A nutrient-limited screen unmasks rifabutin hyperactivity for extensively drug-resistant Acinetobacter baumannii

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Industry screens of large chemical libraries have traditionally relied on rich media to ensure rapid bacterial growth in high-throughput testing. We used eukaryotic, nutrient-limited growth media in a compound screen that unmasked a previously unknown hyperactivity of the old antibiotic, rifabutin (RBT), against highly resistant Acinetobacter baumannii. In nutrient-limited, but not rich, media, RBT was 200-fold more potent than rifampin. RBT was also substantially more effective in vivo. The mechanism of enhanced efficacy was a Trojan horse-like import of RBT, but not rifampin, through fhuE, only in nutrient-limited conditions. These results are of fundamental importance to efforts to discover antibacterial agents.

Discovery of new antibiotics has traditionally occurred via high-throughput screening assays. These assays use rich media, such as Mueller–Hinton II broth (MHII), to enable rapid microbial growth to facilitate the efficiency of the screens. Unfortunately, such methods rarely identify any new candidates6–9. However, we hypothesized that modifying the screening methodology by changing the medium to reflect the in vivo environment better could uncover new activity of drugs with previously unknown efficacy. To test this hypothesis, we designed a nutrient-limited screen using a medium that reflected the in vivo environment better.

We sought to identify new antibiotics active against Acinetobacter baumannii, one of the most drug-resistant bacterial pathogens, which is listed as the top priority of the unmet need for new antibiotics by the World Health Organization7. Blood or lung infections caused by extensively drug-resistant (XDR) A. baumannii causes >50% mortality rate8, and the antimicrobial pipeline for such infections is inadequate9–13.

The ReFRAME compound drug-repurposing library14 was screened against our well-characterized XDR, hypervirulent clinical isolate of A. baumannii HUMC115–19. As the growth medium for our screen, we selected Roswell Park Memorial Institute (RPMI) medium, a defined eukaryotic growth medium containing glucose (2 g l−1), as the only carbon source, and mineral salts. We supplemented the RPMI medium with serum for better simulation of the bloodstream environment10,21. Previous studies have identified the positive benefits of screening for antimicrobial compounds in culture conditions that reflect conditions found in vivo better12,23.

In total, we screened 11,862 small molecules at 20 μM (see Supplementary Dataset 1). The screen resulted in a hit rate of 0.52%; 62 putative hits were identified for follow-up confirmation in a serial dose–response screen. About half the compounds (32/62) were validated to inhibit 50% bacterial growth (IC50) at a concentration <20 μM. These compounds were also assessed for half-maximum mammalian cell cytotoxicity (CC50), and four compounds had a favourable specific antibacterial activity (IC50 < 20 μM) and high selectivity (CC50/IC50 > 10); of these rifabutin (RBT) was the most potent (that is, had the lowest IC50 values, Fig. 1a).

We confirmed the superiority of RBT IC50 values in RPMI medium, compared with a panel of eight rifamycins, using a more sensitive, 11-point dose–response curve (Fig. 1b). RBT was also confirmed to be the most potent and the least toxic rifamycin tested (Fig. 1c).

The minimum inhibitory concentration (MIC) of each drug was determined against A. baumannii HUMC1, a hypervirulent, carbapenem-resistant, XDR strain. In nutrient-limited RPMI medium, the MIC of RBT (0.0156 μg ml−1) was 200-fold lower compared with rifampin (RIF; 3.125 μg ml−1) (Fig. 1e). However, there was no difference in MIC when the bacteria were cultured in nutrient-rich MHII medium (MIC = 3.125 μg ml−1 for both RBT and RIF) (Fig. 1d). This was in contrast to A. baumannii, where we did not find a universal improvement of RBT potency against other bacterial species tested (Fig. 1d and see Extended Data Fig. 1).

We sought to understand the drivers responsible for the change in RBT potency in RPMI versus MHII medium. Modulating the growth rate (temperature), carbon metabolism (glucose supplementation) or activity of cellular efflux pumps (efflux pump inhibitors) did not affect the MIC shift in nutrient-rich versus nutrient-limited media. (Table 1). To distinguish whether the discrepant RBT MICs in RPMI versus MHII medium was due to inhibition of the drug’s activity by MHII medium or enhancement of its activity by RPMI medium, we mixed RPMI and MHII media in equal parts, and used this medium to test the MIC. The MIC of RBT was 8 μg ml−1 in the hybrid medium, similar to the rich medium and much higher than the nutrient-limited medium (Table 1). Thus, the rich MHII medium antagonized the hyperactivity of RBT.
seen in rich medium, enabling a greater number of surviving bacteria. We hypothesized that inhibition of RBT’s hyperactivity was related to a reduction in uptake of the antibiotic into the bacterial cell in rich, nutrient-limited medium (Table 1). We found that adding amino acids to nutrient-limited medium reduced RBT entry into the bacterial cell, to levels similar to those in rich medium, enabling a greater number of surviving bacteria and increased transcription (Fig. 2b–d).

As free, hydrophobic amino acids were mediating the antagonism of RBT activity in RPMI medium, we hypothesized that RBT, and not RIF, was entering the bacterial cell through amino acid transporters. We evaluated single-gene, transposon-disrupted mutants of known amino acid transporters, including apoP, trvP, hisM, mtr, HisP and hisJ, but we did not observe a difference in MICs (see Extended Data Fig. 3).

Therefore, to identify the gene(s) responsible for the hypersensitivity phenotype in the RPMI medium, we selected for HUMC1 spontaneous mutants that had lost their hypersensitivity phenotype to RBT. We passaged bacteria on RPMI agar plates supplemented with 1 µg ml⁻¹ of RBT, and then sequenced genomes of the resulting mutants (see Extended Data Fig. 4). Only fluE-disrupted mutants displayed a shift of the RBT MIC from 0.05 µg ml⁻¹ of the parent strain to 3.13 µg ml⁻¹ of the mutant (see Extended Data Fig. 4). A second, independent mutant selection experiment also identified resistant mutants with fluE mutations (see Extended Data Fig. 5). Defined fluE mutants were also generated to confirm the observed phenotype (see Extended Data Fig. 6).

FluE has been previously described as an iron-regulated, outer membrane protein transporter that is involved with iron acquisition in bacteria. We found that fluE expression was upregulated in low iron conditions (RPMI and iron-depleted MHI media), and expression was downregulated at high iron conditions (RPMI + iron citrate).
and MHII media) (Fig. 2e). The addition of hydrophobic amino acids did not alter \( fhuE \) expression. The lack of change of expression of \( fhuE \), but inhibition of uptake of RBT mediated by amino acids even in low iron conditions (in which \( fhuE \) is expressed), indicated that amino acids worked by competitive inhibition of RBT uptake via the expressed transporter, rather than by altering its expression. Thus RBT hyperactivity requires both low iron and low free amino acid conditions.

