A synthetic mammalian gene circuit reveals antituberculosis compounds

Wilfried Weber*, Ronald Schoenmakers*, Bettina Keller*, Marc Gitzinger*, Thomas Grau†, Marie Daoud-El Baba‡, Peter Sander†§, and Martin Fussenegger*¶

*Department of Biosystems Science and Engineering (D-BSSE), Eidgenössische Technische Hochschule Zurich, Mattenstrasse 26, CH-4058 Basel, Switzerland; †Institute for Medical Microbiology, University of Zurich, Gloriastrasse 30/32, CH-8006 Zurich, Switzerland; ‡Université de Lyon, F-69622 Lyon, France; and §National Center for Mycobacteria, Gloriastrasse 30, CH-8006 Zurich, Switzerland

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Synthetic biology provides insight into natural gene-network dynamics and enables assembly of engineered transcription circuitries for production of difficult-to-access therapeutic molecules. In *Mycobacterium tuberculosis* EthR binds to a specific operator (O_{ethR}) thereby repressing *ethA* and preventing EthA-catalyzed conversion of the prodrug ethionamide, which increases the resistance of the pathogen to this last-line-of-defense treatment. We have designed a synthetic mammalian gene circuit that senses the EthR–O_{ethR} interaction in human cells and produces a quantitative reporter gene expression readout. Challenging of the synthetic network with compounds of a rationally designed chemical library revealed 2-phenylethyl-butyrate as a nontoxic substance that abolished EthR's repressor function inside human cells, in mice, and within *M. tuberculosis* where it triggered derepression of *ethA* and increased the sensitivity of this pathogen to ethionamide. The discovery of antituberculosis compounds by using synthetic mammalian gene circuits may establish a new line of defense against multidrug-resistant *M. tuberculosis*.

genetic engineering | biology | antibiotic | ethionamide | Mycobacterium

Up to 9 million people contract tuberculosis every year and 50 million people are presently infected with *Mycobacterium tuberculosis* resistant to both first-line drugs isoniazid and rifampicin (1) [World Health Organization (WHO), fact sheet no. 104, March 2007]. Ethionamide, a structural analogue of isoniazid, is currently the last line of defense in the treatment of multidrug-resistant tuberculosis (MDR-TB). During 35 years of its clinical use, ethionamide has fortunately elicited little cross-resistance with isoniazid because both prodrugs have to be activated by different mycobacterial enzymes to develop their antimicrobial activity (2). Ethionamide is activated by the Baeyer–Villiger monooxygenase EthA, which converts the prodrug into an antimycobacterial nicotinamide adenine dinucleotide derivative (3, 4). Because *ethA* is repressed by EthR (5), ethionamide-based tuberculosis therapy is often unsuccessful even when prescribed at high hepatotoxic doses (6). Therefore, compounds preventing EthR from binding to the *ethA* promoter could increase the sensitivity of multidrug-resistant *M. tuberculosis* to ethionamide and make tuberculosis treatment safer, more efficient, and affordable. Crystallography-based structural analysis implied that hexadecylocanoate copurifying with EthR could abolish EthR's operator-binding capacity (7). However, hexadecyloctanoate turned out to be too hydrophobic to confirm this hypothesis in any cell/microbial culture system suggesting that it remains a nontrivial challenge to discover bioavailable EthR-binding compounds. Because *M. tuberculosis* is an intracellular pathogen, EthR inhibitors do not only have to specifically target the bacterial repressor, but also need to reach the cytosol without eliciting any cytotoxic effect. Therefore, integrated screening approaches assessing specificity, bioavailability, and cytotoxicity in a single assay are expected to rapidly reveal valid drug candidates. Although synthetic mammalian gene networks designed so far, including epigenetic toggle switches (8), hysteresis networks (9), time-delay circuits (10), and synthetic ecosystems (11), have resulted in important information on the dynamics of physiologic control systems, the EthR-based gene circuit pioneers a direction with a more practical purpose: providing a generic screening platform to discover drug candidates with the potential to efficiently kill *M. tuberculosis*, the causative agent of one of the most devastating human diseases.

