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Organism Engineering for the Bioproduction of the Triaminotrinitrobenzene (TATB) Precursor Phloroglucinol (PG)

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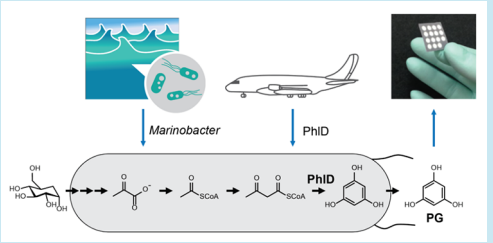
Supporting Information

ABSTRACT: Organism engineering requires the selection of an appropriate chassis, editing its genome, combining traits from different source species, and controlling genes with synthetic circuits. When a strain is needed for a new target objective, for example, to produce a chemical-of-need, the best strains, genes, techniques, software, and expertise may be distributed across laboratories. Here, we report a project where we were assigned phloroglucinol (PG) as a target, and then combined unique capabilities across the United States Army, Navy, and Air Force service laboratories with the shared goal of designing an organism to produce this molecule. In addition to the laboratory strain *Escherichia coli*, organisms were screened from soil and seawater. Putative PG-producing enzymes were mined from a strain bank of bacteria isolated from aircraft and fuel depots. The best enzyme was introduced into the ocean strain *Marinobacter atlanticus* CP1 with its genome edited to redirect carbon flux from natural fatty acid ester (FAE) production. PG production was also attempted in *Bacillus subtilis* and *Clostridium acetobutylicum*. A genetic circuit was constructed in *E. coli* that responds to PG accumulation, which was then ported to an *in vitro* paper-based system that could serve as a platform for future low-cost strain screening or for in-field sensing. Collectively, these efforts show how distributed biotechnology laboratories with domain-specific expertise can be marshalled to quickly provide a solution for a targeted organism engineering project, and highlights data and material sharing protocols needed to accelerate future efforts.



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Biotechnology offers solutions to national security challenges, whether through new routes to materials, responses to rapidly emerging health threats, or providing innovative

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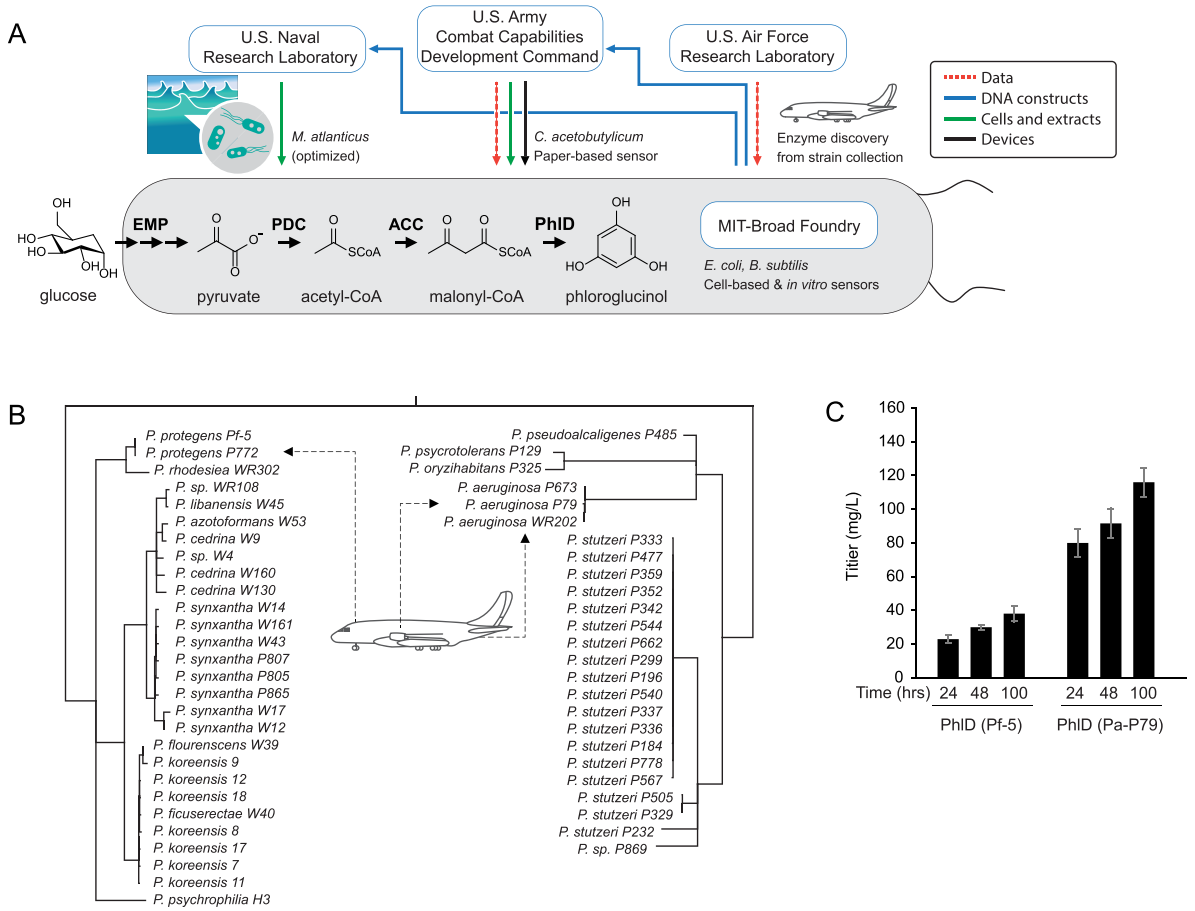