Of 43 \( A. baumannii \) clinical isolates tested in vitro, 65% had a hypersensitive phenotype in nutrient-limited media, whereas the remainder had MICs similar between nutrient-limited and rich media (that is, the RBT remained active, still exhibiting microbial killing at concentrations achievable in vivo, even for non-hypersusceptible strains). We sought to determine whether differences in the amino acid sequences of the FhuE protein in \( A. baumannii \) accounted for these susceptibility differences. Using a panel of \( A. baumannii \) clinical isolates from the Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) Antibiotic Resistance Isolate Bank, we created a phylogenetic clustering based on the amino acid sequence of the FhuE protein. The outer ring was represented predominantly by isolates that were hypersusceptible (MIC \(< 0.05 \mu g ml{ }^{-1} \)) to RBT in RPMI. The inner ring was made up of isolates that were found to have a higher MIC (\( > 3.13 \mu g ml{ }^{-1} \)) to RBT in RPMI medium (see Extended Data Fig. 7). Hence, FhuE amino acid sequences correlated with the hypersensitive phenotype.

To confirm that the expression of the \( fhuE \) isoform from HUMC1 (\( fhuE^{HUMC1} \)) was sufficient to reproduce the RBT hypersensitivity phenotype, we created a \( fhuE^{HUMC1} \) overexpression construct, under the control of an inducible promoter, in a strain disrupted of \( fhuE \) or its wild-type parent. We showed that overexpression of \( fhuE^{HUMC1} \) resulted in increased ATCC17978 sensitivity to RBT and could complement the \( \Delta fhuE \) mutant (Fig. 2f and see Extended Data Fig. 8). Overexpression of \( fhuE^{HUMC1} \) in LAC-4, a strain that encodes a different \( fhuE \) isoform that clusters with the non-hypersensitive group, also increased sensitivity to RBT (Fig. 2c). Thus, \( fhuE^{HUMC1} \) expression was sufficient to recapitulate the hypersensitivity phenotype.

The discrepancy between RBT activity against \( A. baumannii \) in nutrient-limited RPMI versus rich MHII medium led to a critical question: which medium was better at predicting the in vivo efficacy of RBT? In an immunocompromised neutropenic lung infection model, we observed a substantial reduction in c.f.u. in mice infected with \( A. baumannii \) UNT091-1 (wild type) and treated with RBT compared with RIF (Fig. 3a). Furthermore, the effect of RBT was blunted in mice infected with the UNT091-1::\( \Delta fhuE \) strain (Fig. 3a), supporting the role of \( fhuE \) in mediating RBT sensitivity both in vitro and in vivo.

We next tested the RBT efficacy in a normal, immunocompetent, \( A. baumannii \) pneumonia infection model. Mice were infected with \( A. baumannii \) HUMC1 and treated with \( 10 \) mg kg\(^{-1} \) d\(^{-1} \) of RIF or RBT. At 24 h, RBT-treated mice had a log\(_7\) reduction in the median number of c.f.u. in mice infected with \( A. baumannii \) UNT091-1 (wild type) and treated with RBT compared with RIF (Fig. 3b). RIF was partially effective versus placebo at that dose. (Fig. 3b). We then lowered the dose of both drugs to \( 5 \) mg kg\(^{-1} \) d\(^{-1} \) to determine whether RBT-enhanced potency would be unmasked. At the lower dose, RBT-treated mice had significantly reduced numbers of c.f.u. in the lung and markedly improved survival versus RIF (Fig. 3c). No difference was observed between the RIF and PBS control groups.

Next, we tested whether RBT was more effective than RIF in an immunocompetent intravenous model of infection. Mice were infected intravenously with HUMC1 and treated with \( 10 \) mg kg\(^{-1} \) d\(^{-1} \) of RIF or RBT. RBT-treated mice had significantly lower numbers of c.f.u. and improved survival (Fig. 3d). Last, we found that RBT was fully protective when dosed at 300× lower than an RIF dose that was minimally protective (Fig. 3f). Similarly, we also observed that RBT was protective in a \( G. mellonella \) infection model at significantly lower doses compared with RIF (see Extended Data Fig. 9a).

However, there was no difference in efficacy of RBT or RIF in either the mice or the \( G. mellonella \) infection models against \( A. baumannii \) LAC-4, a strain that has a distinct FhuE isoform and no difference in RBT/RIF MICs in nutrient-depleted medium (Fig. 3e and see Extended Data Fig. 9b,c).

RBT would probably be used clinically in combination with a second antibiotic, a practice that is standard in the use of the rifamycin antibiotics. We measured the spontaneous frequency of resistance emergence to RBT in vitro by culturing \( A. baumannii \) HUMC1 overnight in MHII or RPMI medium, and plating bacteria on agar plates supplemented with RBT alone, colistin (COL) alone or RBT+COL. We were unable to select for any

### Table 1 | MICs were performed against HUMC1 to determine which component of the MHII medium inhibits the activity of RBT

| Efflux pump inhibitor | MHII | RPMI |
|-----------------------|------|------|
| Carbonyl cyanide 3-chlorophenylhydrazone (2 µg ml\(^{-1} \)) | 1.56 | - |
| Thoridazine (8 µg ml\(^{-1} \)) | 1.56 | - |
| Verapamil (50 µg ml\(^{-1} \)) | 1.56 | - |

| Fractionated MHII spiked into RPMI medium | MHII | RPMI |
|-------------------------------------------|------|------|
| 10-30 kDa MHII fraction                  | 8.00 | 8.00 |
| <10 kDa MHII fraction                    | 8.00 | 8.00 |

| Acetonitrile treated <10 kDa MHII fraction | MHII | RPMI |
|-------------------------------------------|------|------|
| Aqueous layer                             | 0.03 | -    |
| Organic layer                             | 4.00 | -    |
| Organic layer, proteinase K digested      | 4.00 | -    |
| Organic layer, sodium periodate treated   | 4.00 | -    |

| Amino acid supplementation RPMI          | MHII | RPMI |
|------------------------------------------|------|------|
| Non-essential amino acid solution        | 0.78 | -    |
| Essential amino acid solution            | 1.56 | -    |
| L-Arginine (0.5 mM)                      | 0.05 | -    |
| Glutamic acid (0.5 mM)                   | 0.05 | -    |
| Glycine (0.5 mM)                         | 0.05 | -    |
| Leucine (1mM)                            | 0.05 | -    |
| L-Histidine (0.5 mM)                     | 1.56 | -    |
| L-Tryptophan (0.125 mM)                  | 1.56 | -    |

| Iron supplementation | MHII | RPMI |
|----------------------|------|------|
| + 400 µM iron citrate| 3.13 | 3.13 |