Results

Design of an EthR-Based Synthetic Mammalian Gene Circuit. Structural analysis (7, 12) classifying EthR as a TetR/CamR family repressor suggested the existence of compounds that could modulate the affinity of EthR for its O_{ethR} operator (13, 14). Adopting a synthetic biology approach we have designed a gene network whose topology enabled detection of EthR-binding molecules inside human cells, thereby scoring for noncytotoxic and bioavailable compounds accessing the pathogenic habitat of *M. tuberculosis* (Fig. 1*a*). The gene circuit consists of a synthetic transactivator, EthR, fused to the VP16 transactivation domain of *Herpes simplex* (pWW489, P_{SV40}-EthR-VP16-pA), which induces SEAP (human placental secreted alkaline phosphatase) expression in human embryonic kidney cells (HEK-293) after binding to a chimeric promoter containing the EthR-specific operator (O_{ethR}) 5′ of a minimal *Drosophila* heat shock protein 70 promoter ($P_{hsp90min}$; pWW491, O_{ethR}-$P_{hsp70min}$-SEAP-pA) (8.2 ± 0.8 units/liter; background level of pWW491, 0.4 ± 0.1 units/liter) (Fig. 1*a*). Cell-permeable EthR-interacting compounds were expected to release EthR-VP16 from O_{ethR}-$P_{hsp70min}$, thereby repressing SEAP production to basal levels (Fig. 1*a*). Interestingly, hexadecyloctanoate (10 mM), identified in crystallography studies to compromise EthR's DNA-binding capacity (7), failed to decrease SEAP expression in HEK-293 cells containing pWW489 and pWW491 (data not shown), which is probably because of its highly lipophilic structure (ClogP = 11.29). Therefore, it remains a nontrivial challenge to discover bioavailable EthR-binding compounds as they must be sufficiently lipophilic to fit into the hydrophobic tunnel of EthR (7) while being hydrophilic enough to reach therapeutic levels in the bloodstream and inside infected cells.

Discovery of Compounds Affecting the DNA-Binding Affinity of EthR. Capitalizing on crystallography data describing EthR's small-molecule binding site as "hydrophobic tunnel-like cavity fitting a lipophilic ligand" (7, 12) and on the observation that repressors

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¶To whom correspondence should be addressed. E-mail: fussenegger@bsse.ethz.ch.

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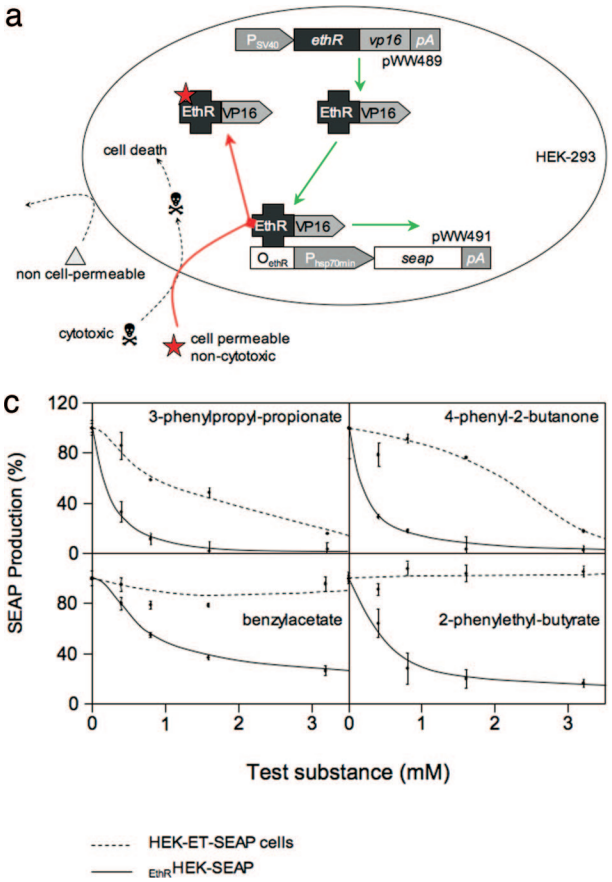

b

Compound	Structure	Formula	M_w	ClogP
Methyl-hexanoate		$C_7H_{14}O_2$	130.18	2.298
1-Butyl-propionate		$C_7H_{14}O_2$	130.18	2.298
1-pentyl-acetate		$C_7H_{14}O_2$	130.18	2.298
4-phenyl-2-butanone		$C_{10}H_{12}O$	148.20	1.889
Methylphenyl-acetate		$C_9H_{10}O_2$	150.17	1.820
Benzyl-acetate		$C_9H_{10}O_2$	150.17	1.960
2-phenylethyl-acetate		$C_{10}H_{12}O_2$	164.20	2.279
2-phenylethyl-propionate		$C_{11}H_{14}O_2$	178.23	2.808
2-phenylethyl-butyrate		$C_{12}H_{16}O_2$	192.25	3.337
3-phenylpropyl-propionate		$C_{12}H_{16}O_2$	192.25	3.187
2-phenylethyl-isopentanoate		$C_{13}H_{18}O_2$	206.28	3.736
Hexadecyl-octanoate		$C_{24}H_{48}O_2$	368.64	11.29

