the odds of detecting novel FASII inhibitors.

While the reporter screens described here respond to reduction in the flux through the FASII pathway, they may not detect inhibitors of each of the steps in the pathway for both genetic and biochemical reasons. First, there is redundancy of genes encoding enzymes for some pathway steps in *P. aeruginosa*. *P. aeruginosa* contains two AcpP proteins, two enoyl-ACP reductases (a triclosan-sensitive FabI and an insensitive FabV [39]), as well as multiple condensing enzymes (38). Three of the condensing enzymes appear to be critical in *P. aeruginosa*, controlling initiation condensation (FabY) and elongation condensation for saturated (FabF) and unsaturated (FabB) fatty acids (45, 46). Each of these three condensing enzymes is a potential drug target. The others are nonessential for *P. aeruginosa* growth in laboratory media and include a bypass condensation enzyme (FabH3) utilizing C_8 -CoA (47), an enzyme used for quinoline signal synthesis (FabH1; also called PqsD) not directly related to essential cell membrane synthesis (48), and two that are of unknown function and upregulated weakly in response to FASII inhibition (FabF2, FabH2). Second, some FASII steps appear to catalyze rapid equilibrium reactions, reducing the likelihood that inhibitors can be identified. These include the FabD and FabZ enzymes (49). Therefore, the most likely *P. aeruginosa* FASII steps to yield inhibitors are ACC, the elongation cycle-condensing enzymes FabB and FabF, the reductase FabG, and the initiation-condensing enzyme FabY (49).

The specificity of the screen is limited by the fact that other cellular stresses in addition to FASII depletion could lead to upregulation of the reporter. Because it is not feasible to examine every possible stressor, it is crucial to evaluate primary hits in secondary assays. In this case, we used additional luminescent reporter assays to rapidly identify compounds that affect only FASII-related reporters. Then, we applied another cellular assay, the relative inhibition of the incorporation of radiolabeled precursors

into lipids and DNA. Inhibitors that demonstrated selectivity for inhibition of lipid synthesis were evaluated further using the genetic approach of resistance mutation selection. The selection and mapping of mutations conferring SABA resistance in *E. coli* indicates that the molecular target of SABA analogs is the biotin carboxylase AccC. Furthermore, our observation that the addition of any of the resistant *accC* alleles in *E. coli* Δ *tolC* strains on an extrachromosomal plasmid results in SABA resistance indicates that *accC* is the only SABA target in the cell necessary for growth inhibition. While this approach is very powerful, it is not applicable if the compound does not exhibit an MIC. In such cases, biochemical assays will be required to identify the targeted protein. In this case, we used biochemical assays to provide support for AccC as the target of the SABA series, and the results are quite consistent with this hypothesis. Further research is under way to fully characterize the mechanism of SABA inhibition of ACC.

At least two other AccC inhibitors, PYP and a benzimidazole, have been described and cocrystallized with AccC (PDB accession numbers 2V58 [30] and 3JZF [50], respectively). Both inhibitors bind in or near the ATP binding site, and four codons that can be mutated to give rise to PYP resistance have been identified (30). These residues line the ATP binding site and are distinct from the three residues that are altered in the SABA-resistant mutants (see Fig. S3 in the supplemental material). The sites of SABA-resistant mutations are located on the other side of the protein and appear to surround a cleft that may provide access for substrates (see Fig. S6 in the supplemental material). These mutations do not confer resistance to PYP (Table 2; also, see Fig. S3 in the supplemental material). Thus, we hypothesize that the SABA inhibitors bind AccC at a site distinct from those for PYP and the benzimidazole and likely do not interfere with ATP binding. The novelty of the SABA scaffold and its potentially unique binding site on AccC suggest the potential clinical utility for this series if the antibacterial activity can be optimized. Clearly, chemical optimization must include efforts to reduce efflux from Gram-negative bacteria, and application of the cellular screen configured in an efflux-competent host will provide an extremely sensitive cellular luminescent assay to identify analogs capable of escaping efflux.

In conclusion, the characterization of two SABA primary

FIG 6 Effect of wild-type and SABA-resistant *accC* alleles on the growth of *P. aeruginosa* PAO397 in the presence of inhibitors. The extent of growth was measured at 24 h for PAO397 carrying the endogenous chromosomal copy of the wild-type *accC* gene (A) or for PAO397 engineered to carry the wild-type *P. aeruginosa* *accC* gene (MDM2402) (B), the *E. coli* wild-type *accC* allele (MDM2363) (C), or the *E. coli* SABA-resistant *accC*(F193V) allele (MDM2365) (D), all under *lac* regulation, on the pUCP24GW-*lacI*^q*lacPO* plasmid in place of the chromosomal copy of the *accC* gene. Cells were incubated overnight in the presence of a concentration range of TLM (series 1), ciprofloxacin (series 2), or SABA-1 (series 3) in the presence of the indicated IPTG concentration (see the key in panel A2), and growth was measured as OD₆₀₀.

screening hits demonstrates that the novel screens described here provide sensitive detection of new chemical entities targeting the FASII pathway. As cellular pathway screens, they offer the potential to identify new cell-permeant inhibitors of a variety of FASII pathway steps. By using an efflux competent host strain, the reporter may also allow detection of new classes of FASII inhibitors that can penetrate the *P. aeruginosa* wall and membrane and also evade efflux; at least, such reporter strains will provide a rapid screening tool for identification of analogs that are less subject to efflux pumps in *P. aeruginosa*.

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