The MICs for CCCP and thiordazine alone are 8 and 64 µg ml\(^{-1} \), respectively.
Fig. 2 | The role of fhuE and sensitivity to RBT. The protein FhuE is predicted to be involved in iron transport. a, Iron was removed from MHII medium by the addition of transferrin or Chelex 100 to make the iron-depleted MHII formulation. The addition of 400 μM iron citrate to RPMI medium resulted in a larger MIC. All conditions (n = 3 independent replicates) were tested against A. baumannii HUMC1. Statistical comparisons were determined by one-way analysis of variance compared with the MHII group. P = 0.00134 (column 1 versus column 2), 0.000105 (column 1 versus column 5) and 0.000096 (column 1 versus column 7). b, An overnight culture of A. baumannii HUMC1 was subcultured in fresh RPMI medium, with or without supplemented amino acids (AAs). The intracellular concentration of RBT was measured by MS and normalized to 108 c.f.u. (drug treated: non-drug treated control). c, The number of viable cells was determined by plating serial dilutions on agar plates at 0, 1, 8, and 24 h and enumerating c.f.u. (n = 3 independent replicates). d, A GFP-expressing reporter strain of A. baumannii ATCC17978 was cultured in RPMI medium, with or without supplemented amino acids, and challenged with RBT. The addition of amino acids (open symbols) resulted in less inhibition of GFP fluorescence, which is consistent with the higher MICs observed in these same conditions. The data represent two experiments. e, Expression of fhuE (n = 3 independent replicates) was measured by quantitative PCR (normalized to the housekeeping gene rpoD) in various culture conditions. f, fhuE<sup>ATCC17978</sup> was then cloned from A. baumannii HUMC1 (hypersensitive strain) in pVRL2Z under the control of an arabinose-inducible promoter. MICs (n = 3 independent replicates) were done with and without arabinose induction in MHII medium. Median and interquartile ranges are plotted for b–f. TN-disrupted, transposon-disrupted.
spontaneous mutants in the double-selective plates (Fig. 4a,b). In addition, we found that RBT and COL interacted synergistically in vitro (see Extended Data Fig. 10). We determined the spontaneous frequency of resistance emergence to RBT in vivo by infecting mice, then harvesting blood and kidneys at 16.5 h post-infection, and finally plating samples on agar plates supplemented with RIF or PBS.
Emergence of resistant mutants

**Fig. 4 | RBT and COL combination therapy.** Selection of antibiotic-resistant mutants by high inoculum plating. **a, b,** A. baumannii HUMC1 (n = 3 independent replicates) was cultured in MHII medium (**a**) or RPMI medium (**b**) overnight, and mutants were selected by plating bacteria on drug plates containing 8 µg ml⁻¹ of RBT, 16 µg ml⁻¹ of COL or a combination of both antibiotics. The addition of COL can suppress the emergence of RBT resistance compared with the RBT alone group on RPMI agar (Kruskal–Wallis P = 0.046). Median and interquartile ranges are plotted. **c,** C3H mice (n = 10 per group) were infected intravenously with A. baumannii HUMC1 IV and treated with subtherapeutic doses of PBS, COL (0.005 mg kg⁻¹) alone, RBT (0.05 mg kg⁻¹) alone or the combination of COL + RBT. Mice were treated once daily for 3 d. Only the RBT + COL treatment group was significantly improved compared with the PBS control group (log(rank) *P = 0.02).

alone or RBT alone. The frequency of spontaneous RBT resistance emergence was <1.7 × 10⁻³ and <3.7 × 10⁻⁴ c.f.u. in vivo (Table 2) in the blood and kidneys, respectively. These values were about 10-fold less compared with the frequency of spontaneous RIF resistance emergence, which was 8.4 × 10⁻² and 1.2 × 10⁻² c.f.u. in the blood and kidneys, respectively. Furthermore, we found that the treatment combination RBT + COL was synergistic in vitro, even with the less sensitive ΔfluE mutant (see Extended Data Fig. 10), and that the combination of RBT + COL was more effective in vivo (Fig. 4c).

Thus, these collective experiments confirmed that RBT has an excellent activity in vivo and is more potent when compared with Rif, and that, in vitro, MIC results in nutrient-limited medium predicted in vivo efficacy better than in vitro MIC results in rich medium. The superiority of RBT over Rif is highly significant due to a recent, randomized, controlled clinical trial that found a non-significant trend to improved clinical cure, with a significant improvement in microbiological eradication when adding Rif to COL for treatment of XDR A. baumannii infections²⁸. Given that Rif had some effect clinically, and that RBT appears to be far more potent and effective in preclinical models, we believe that the addition of adjunct RBT, compared with Rif, could serve as a critically needed therapeutic option for patients with infections caused by XDR A. baumannii, potentially resulting in superior survival compared with the current standard of care. Thus, studies with RBT are under way to evaluate the clinical utility of this promising drug for the treatment of A. baumannii infections for which there are currently limited treatment options.

Finally, high-throughput screens of small molecules have been standard practice for the pharmaceutical industry for decades. These libraries, which may contain millions of compounds, have been screened many times; however, new screens of the same library rarely identify novel compounds²⁹⁻³¹. Our results demonstrate that, using more physiologically relevant, nutrient-limited media has the potential to identify many additional treatments for antibiotic-resistant bacterial infections that have been missed to date due to screens of these large libraries based on rich culture media²³⁻³⁵. Thus, repeat screening of these large libraries in nutrient-limited media may offer a promising, critically needed approach to identifying antimicrobial agents with which to combat the crisis of antibiotic-resistant infections.

**Methods**

**Ethics statement.** All animal work was conducted following approval by the Institutional Animal Care and Use Committee (protocol no. 20882) at the University of Southern California, in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Infected mice develop weight loss, ruffled fur, poor appetite, decreased ambulation, huddling behaviour and low body temperature. Mice that display huddling behaviour and are poorly mobile will be weighed once daily. Weight loss of >15% body weight will trigger euthanasia. Mice were monitored at least twice daily for 7 d. Soft bedding and other enrichment devices were provided as recommended by the veterinary staff. Nutritional supplements, such as hydrogel packs, were provided as needed.

**Source of bacterial isolates.** The complete list of bacterial isolates tested is listed in Supplementary Dataset 1. Strains were obtained from the CDC Antimicrobial Resistance Isolate Bank, the She lab, the Spellberg lab and BioVersys.

**Bacterial culture.** Working solutions of bacteria were prepared using frozen stocks of A. baumannii strains as previously published, or by inoculating a fresh overnight culture in tryptic soy broth (TSB) and incubating at 37°C and 200g. The overnight culture was diluted 1:100 and then subcultured in MHII medium at 37°C and 200g until the culture reached an absorbance at 600 nm (OD₆₀₀) of 0.5. For some assays, bacteria were also subcultured in RPMI 1640 medium (Gibco, catalogue no. 11875119).