Fig. 1. EthR-based synthetic gene network in mammalian cells. (*a*) A gene fusion of *ethR* with the *Herpes simplex*-derived *vp16* transactivation domain is expressed under the control of the simian virus 40 promoter (P_{SV40}, plasmid pWW489) in HEK-293. The chimeric transactivator EthR-VP16 binds to its operator O_{ethR} thereby activating transcription from the minimal *Drosophila* heat shock 70 promoter ($P_{hsp70min}$), driving expression of human placental secreted alkaline phosphatase (*seap*, plasmid pWW491). In the presence of a cell-permeable, noncytotoxic inducer, binding of EthR-VP16 to the promoter is inhibited, thereby resulting in transcriptional silence (red lines). Non-cell-permeable or cytotoxic compounds are automatically excluded from the hit list. (*b*) Compounds selected for testing as potential inducers of EthR. The ClogP value indicates the calculated distribution coefficient between *n*-octanol and water. (*c*) Screening of a rationally designed compound library by using the EthR-based gene network. 30,000 HEK-293 containing either the EthR-based gene network (pWW489 and pWW491, $_{EthR}$-HEK-SEAP cells) or an isogenic constitutive SEAP expression network (pWW35 and pWW37, HEK-ET-SEAP cells) were cultivated for 48 h in the presence of potential inducers before SEAP profiling. SEAP expression was normalized to 100%.

are often feedback-controlled by the products of their target gene (15, 16), we have synthesized a library of hydrophilic esters (ClogP < 4), a substance class, which is the main product of EthA-catalyzed Baeyer–Villiger oxidation (Fig. 1*b*). When HEK-293 populations containing the EthR-based gene circuit (Fig. 1*a*) were exposed to 0–3.2 mM individual library components, only benzylacetate, 3-phenylpropyl-propionate, 2-phenylethyl-butyrate, and 4-phenyl-2-butanone [a ketone class control (7)] induced a significant decrease in SEAP expression, suggesting that these compounds may trigger the release of EthR from O_{ethR} (Fig. 1*c*). However, because 3-phenylpropyl-propionate and 4-phenyl-2-butanone were also reducing SEAP levels of HEK-ET-SEAP cells transgenic for constitutive SEAP expression (Fig. 1*c*), these substances were considered cytotoxic at EthR-releasing concentrations [SEAP production and viability of HEK-ET-SEAP cells were shown to correlate; supporting information (SI) Fig. S1]. Therefore, only benzylacetate (IC_{50} = 1 mM) and 2-phenylethyl-butyrate (IC_{50} = 0.5 mM) were used for further studies (Fig. 1*c*).

Validation of EthR-Modulating Compounds in Bacteria and *in Vitro*. When exposing *Escherichia coli* engineered for P_{ethR}-driven GFP expression to benzylacetate and 2-phenylethyl-butyrate concentrations used for the aforementioned experiments with mammalian cells, dose-dependent GFP expression could be observed, indicating that these compounds were also able to trigger release of EthR from O_{ethR} in prokaryotes (Fig. 2 *a* and *b*; see Fig. S2 for expression constructs and experimental setup). Similar to mammalian cells, hexadecyloctanoate was unable to modulate EthR's P_{ethR}-binding affinity in *E. coli* at concentrations up to 10 mM (data not shown). Benzylacetate efficiently released EthR from its operator but did so only at elevated concentrations (10 mM) that are known to be mutagenic and likely incompatible with future therapeutic use (Material Safety Data Sheet, Sigma). We have therefore focused on 2-phenylethyl-butyrate and further characterized the adjustable EthR-O_{ethR} release capacity of this licensed food additive in a cell-free ELISA system (Fig. 2*c*): Hexahistidine-tagged EthR (EthR-His_6) was allowed to bind to agarose beads containing immobilized O_{ethR} in the presence of increasing concentrations of 2-phenylethyl-butyrate and O_{ethR}-interacting EthR-His_6 was quantified after a washing step by using a His_6-specific horseradish peroxidase-coupled antibody and a standard assay system (Fig. 2*c*). The inverse correlation of 2-phenylethyl-butyrate and O_{ethR}-bound EthR indicates that this compound is able to induce release of EthR from its operator. The specificity of 2-phenylethyl-butyrate's EthR-releasing capacity was confirmed by an identical control experiment with an unrelated repressor–operator interaction (Fig. S3).