**High-throughput compound screening.** Compounds from the 10 mM ReFRAME library stock²⁶ were acoustically transferred using the Echo 555 Liquid Handler (Labcyte Inc.) at 20µM final assay concentration into 1,536-well plates. Log(phase growth) A. baumannii HUMC1 was diluted in assay medium (MHII or RPMI medium with and without 50% normal mouse serum) and dispensed into assay plates using the MultiFlo FX Multi-Mode Dispenser (BioTek). Bacterial viability was assessed 24 h later using the BacTiter-Glo Microbial Cell Viability Assay (Promega) and the PHERAstar microplate reader (BMG Labtech). Positive
controls included 10 μM COL and doxycycline. The assay was normalized to neutral and positive controls, and putative hits were selected based on a 50% reduction in viability. Putative hits were re-tested in single-point triplicate and on reconfirmation were further tested in an 8-point, 1:3-dose–response, and counter-screened against mammalian HepG2 and HEK293T cell lines using a 72-h CellTiter-Glo Luminescent Cell Viability Assay (Promega). For the mammalian cytotoxicity counter screen, 40 μM puroycin (Sigma) was used as the positive control and data were normalized as for the primary assay. All data were uploaded to and analysed in Genedra Screen v.13.0.1-standard. replicate data were analysed using median condensing. Dose–response curves were fitted with the four-parameter Hill equation.

Antibiotic preparation. RIF (Sigma, catalogue no. R3501-1G) and RBT (Sigma, catalogue no. R3330-25MG) were dissolved in dimethyl sulfoxide (DMSO). The working solution of antibiotic was prepared at twice the desired final drug concentration of the starting well. The antibiotic working solution dilutions were prepared in the respective media used for the MIC—MHII or RPMI medium.

MIC protocol. Unless otherwise indicated, the standard broth microdilution method was used to determine MICs. The medium used for the MIC assays performed in the present study was either MHII or RPMI 1640 medium. Briefly, 100 μl of medium, RPMI or MHII, was added to the wells in columns 2–10. Column 11 served as a positive growth control and contained only bacteria and medium. Column 12 served as the negative control and contained only culture medium without bacteria. Next, 200 μl of a 2× RBT or RIF working solution was added to the wells in column 1. Twofold serial dilutions of the antibiotic were performed through column 10. Next, 100 μl of a 1×10^6 c.f.u. ml^−1 working solution of bacteria was added to each of the wells in columns 1–11. The inoculum concentration was confirmed by plating serial dilutions on TSA plates. MIC plates were incubated at 35 ± 2 °C without shaking and the results were recorded at 24 h. To test the effect of the individual components of the MHII medium, MHII fractions (size separation, acetonitrile extracted, proteinase K digested or sodium periodate oxidized), 10 μl of the purified fraction was added to the appropriate wells.

As indicated, amino acids were used to supplement the medium for MIC testing. Mixed amino acids were prepared by adding 10 μl of Gibco MEM Amino Acids Solution (Thermo Fisher Scientific, catalogue no. 11130051) and Gibco MEM Non-Essential Amino Acids (Thermo Fisher Scientific, catalogue no. 11140050) to each well in the MIC assay. In addition, the effect of individual amino acids on the MIC was tested by the addition of purified amino acids at the same concentration contained in the Gibco mixed amino acid solution listed above. Amino acids were prepared as fresh and filter-sterilized solutions before use. For some MICS, efflux pump inhibitors were added to the medium. Efflux pump inhibitor MICS were performed to identify subinhibitory concentrations of the efflux pump inhibitors. The final concentration of inhibitors used were as follows: verapamil (50 μmML^−1), thioridazine (8 μmML^−1) and CCCP (2 μmML^−1).

For MICS involving the fluoroquinolones overexpression strains, the respective stains were cultured overnight and subcultured in low-salt Luria broth (LB) supplemented with 250 μmML^−1 of zeocin at 37 °C and 200 μg to maintain the overexpression plasmid. MICS assays were done in MHII or RPMI medium supplemented with 20 μgML^−1 of zeocin to maintain the plasmid. Expression of fluoroquinolones was induced by the addition of arabinose at the final concentrations of 8%, 4%, 1% or no arabinose as a control.

Fractional inhibitory concentration index. Drug–drug interaction between RBT or RIF and COL in MHII medium. The drug–drug interactions were evaluated by calculating the fractional inhibitory concentration index (FICI). FICI = (MIC (drug A) + MIC (drug B))/MIC (drug A) + MIC (drug B), in which C_A and C_B are drug concentrations of drug A and drug B in combination, and MIC_A and MIC_B are the MICs of drug A and drug B alone. Synergy was defined as FICI ≤ 0.5; no interaction was defined as FICI = 0.5–4.0 and antagonism was defined as FICI > 4.0.

Fractionation of MHII medium. Size fractionation. MHII medium (10X) was used for the fractionation to maximize the concentration of the MHII components. The medium was filtered through a 0.22-μm filter and then the medium was run through centrifugal filtration columns with 10- and 30-kDa molecular mass cut-offs at 12,000 g for 20 min. The >30-kDa fraction was collected and reserved for experimentation. The flow through was collected and transferred to a column with a 10-kDa molecular mass cut-off. The centrifugation step was repeated as previously stated. The <10-kDa flow through was collected from this column. The fraction that was 10 < x < 30 kDa was collected as well.

Acetonitrile extraction. The organic and inorganic layers were separated with a liquid–liquid extraction by mixing the purified MHII fraction 1:1 with 100% acetonitrile. The sample was vortexed thoroughly and centrifuged at maximum speed for 10 min. The aqueous and organic layers were transferred to clean microcentrifuge tubes. To ensure the removal of any residual acetonitrile, the extracted MHII sample was dried using a SpeedVac and then resuspended in the original volume using sterile molecular-grade H2O.

Proteinase K digestion. Proteinase K (30 μl) (Invitrogen, catalogue no. 46-7603) was added to 1 ml of the MHII fraction <10kDa (organic extract or non-extracted as a control). The medium was incubated at 65 °C for 1 h. To inactivate the proteinase K, the sample was then incubated at 80 °C for 15 min.

Sodium periodate oxidation. Sodium periodate oxidation was performed as previously described. Briefly, to oxidize the carbohydrates in the organic layer of the medium, sodium periodate was added to the <10 fraction at a final concentration of 10 mM. The sample was incubated at room temperature for 30 min. After incubation, the sodium periodate was quenched using 0.1 ml of 50% glycerol for every 1 ml of reaction. The sample was incubated at room temperature for 1 h before downstream application.

Time-kill assays. Time-kill assays were performed in a 96-well, U-bottomed plate using the same plate set-up as for the MIC assay. We measured viable cell counts at 1, 8 and 24 h. At each time point, the contents of an individual well were collected and c.f.u. were determined by plating serial dilutions on TSA plates and incubating overnight at 37 °C. As a control, the remaining wells of the 96-well plate were returned to the incubator after each sample collection, and an MIC was determined as described in MIC protocol.

Mass spectrophotometry. Extractions and measurements were done as previously described. A log(Phase) A. baumannii culture was incubated in 0.79 or 0.38 μmML^−1 of RBT in the presence or absence of an amino acid mixture at 37 °C. Bacteria were harvested at 0, 1, 8 and 24 h and c.f.u. were determined by plating serial dilutions on agar medium. The cell-free supernatant was collected by filtration through a 0.22-μm filter. RBT were extracted by adding LC–MS-grade acetonitrile:methanol:water (40:40:20) solution that was precooled to −40 °C. LC–MS differentiation and detection of RBT were performed using a Cogent Diamond Hydride Type C column (Microsolve Technologies) with an Agilent 1200 Liquid Chromatography system as published previously. An isotropic pump was used for continuous infusion of a reference mass solution to allow mass axis calibration. A detected ion was validated based on unique, accurate, mass-retention time identifiers.

Selection of spontaneous RBT-resistant mutants and whole-genome sequencing. The RBT-hypersensitive strain HUMC1 was cultured overnight in 50 ml RPMI medium. Bacteria were plated on RPMI agar plates supplemented with 1 μmML^−1 of RBT. Individual colonies were selected and then counter-screened in RPMI agar plates supplemented with 25 μmML^−1 of RBT.

Colonies that grew on the RPMI agar plates supplemented with 1 μmML^−1, but not 25 μmML^−1, of RBT were processed for whole-genome sequencing. Sequencing libraries for the selected mutants and parent strain were prepared at the USC Molecular Genomics Core. Libraries were simultaneously prepared from extracted genomic DNA using the Illumina Nextera XT library prep kit according to the manufacturer’s protocol (Illumina, catalogue no. FC-131-1024).
Prepared libraries were sequenced on the Illumina MiSeq v2 at 2× 150 cycles. Assembled reads were aligned to the previously published A. baumannii HUMC1 sequence (National Center for Biotechnology Information (NCBI) reference sequence NZ_LQKO10000007.1). The FASTQ files were uploaded to Partek Flow software, v.6.0 (Partek, Inc.) on a Linux-based high-performance computing system at Pennsylvania State University College of Medicine, adaptor trimmed and remapped to the HUMC1 reference sequence (NZ_LQKO10000007.1) using the alignment algorithm (BWA-MEM) with a few modifications (mismatch penalty 4, gap open penalty 6, clipping penalty 5 and alignment score cut-off 30) for short read mapping. After alignment, annotated variants were saved.

FlhE phylogenetic tree. The maximum-likelihood phylogenetic tree of 43 FlhE amino acid sequences were constructed using the CLC Genomics Workbench 12 (Qagen) software. Nucleotide sequences were generated by assembling sequence read archive reads of the 41 clinical isolates from the CDC and FDA Antimicrobial Resistance Isolate Bank and aligning to the HUMC1 and the LAC-4 flhE gene. Numbers at the base of the nodes correspond to bootstrap (10,000) probability >90%. The scale indicates the number of amino acid substitutions per site.

Overexpression construct of flhE<sup>ΔfhuE</sup>. The ORF of the flhE gene was cloned into the pVRL2Z plasmid under the control of the arabinose-inducible promoter. HUMC1 genomic DNA and pVRL2Z plasmid DNA were extracted using the Quick-DNA Fungal/Bacterial Kits (Zymo, catalogue no. D6065) and the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, catalogue no. K0502), respectively. The flhE gene from pVRL2Z PCR using oligonucleotide primers (5′-taagcAGATGATCCTGAGGAGTGGTCTCAGTTC-3′/5′-taagcGCGGCGCCCTGCTTCTGACCTTGGCCTG-3′) and purified using PureLink PCR Purification Kit (Thermo Fisher Scientific, catalogue no. K310001). The Q5 High-Fidelity 2x Master Mix (New England Biolabs, catalogue no. M0492S) was used for all cloning-related PCRs. The vector and insert were both digested with NdeI/EcoRI (New England Biolabs, catalogue nos. R0189S and R01015), digested products were purified and the vector/insert was ligated using T4 ligase (New England Biolabs, catalogue no. M0202S).

Transformation of A. baumannii AB5674, ATCC17978 and LAC-4 was performed by electroporation using freshly prepared competent cells. Transformants were selected on low-salt LB agar supplemented with 250 µg ml<sup>−1</sup> of zeocin. Transformants were confirmed by reisolating the plasmid by miniprep, digestion of the plasmid DNA using NotI/EcoRI and visualization of the digested products on an agarose gel.

Electrocomptent cells were prepared by subculturing bacteria to log (phase growth) in TSB at 37 °C and 200 r.p.m. for 5 min at 4 °C. Bacteria were washed 5× by pipetting up and down. Cells were collected by centrifugation at 16,000 g for 10 min at 4 °C and the cell pellet was washed in 1 ml ice-cold 10% glycerol. 1 mM isopropyl-β-D-1-thiogalactopyranoside and 200 µg ml<sup>−1</sup> of 3′-azido-3′-deoxythymidine to select for plasmid removal from the genome. Scarless deletion of flhE was screened with primers 5′-TACCTTACCCCTTGGCCTGAC-3′/5′-AAAGATACGACCAACCGCGC-3′ and confirmed by DNA sequencing (Microsynth AG).

GFP expression. Transformation of A. baumannii ATCC17978 was performed by electroporation using freshly prepared competent cells. Transformants were selected on TSA agar supplemented with 10 µg ml<sup>−1</sup> of gentamicin (Sigma, catalogue no. G12727). Transformants were confirmed by green fluorescent protein (GFP) expression using a fluorescent plate reader.

Transformants were then subcultured in RPMI medium supplemented with 10 µg ml<sup>−1</sup> of gentamicin and 4% arabinose. Bacteria (1×10<sup>6</sup> c.f.u.) and the appropriate concentration of RBT were added to RPMI medium supplemented with 10 µg ml<sup>−1</sup> of gentamicin and 4% arabinose, up to the total volume of 200 µl. Amino acids (10 µl) were added in appropriate samples as well. GFP expression was measured using flow cytometry analysis every 30 min.

G. mellonella infection model. G. mellonella larvae (10 per group) were infected with log (phase growth) (OD<sub>600</sub> = 0.5) bacteria redistributed in phosphate-buffered saline (PBS), and larval size was recorded to ensure a right-angled second proleg. The infected larvae were subsequently treated with RBT or RIF 1 h after infection with the 10-µl injection of the specified antibiotic dose in the left second proleg. The injected larvae were incubated at 37 °C in 90 mm plastic Petri dishes and monitored for survival for 72 h. Larvae were considered dead when they did not move in response to stimulus with a pipette tip.

In vitro mutant selection. Spontaneous RBT- and COL-resistant mutants were selected by high inoculum plating on selective agar plates supplemented with 8 µg ml<sup>−1</sup> of RBT, 16 µg ml<sup>−1</sup> of COL or a combination of both antibiotics. Overexpression of flhE gene from HUMC1 were grown in 20 ml M9 or RPMI medium at 37 °C and 200 g. For bacteria cultured in MHI medium, 100 µl of culture was plated directly on selective plates to enumerate the antibiotic-resistant population, and serial dilutions were plated on non-selective plates to enumerate the total population. As the bacteria grow to a lower density in RPMI medium, the bacteria were first concentrated by 10% centrifugation before plating on non-selective and selective agar plates as described above. All experiments were done in triplicate.