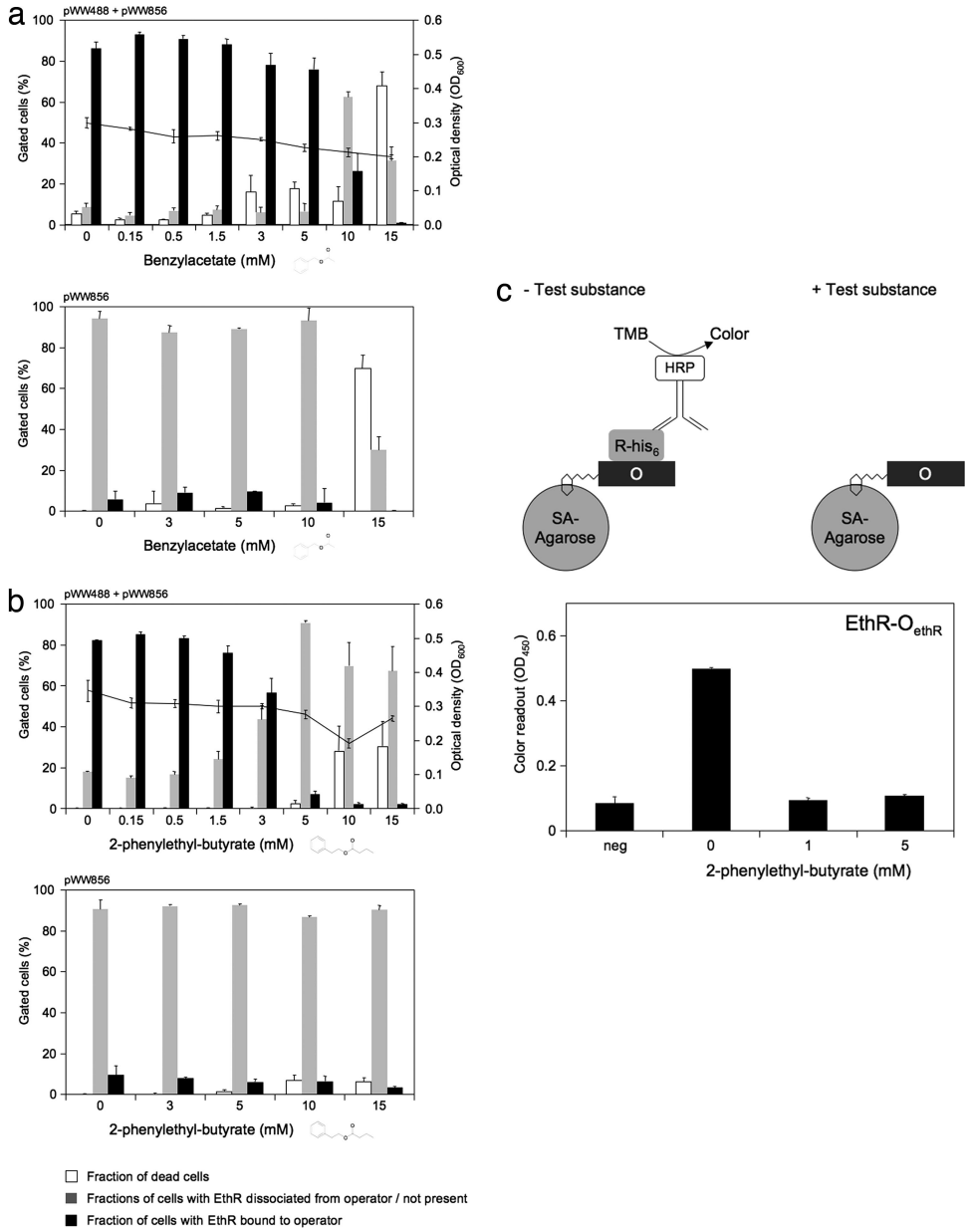

Fig. 2. Validation of the inducer in bacteria and in a cell-free system. (*a*) Effect of benzylacetate in *E. coli*. *E. coli* BL21(DE3), transformed with pWW488 and pWW856 (see Fig. S2*a*), was grown in the presence of IPTG (to induce EthR expression) at the indicated benzylacetate concentrations for 5.5 h before analyzing the cells by FACS (for FACS gates and parameters; see Fig. S2*b*). The optical density at 600 nm (OD_{600}) after the growth period is indicated as well. As a control, *E. coli* BL21(DE3) transformed with pWW856 alone were used in parallel. (*b*) Effect of 2-phenylethyl-butyrate in *E. coli*. Experimental setup as described in *a*. (*c*) Impact of 2-phenylethyl-butyrate on the interaction between EthR and O_{ethR} *in vitro*. Biotinylated operator O_{ethR} (*O*) immobilized on streptavidin-agarose beads (SA-Agarose) was incubated in the presence or absence of 2-phenylethyl-butyrate at the indicated concentrations in a cell lysate of *E. coli* BL21(DE3) transformed with pWW862 (P_{T7}-*ethR-his*$_6$-term) for production of hexahistidine-tagged EthR (R-his_6). After washing, his_6-tagged EthR was detected by a monoclonal anti-his_6 antibody coupled to horseradish peroxidase (HRP), resulting in the conversion of 3,3′,5,5′-tetramethylbenzidine (TMB) to a colored formazane. As negative controls, nonrecombinant cell lysate was used (neg).

2-Phenylethyl-butyrate Modulates EthR Activity in Mice. To evaluate whether the licensed food additive 2-phenylethyl-butyrate [Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives, JECFA no. 991], retains its regulating activity *in vivo*, we stably transfected the EthR-based gene circuit into HEK-293 cells ($_{EthR}$HEK-SEAP, transgenic for pWW489 and pWW491; see Fig. S4 *a* and *b* for clonal variation, Fig. S4*c* for adjustability, and Fig. S4*d* for reversibility of the gene circuit). $_{EthR}$HEK-SEAP cells were microencapsulated and implanted i.p. into mice. Animals treated with 2-phenylethyl-butyrate showed significantly reduced SEAP serum levels (without 2-phenylethyl-butyrate, 420 ± 43 milliunits/liter; with 625 μl/kg 2-phenylethyl-butyrate, 207 ± 29 milliunits/liter) suggesting that this compound was bioavailable and reached EthR-inactivating concentrations inside target cells. Mice with implanted control cells harboring only pWW491 showed background SEAP expression (84 ± 16 milliunits/liter).

2-Phenylethyl-butyrate Increases the Sensitivity of *Mycobacterium bovis* and *M. tuberculosis* to Ethionamide. Growth of *M. tuberculosis* is significantly impaired in the presence of ethionamide because of EthA-mediated conversion of this prodrug into an antimyco-

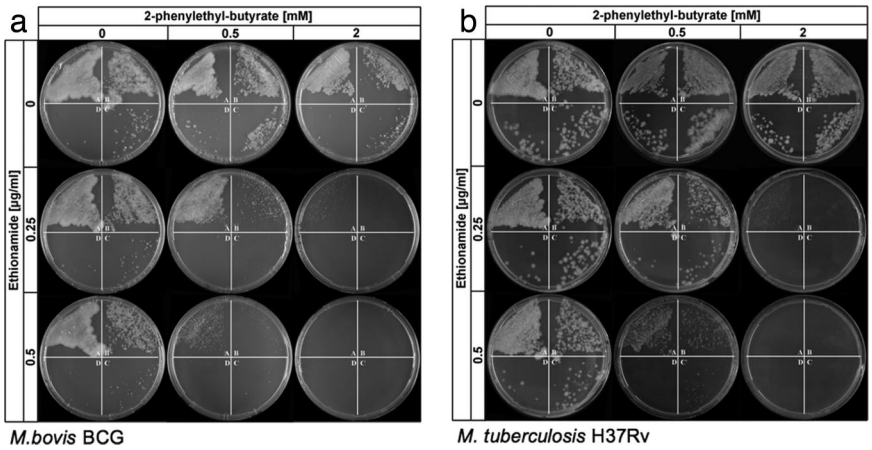

Fig. 3. Effect of 2-phenylethyl-butyrate and ethionamide on *M. bovis* bacillus Calmette–Guérin and *M. tuberculosis*. (*a*) Synergistic effect of 2-phenylethyl-butyrate and ethionamide (ETH) on the growth inhibition of *M. bovis* bacillus Calmette–Guérin. A–D correspond to serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of an *M. bovis* bacillus Calmette–Guérin settling culture (OD_{600}: 0.6). (*b*) Synergistic effect of 2-phenylethyl-butyrate and ethionamide (ETH) on growth inhibition of *M. tuberculosis* H37Rv. A–D correspond to serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of an *M. tuberculosis* H37Rv settling culture (OD_{600}: 0.4).