Mouse studies. Mice were randomly assigned to treatment groups, which were not blinded.

Intravenous infection. A. baumannii HUMC1 frozen stock was prepared as described previously<sup>18</sup>. Frozen stocks of HUMC1 were thawed and diluted in PBS to adjust the bacterial density as needed for infection. Male C3HeB/FeJ mice, aged 8–12 weeks, were infected with 2×10<sup>6</sup> c.f.u. via tail vein injection, and the inoculum bacterial density was confirmed by plating serial dilutions on TSA plates and incubating overnight at 37 °C.

Oral aspiration infection. Mice were infected as previously described<sup>18</sup>. Single colonies of A. baumannii HUMC1 grown on TSA were used to inoculate TSB and bacteria were cultured overnight at 37 °C and 200g. The next day, the bacteria were subcultured by diluting overnight to 1:100 in fresh TSB and cultured for 4 h at 37 °C and 200g. The subculture was washed with PBS three times and adjusted to an OD<sub>600</sub> of 0.5. The inoculum was concentrated to 2×10<sup>8</sup> c.f.u. ml<sup>−1</sup> and a 9- to 10-week-old male C3HeB/FeJ male mouse was infected with 50 µl (1×10<sup>6</sup> c.f.u.) of inoculum via oral aspiration. The inoculum c.f.u. were confirmed by plating on TSA plates and incubating overnight at 37 °C.

Neutropenic pneumonia infection. Female CD-1 mice were made neutropenic by the administration of 150 and 100 mg kg<sup>−1</sup> of cyclophosphamide, intraperitoneally, at 4 and 1 day before infection. Frozen stock of A. baumannii Bioversys UNT091-1 and UNT091-1::flhE was plated on TSA and incubated overnight at 37 °C. The plate growth was concentrated into 10<sup>5</sup> c.f.u. ml<sup>−1</sup> (OD 600 nm), and then 10-fold serial diluted in fresh TSB; this log<sub>10</sub> (dilution) was used as the infecting inoculum. The infecting inoculum was approximately 6–7 log<sub>10</sub> (c.f.u.) per animal. In addition, the OD-adjusted plate suspension was 10-fold serially diluted and spot plated on to brain–heart infusion agar/charcoal plates to determine the input of c.f.u.

Mice were anesthetized by intraperitoneal injection of 0.2 ml of a ketamine-HCl (40 mg per kg birth weight) + xylazine (6 mg per kg birth weight) mixture. Anaesthetized mice were intranasally inoculated with 0.05 ml of the designated inoculum. For intranasal inoculation, drops were placed on to the external nares and then we waited for inhalation. After inoculation, each mouse was placed back into its cage with heat pads for recovery. Plate counts were performed to confirm the exact c.f.u. of input for each strain (targeting 6.5 log<sub>10</sub> (c.f.u.) per mouse).

Mice were euthanized by CO<sub>2</sub> inhalation at 26 h post-infection, and the lungs aseptically removed, placed in cold PBS, homogenized, serially diluted and spot plated on brain–heart infusion agar/charcoal plates for determination of bacterial lung titres (log<sub>10</sub> (c.f.u.) per lung). Colony counts were performed on the agar plates. The number of colonies was converted to c.f.u. per lung by multiplying the number of colonies by the volume of the lung homogenate spotted, and the dilution at which the colonies were counted (5–50 colonies per spot). All count data were transformed into log<sub>10</sub> (c.f.u.) per lung for calculation of the mean and s.d. The limit of detection was log<sub>10</sub> = 2.35.
Mice were inoculated intranasally with equivalent titres (6.75 and 6.90 log_{10}(c.f.u.)) of each of the two A. baumannii strains and the treatment (single intravenous dose) with RBT and RIF was administered at 2 h post-infection. 

**Antibiotic treatments.** RIF (Sigma, catalogue no. R3501-1G) and RBT (Sigma, catalogue no. R330-25MG) were dissolved in DMSO. The working solution of antibiotics was prepared fresh daily. The appropriate concentration of antibiotic working solution was prepared in PBS with 10% DMSO, and administered by oral gavage. The control mice received the same volume of PBS with 10% DMSO without any drug. RIF, RBT and the control were administered once a day for 2 d, starting the day of infection.

**Blood colony-forming units.** Blood (50–100μl) was collected by a tail nick at the indicated time points post-oral aspiration or intravenous infection. Blood samples were serially diluted in PBS and plated on TSA plates. Agar plates were incubated overnight at 37°C and the c.f.u. were counted the next day.

**Lung colony-forming units.** At 18 h post-infection, lungs were harvested, weighed and homogenized in sterile PBS. Lung homogenates were serially diluted in PBS and plated on TSA plates. The plates are incubated overnight at 37°C and the c.f.u. were counted the next day.

**Mutant selection.** Male C3HeB/FeJ mice were infected with 2.7×10^9 c.f.u. of A. baumannii HU-MC1. Mice did not receive RIF or RBT treatment. Blood and kidneys were collected 16.5 h post-infection and samples were plated on TSA or RPMI agar plates alone or supplemented with 8 µg ml^−1 of RIF or RBT.

**Statistics.** The bacterial burden was compared using the Mann–Whitney test. Time to death was compared using the log(rank) test. P values <0.05 were considered significant.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** Screening data are available on ReFRAMEdb.org. Genome sequencing data are provided with the paper.
38. Nielsen, T. B., Yan, J., Luna, B. & Spellberg, B. Murine oropharyngeal aspiration model of ventilator-associated and hospital-acquired bacterial pneumonia. *J. Vis. Exp.* **136**, e57672 (2018).

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**Author contributions**
B.L., M.B., V.T., C.M. and B.S. designed the experiments and wrote the manuscript. M.B. and C.M. helped design the HTS assay and performed the compound screening and analysis. B.L., A.U., J.Y., T.N., P.L., J.C., W.K., H.E., N.S., C.K., S.L. and G.D. participated in performing experiments, contributed intellectually and interpreted results. B.L., A.U., P.L. and R.S. conducted the MIC testing. J.L., W.K. and H.E. conducted the LC–MS/MS experiments. J.Y., T.B.N., P.L. and B.L. conducted the in vivo experiments.

**Competing interests**
B.L., B.S. and T.N. own equity in ExBaq. The University of Southern California has a financial interest in ExBaq. G.E.D., V.T., C.K. and S.L. own equity in BioVersys.

**Additional information**
Extended data is available for this paper at https://doi.org/10.1038/s41564-020-0737-6. Supplementary information is available for this paper at https://doi.org/10.1038/s41564-020-0737-6.