bacterial nicotinamide adenine dinucleotide derivative (3, 17). EthR-mediated repression of *ethA* transcription requires rather high clinical doses of ethionamide [up to 1g/day (6, 18)], which is associated with severe side effects including neurotoxicity (19) and fatal hepatotoxicity (6), yet is often still insufficient to reach minimum inhibitory levels in the bloodstream (20). Therefore, 2-phenylethyl-butyrate-triggered dissociation of EthR from the *ethA* promoter resulting in derepression of *ethA*, which was confirmed by quantitative RT-PCR of ethA transcripts (2.93 ± 0.04- and 9.7 ± 1.7-fold increase in the presence of 0.5 and 2 mM 2-phenylethyl-butyrate, respectively), may increase the sensitivity of *Mycobacterium* to ethionamide-based therapy. Growth of *M. bovis* bacillus Calmette–Guérin and *M. tuberculosis* H37Rv in the presence of subinhibitory ethionamide concentrations (0.25 and 0.5 μg/ml), which are easily reached by therapeutic doses [c_{max} (250 mg oral) = 2 μg/ml; $t_{1/2}$ = 2 h (18)] was dose-dependently inhibited by 0.5 and 2 mM 2-phenylethyl-butyrate (Fig. 3). Because 2-phenylethyl-butyrate alone did not show any growth inhibitory effect, we suggest that it acted synergistically with ethionamide to kill the pathogen (Fig. 3). 4-Phenyl-2-butanone, which was previously suggested to neutralize EthR, was found to be cytotoxic (Fig. 1*c* and Fig. S1) and did not act synergistically with ethionamide to kill *M. bovis* at concentrations that were higher than the ones at which 2-phenylethyl-butyrate was effective (Fig. S5).

Discussion

Synthetic gene circuits have dramatically increased progress on gene-function relationships in the postgenomic era (8–11, 21–25). They also continue to provide the key parts for synthetic biologists to decipher natural gene network dynamics (8–10) and to reprogram cellular function for production of important precursor drugs (23). We have engineered a synthetic gene network in human cells for screening of functional antimicrobial agents with precise target specificity, undetectable cytotoxicity, and the capacity to reach the cytosol to eliminate intracellular pathogens. The network setup is generic so that it can, in principle, be adapted to essential transcription regulators of other pathogens. With the integration of EthR (4), which controls the resistance of *M. tuberculosis* to ethionamide, into a synthetic mammalian gene network we have been able to identify the licensed food additive 2-phenylethyl-butyrate as a potent inhibitor of EthR in *M. tuberculosis*, as well as *in vivo*, which dramatically increases the sensitivity of this pathogen to the last-line-of-defense drug ethionamide and potentially to other *ethA*-dependent compounds (26). Therefore, 2-phenylethyl-butyrate could set up an efficient and safe line of defense against multidrug-resistant tuberculosis.