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| Species          | Strains           | Rifabutin MIC (µg/mL) |
|------------------|-------------------|-----------------------|
| *P. aeruginosa*  | PAO1              | 8                     |
|                  | ATCC-27853        | 8                     |
| *K. pneumoniae*  | ATCC-43816        | 16                    |
|                  | ATCC-27736        | 16                    |
| *E. coli*        | ATCC-25922        | 4                     |
|                  | ATCC: BAA-2471™   | 8                     |

Extended Data Fig. 1 | MIC of rifabutin in RPMI+10% FCS on non- *A. baumannii* Gram-negative ESKAPE species.
| Strain  | Iron (µM) in RPMI + 10% FCS | MIC (µg/mL) |
|---------|---------------------------|-------------|
| HUMC1   |                           |             |
| 0       | 0.001                     |             |
| 0.1     | 0.002                     |             |
| 0.5     | 0.004                     |             |
| 1       | 0.004                     |             |
| 5       | 0.016                     |             |
| 10      | 0.016                     |             |
| 50      | 1                         |             |
| 100     | 4                         |             |

*Extended Data Fig. 2* | MIC of rifabutin in RPMI + 10% FCS supplemented with increasing amount of ammonium iron(III) citrate.
### Extended Data Fig. 3 | Rifabutin MICs against *A. baumannii* AB5075 transposon disruption mutants.

Mutants were deficient in amino acid transport genes. MIC assay was done in both MHII and RPMI media. AB5075-UW is the parent strain for the transposon mutants.

| Strain     | Disrupted Gene | RBT (µg/mL) | MHII | RPMI |
|------------|----------------|-------------|------|------|
| HUMC1      |                | 6.25        | 0.05 |      |
| AB5075-UW  |                | 6.25        | 0.05 |      |
| AB00188    | aroP           | 3.13        | 0.05 |      |
| AB00190    | aroP           | 6.25        | 0.05 |      |
| AB03015    | aroP           | 6.25        | 0.05 |      |
| AB02204    | aroP           | 6.25        | 0.05 |      |
| AB09979    | aroP           | 0.78        | 0.05 |      |
| AB09980    | tryP           | 3.13        | 0.05 |      |
| AB00215    | tryP           | 6.25        | 0.05 |      |
| AB03612    | hisM           | 3.13        | 0.05 |      |
| AB03616    | hisM           | 6.25        | 0.05 |      |
| AB03618    | hisM           | 6.25        | 0.05 |      |
| AB06383    | hisJ           | 6.25        | 0.05 |      |
| AB07813    | mtr            | 25          | 0.05 |      |
| AB02205    | aroP           | 3.13        | 0.05 |      |
| AB07354    | aroP           | 6.25        | 0.05 |      |
| AB07356    | aroP           | 1.56        | 0.05 |      |
| AB09982    | tryP           | 6.25        | 0.05 |      |
| AB00216    | tryP           | 1.56        | 0.05 |      |
| AB06392    | hisP           | 0.78        | 0.05 |      |
| AB06386    | hisP           | 0.78        | 0.05 |      |
| AB06105    | aroP           | 3.13        | 0.05 |      |
| AB03608    | hisJ           | 3.13        | 0.05 |      |
Extended Data Fig. 4 | Rifabutin MICs against *A. baumannii* ABS075 transposon disruption mutants. MIC assay was done by culturing the bacteria in either MHII or RPMI media.

| Strain  | AB locus | Disrupted gene                                           | MHII | RPMI |
|---------|----------|----------------------------------------------------------|------|------|
| AB05671 | ABUW_2165 | TonB-dependent siderophore receptor (fhuE)               | 3.13 | 3.13 |
| AB05672 | ABUW_2165 |                                                          | 6.25 | 3.13 |
| AB05673 | ABUW_2165 |                                                          | 3.13 | 3.13 |
| AB05674 | ABUW_2165 |                                                          | 3.13 | 1.56 |
| AB10057 | ABUW_3811 | DID                                                      | 3.13 | 0.05 |
| AB10058 | ABUW_3811 |                                                          | 3.13 | 0.05 |
| AB06731 | ABUW_2557 | hypothetical protein                                     | 3.13 | 0.05 |
| AB06732 | ABUW_2557 |                                                          | 6.25 | 0.05 |
| AB06076 | ABUW_2318 | cytosine permease                                        | 3.13 | 0.05 |
| AB06077 | ABUW_2318 |                                                          | 1.56 | 0.05 |
| AB05837 | ABUW_2228 | TonB-dependent receptor                                  | 3.13 | 0.05 |
| AB05838 | ABUW_2228 |                                                          | 3.13 | 0.05 |
| AB05361 | ABUW_2062 | phospholipase D family protein                           | 1.56 | 0.05 |
| AB05362 | ABUW_2062 |                                                          | 1.56 | 0.05 |
| AB07566 | ABUW_2893 | hypothetical protein                                     | 0.78 | 0.05 |
| AB07567 | ABUW_2893 |                                                          | 0.78 | 0.05 |
| AB02034 | ABUW_0745 | hypothetical protein                                     | 0.78 | 0.05 |
| AB02035 | ABUW_0745 |                                                          | 1.56 | 0.05 |
| AB09112 | ABUW_3470 | Zn-dependent hydrolase                                   | 0.78 | 0.05 |
| AB09114 | ABUW_3470 |                                                          | 0.78 | 0.05 |
| AB01188 | ABUW_0449 | hypothetical protein                                     | 1.56 | 0.05 |
| AB01192 | ABUW_0449 |                                                          | 1.56 | 0.05 |
| AB04576 | ABUW_1741 | outer membrane protein assembly factor BamA             | 1.56 | 0.05 |
| AB05479 | ABUW_2100 | LysR family transcriptional regulator                   | 3.13 | 0.05 |
| AB05480 | ABUW_2100 |                                                          | 1.56 | 0.05 |
| AB05756 | ABUW_2194 | acyl-CoA dehydrogenase                                   | 1.56 | 0.05 |
| AB05757 | ABUW_2194 |                                                          | 6.25 | 0.05 |
| AB06047 | ABUW_2307 | biotin synthase                                           | 0.05 | 0.05 |
| AB06049 | ABUW_2307 |                                                          | 0.05 | 0.05 |
| AB06089 | ABUW_2322 | integrase                                                 | 3.13 | 0.05 |
| AB06091 | ABUW_2322 |                                                          | 6.25 | 0.05 |
| AB05348 | ABUW_2057 | alcohol dehydrogenase                                    | 1.56 | 0.05 |
| AB05089 | ABUW_1933 | NADP-dependent fatty aldehyde dehydrogenase             | 1.56 | 0.05 |
| AB04593 | ABUW_1750 | HAD-superfamily hydrolase                                | 3.13 | 0.05 |
| AB04594 | ABUW_1750 |                                                          | 3.13 | 0.05 |
| AB02891 | ABUW_1069 | NADH dehydrogenase                                       | 3.13 | 0.05 |
| AB02892 | ABUW_1069 |                                                          | 6.25 | 0.05 |
| AB06694 | ABUW_2539 | IS4 family transposase                                   | 3.13 | 0.05 |
| AB06695 | ABUW_2539 |                                                          | 3.13 | 0.05 |
| AB01494 | ABUW_0559 | putative phage-related membrane protein                 | 3.13 | 0.05 |
| AB01495 | ABUW_0559 |                                                          | 6.25 | 0.05 |
| AB06821 | ABUW_2586 | allophanate hydrolase                                    | 3.13 | 0.05 |
| AB06822 | ABUW_2586 |                                                          | 3.13 | 0.05 |
| AB04749 | ABUW_1817 | putative peroxidase(α/β hydrolase)                       | 3.13 | 0.05 |
| Collection day | Serial passage medium | Antibiotic pressure | MIC (µg/mL) | WGS mutations |
|----------------|------------------------|---------------------|-------------|---------------|
| Day 14 (96 well plate serial passage) | RPMI + 10% FCS | RBT | Rifabutin in RPMI + 10% FCS: 0.004, Rifampicin in CA-MHB: 4 | 4 | - transposon insertion in AWC45_RS10145 (TonB-dependent siderophore receptor), Arg149fs* |
| | RPMI + 10% FCS | RIF | 0.004 | 8 | ND |
| | CA-MHB | RBT | 0.004 | 4 | ND |
| | CA-MHB | RIF | 0.008 | 16 | ND |
| Day 3 (tube serial passage) | RPMI + 10% FCS | RBT | 0.008 | >32 | - insertion mutation in AWC45_RS10145 (TonB-dependent siderophore receptor), Ile364fs* |
| | RPMI + 10% FCS | RIF | >32 | >32 | ND |
| | CA-MHB | RBT | >32 | >32 | - transposon insertion in AWC45_RS10145 (TonB-dependent siderophore receptor), Ile433fs* |
| | CA-MHB | RIF | 0.12 | >32 | - RpoB S521F mutation |