Materials and Methods

Vector Design. pWW489 (P_{SV40}-*ethR-vp16*-pA) was constructed by PCR-mediated amplification of *ethR* from genomic *M. bovis* DNA by using oligonucleotides OWW400 (5′-gcatccatatgaattccaccatgaccacctccgcggcca-3′) and OWW401 (5′-cgatcgcgcgcggctgtacgcggagcggttctcgccgtaaatgc-3′) followed by restriction and ligation (EcoRI/BssHII) into pWW35 (27). pWW491 (O_{ethR}-$P_{hsp70min}$-SEAP-pA) was obtained by direct cloning of a synthetic O_{ethR} sequence (5′-gacgtcgatccacgctatcaacgtaatgtcgaggccgtcaacgagatgtcgacactatcgacacgtagcctgcagg-3′) (AatII/SbfI) into pMF172 (27). pWW488 (P_{T7}-*ethR-vp16-his*$_6$) was constructed by PCR-mediated amplification of *ethR-vp16* from pWW489 by using oligonucleotides OWW400 and OWW60 (5′-gctctagagcaagcttttaatggtgatggtgatgatgcccaccgtactgtcaattccaag-3′) followed by cloning (NdeI/HindIII) into pRSETmod (28). pWW856 (P_{ethR}-*gfp*-pA) was constructed in three steps: (*i*) *gfp* was PCR-amplified from pLEGFP-N1 (Clontech) by using oligonucleotides OWW848 (5′-ggcttgaattcaaaggagatataccatggtgagcaagggcgag-3′) and OWW849 (5′-ggctttctagacaaaaaacccctcaagacccgtttagaggccccaaggggttatgctagttacttgtacagctcgtccatgccg-3′) and cloned (EcoRI/XbaI) into pWW56 (27) (pWW854). (*ii*) A synthetic O_{ethR} sequence was directly cloned (HindIII/EcoRI) into pWW854 (pWW855). (*iii*) P_{ethR}-*gfp* was excised (BamHI/StuI) from pWW855 and ligated (BamHI/ScaI) into pACYC177 (NEB) (pWW856). pWW862 (P_{T7}-*ethR-his*$_6$) was assembled by annealing oligonucleotides OWW479 (5′-cgcgcatcatcatcatcatcattaagcggccgca-3′) and OWW480 (5′agcttgcggccgcttaatgatgatgatgatg-3′) and cloning the double-stranded DNA BssHII/HindIII into pWW488. pWW871 (5′LTR-Ψ^+-*ethR-vp16*-P_{PGK}-*neo*^{R}-3′LTR) was designed by cloning *ethR-vp16* of pWW489 (EcoRI/BamHI) into pMSCVneo (Clontech). pWW35 (P_{SV40}-*E-vp16*-pA), pWW37 (ETR-$P_{hCMVmin}$-*seap*-pA) and pWW313 (P_{T7}-E-his_6-pA) have been described (28).

Cell Culture. Human embryonic kidney cells (HEK-293, American Type Culture Collection CRL-1573) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FCS (Pan Biotech GmbH, catalog no. 3302, lot P231902) and 1% of a penicillin/streptomycin solution (Sigma, catalog no. 4458). Cells were transfected by using standard calcium phosphate procedures (29) and retroviral particles were produced according to the manufacturer's protocol (Clontech). $_{EthR}$HEK, transgenic for constitutive EthR-VP16 expression, was constructed by transducing HEK-293 with pWW871-derived retroviral particles followed by selection in DMEM containing 200 μg/ml neomycin and single-cell cloning. Cotransfection of $_{EthR}$HEK with pWW491 and pPUR (Clontech), subsequent selection in 200 μg/ml neomycin, 1 μg/ml puromycin followed by single-cell cloning resulted in $_{EthR}$HEK-SEAP. The cell line HEK-ET-SEAP, transgenic for constitutive SEAP expression, was described in ref. 27.

SEAP was quantified in cell culture supernatants by using a *p*-nitrophenyl-phosphate-based assay (30) and in mouse serum by employing a commercial chemiluminescence test (Roche Applied Science, catalog no. 11779842001).

The impact of chemicals on the viability of human cells was assessed by using the WST-1 cell proliferation assay according the to manufacturer's protocol (Roche Applied Science, catalog no. 05015944001).

Chemicals. Pentyl-acetate, methyl-phenylacetate, 2-phenylethyl-acetate, 4-phenyl-2-butanone (all Fluka) and 2-phenylethyl-butyrate (Sigma) were commercially obtained. Methyl-hexanoate was obtained by reacting hexanoic acid with thionylchloride in methanol. Butyl-propionate, 2-phenylethyl-propionate, 2-phenylethyl-isopentanoate, and 3-phenylpropyl-propionate were prepared by reacting the corresponding alcohol with the acid chloride in dichloromethane by using triethylamine as a base. Hexadecyloctanoate and benzylacetate were synthesized from the bromide and the acid with K_2CO_3 as the base in dimethylformamide. All esters were purified either by column chromatography (silica, ethylacetate/hexane) or distillation. ClogP was determined by using Chemdraw Ultra 10.0 (CambridgeSoft). Erythromycin (Sigma, catalog no. E5389) was used as a 1,000× stock solution of 5 mg/ml in ethanol. Ethionamide was purchased from Sigma (catalog no. E6005) and prepared as 200× stock solution in DMSO.