Extended Data Fig. 5 | MIC of rifabutin and rifampicin on HUMC1 and the serial passaged mutants, and WGS of mutants with increased rifabutin MIC.
## MIC of rifabutin and rifampicin on the *fhuE* deleted mutants and their parental strains.

| Strains               | MIC (μg/mL) | Rifabutin in RPMI + 10% FCS | Rifampicin in CA-MHB |
|-----------------------|-------------|-----------------------------|----------------------|
| HUMC1                 | 0.002       |                             | 32                   |
| HUMC1 ΔfhuE           | 2           |                             | 32                   |
| UNT091-1              | 0.002       |                             | 2                    |
| UNT091-1 ΔfhuE        | 0.5         |                             | 4                    |

**Extended Data Fig. 6 | MIC of rifabutin and rifampicin on the *fhuE* deleted mutants and their parental strains.**
Extended Data Fig. 7 | Clustering of fhuE by amino acid sequence. MICs were done for 43 A. baumannii clinical isolates to determine if the isolates were hypersensitive to RBT in RPMI medium. 28 of the 33 isolates in the outer rings exhibit the hypersensitive phenotype (open circles) in RPMI (RBT MIC = 0.05µg/mL). 9 of 10 isolates in the inner ring are not hypersensitive (filled-in shapes). Of the 41 carbapenem-resistant isolates tested, 26 of those are hypersensitive to RBT.
### Extended Data Fig. 8 | MIC of rifabutin on the plasmid mediated fhuE expressing ATCC-17978 strains.

| Strain          | Plasmid                | Rifabutin MIC (µg/mL) |
|-----------------|------------------------|-----------------------|
|                 | MHII no IPTG           | MHII 1 mM IPTG        |
| *A. baumannii*  | no plasmid             | 4                     | 4                     |
| ATCC 17978      | empty plasmid          | 1                     | 2                     |
|                 | FhuE expressing plasmid| 0.016                 | 0.002                 |
Extended Data Fig. 9 | RPMI MIC predicts in vivo response to treatment. *Galleria mellonella* larvae (10 per group) were infected with *A. baumannii*. **a,** Larvae were infected with strain HUMC1 at 1.6 x10^4 CFU/larvae and treated with rifabutin (plain lines) or rifampicin (dashed lines) at 0.1 mg/kg (blue lines), 1 mg/kg (red lines) and 10 mg/kg (green lines) and survival was measured over 72 hours. **b,** *G. mellonella* larvae were infected with *A. baumannii* LAC-4 and treated with RBt or **c,** RIF. Consistent with the RPMI MIC data, there was no difference in outcomes based on treatment.
Extended Data Fig. 10 | Drug–drug interaction between rifabutin or rifampin and colistin in MHiI The drug–drug interaction were evaluated by calculating the fractional inhibitory concentration index (FICI). FICI = FICA + FICB = (CA/MICA) + (CB/MICB), in which CA and CB are drug concentration of drug A and drug B in combination and MICA and MICB are the MIC of drug A and drug B alone. Synergy was defined as FICI ≤ 0.5, no interaction was defined as FICI > 0.5–4.0 and antagonism was defined as FICI > 4.0.

| Strain             | Drug     | FICI | Result |
|--------------------|----------|------|--------|
| HUMC1              | rifabutin| 0.46 | synergy|
| HUMC1              | rifampin | 0.58 | no interaction |
| HUMC1 ΔfhuE mutant | rifabutin| 0.46 | synergy|
| LAC-4              | rifabutin| 0.46 | synergy|
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  Give P values as exact values whenever suitable.
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- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
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Data collection  No Software was used

Data analysis  Graphpad Prism (version 7) was used for statistical tests.
  The maximum-Likelihood phylogenetic tree was constructed using the CLC Genomics Workbench 12 (Qiagen) software

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| Sample size | Mice experiments involved 6-10 mice per group (group size is indicated in the figure legend). These group sizes have traditionally been sufficient to identify a difference in survival (log-rank test) and CFUs (mann-whitney). |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Data was reported for all experiments in which positive and negative controls performed as expected. Individual data points were not censored or excluded. |
| Replication | Key in vivo experiments were replicated in different infection models (blood vs lung infection models) in mice. Additionally, a G. mellonella model was used as a secondary in vivo model. All in vivo experiments correlated with each other. To confirm that A. baumannii was hypersensitive to RBT, antimicrobial susceptibility tests were done at 5 labs (Calibr, BioVersys, Spellberg, Luna, and She labs at USC). To confirm that the phenomena was not strain specific, over 40 clinical isolates were tested. |
| Randomization | For in vivo experiments, mice were assigned to groups at random. |
| Blinding | Investigators were not blinded to the study. |

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| ☒ ☒ | Animals and other organisms |
| ☒ | Human research participants |
| ☒ | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

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| Laboratory animals | 8-10 week old male C3HeB/FeJ (Jackson stock # 000658) or outbred mice were used. The strain of mice is provided in the figure legend. |
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