FACS Analysis. *E. coli* BL21(DE3) (Invitrogen), transformed with pWW488 and pWW856 or pWW856 alone, were grown overnight in Luria Bertani (LB) media containing 100 μg/ml ampicillin and 30 μg/ml kanamycin (for pWW856-transformed cells only). Then, 150 μl of *E. coli* suspension (OD_{600} 1.3) was added to 2 ml of fresh LB media containing antibiotics and 1 mM isopropyl β-D-thiogalactoside (IPTG) where indicated. After growth for 5.5 h at 37°C, 500 μl of the suspension was transferred to a new tube and centrifuged for 3min at 800 × *g*. The pellet was washed twice with 1 ml of PBS and resuspended in 2 ml of PBS for FACS analysis (>10,000 cells per sample), which was performed on a Cytomics FC500 (Beckman Coulter) with 405 nm used for excitation and 510 nm for emission. FACS gates are shown in Fig. S2

ELISA. The EthR-his_6-specific ELISA was performed as previously described for the E-his_6-based ELISA (28) with the exception that the biotinylated operator sequence (O_{ethR}) was immobilized on streptavidin agarose beads (Novagen, catalog no. 69203) instead of a microtiter plate.

***In Vivo* Methods.** $_{EthR}$HEK-SEAP was encapsulated in alginate-poly(L-lysine)-alginate capsules (200 cells per capsule) as described in ref. 27. Female OF1 (Oncins France souche 1, Charles River Laboratories) mice were injected i.p. with 700 μl of capsule suspension containing 2×10^6 cells. One and 25 h post-capsule implantation, the mice were injected with 2-phenylethyl-butyrate at the indicated concentration [the injection volume was adjusted to 100 μl by adding canola oil (Migros)]. Forty-eight hours post-capsule implantation, serum samples were analyzed for SEAP expression. Dissection of the animals revealed no inflammation at the injection site. Animal experiments were conducted by M.D.-E. at the Institut Universitaire de Technologie A (Lyon, France) in accordance with European Community legislation (86/609/EEC) and approved by the French Republic (no. 69266310). For each experimental condition mean values including the standard deviation of at least eight mice are indicated.

Mycobacteria Cultivation and Susceptibility Testing. *M. tuberculosis* H37Rv (ATCC27294) and *M. bovis* bacillus Calmette–Guérin no. 1721, a streptomycin-resistant derivative of bacillus Calmette–Guérin Pasteur, carrying a nonrestrictive *rpsL* mutation (K42R) (31) were grown in Middlebrook 7H9 supplemented with oleic acid, albumin, dextrose, catalase (Difco) and Tween 80 (0.05%) until midlog phase. Tenfold serial dilutions (20 μl) were streaked on Middlebrook 7H10-OADC agar plates containing solvent (DMSO, 200-fold dilution), ethionamide (0.25–0.5 μg/ml) and 2-phenylethyl-butyrate (0.5 or 2 mM) where indicated. Plates were incubated at 37°C and growth was documented after 2 and 3 weeks.

For quantitative analysis of *ethA* transcripts, *M. tuberculosis* H37Rv were treated at midlog phase with DMSO (40-fold dilution) or 2-phenylethyl-butyrate (0.5 or 2 mM) for 24 h at 37°C, harvested by centrifugation (4,400 × *g*, 10min, 4°C) and total RNA was extracted by using the RiboPure-Bacteria Kit (Ambion, catalog no. AM1925) according to the manufacturer's protocol. Total RNA was reverse transcribed (25°C, 10 min; 48°C, 30min; 95°C, 5min) by using TaqMan reverse transcription reagents (Applied Biosystems, catalog no. N8080234) and *ethA*-specific cDNA was quantified on an Applied Biosystems 7500 real-time PCR device by using the following ethA-specific primers: forward primer, 5′-cgatcacgacgatgttcttagc-3′; reverse primer, 5′-tcgggccgatcatc-cat-3′; probe labeled with 5′FAM and 3′TAMRA, 5′-FAM-cgtagtcgaggtc-ctcgggccagt-TAMRA-3′. All samples were standardized by using the *M. tuberculosis sigA* gene [Rv2703 (32)] and the following *sigA*-specific primers: forward primer, 5′-ccgatgacgacgaggagatc-3′; reverse primer, 5′-ggcctc-cgactcgtcttca-3′; probe labeled with 5′FAM and 3′TAMRA dyes, 5′-FAM-aaggacaaggcctccggtgatttcg-TAMRA-3′.

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