Figure 1. Production of PG in *E. coli* and strategy for optimization. (A) Combined resources used to optimize PG production and sensing. (B) Phylogenetic tree of *Pseudomonas* isolates from aircraft, based on sequence similarity of the 16S ribosomal subunit. The *P. protegens* Pf-5 strain is included as a reference (top left). Arrows indicate the sources of the three strains in which *phlD* genes were detected via PCR. (C) PG production by *E. coli* carrying different enzymes. Cultures were grown for 24, 48, and 100 h in EZ Rich medium before PG purification and quantification by LC−MS analysis (Supplementary Methods). Error bars represent the standard deviation of measured titers from five independent experiments performed on different days.

strategic options.[1−3] Living organisms can be designed to make molecules or act as autonomous agents that function as human therapeutics or in the environment.[4−8] Organism design has become a sophisticated endeavor, involving computer aided design (CAD) software operating at the molecular, system, and genome levels, data-guided decision making, and artificial intelligence (AI).[9−15] Early in the design process, a strain must be selected to serve as the starting chassis. While there are strain banks that have 10 000s of bacteria and fungi, many with unique attributes,[16−21] identifying a starting host for an engineering effort is often based on access and institutional experience. Data access can also be limiting, including characterized genetic parts, experimental protocols (culturing, manipulation, *etc.*), and -omics information (genome, transcript/protein levels, metabolic maps).[22−29] When confronted with a new challenge, particularly one that is time sensitive, it is critical to be able to draw on expertise, data and software, and physical resources from multiple facilities and organizations during the design process. While other engineering fields routinely coordinate specialties for a design project, this is at a nascent stage in biotechnology.

Pressure tests are a means to evaluate readiness to act on an emerging need. Over several years, the Foundry at MIT was tested by receiving lists of desired target molecules for which producing strains had to be built in months.[5] In some cases, strains that made the molecules had been described in the literature but access was restricted. For example, we were asked to make a toxin with potential as a cancer chemotherapeutic, for which there was a published natural producer[30] that was not shared citing intellectual property concerns. Applying bioinformatics tools, we rapidly identified an alternative strain that produces the same compound from a commercial strain bank.[31] Similarly, when we were asked to produce a putative rocket fuel precursor, we were denied access to a published strain.[32] However, we were able to reconstruct this strain in 3 weeks (Supplementary Figure S1).

One such compound we were asked to make is phloroglucinol (PG), which is a flexible chemical precursor used to make pharmaceuticals, cosmetics, textiles, dyes, and energetic materials.[33,34] It has garnered interest as a chemical precursor to the powerful, yet insensitive, explosive triaminotrinitrobenzene (TATB), used as part of some nuclear weapon designs.[35−38] Compared to other TATB precursors, PG is less volatile and easier to transport, but its chemical synthesis is inefficient.[39] A biological route could provide access with fewer waste streams. PG is naturally produced by pseudomonads, and genetic engineering has been applied to move its production to heterologous hosts.[40] Only a single enzyme, the type III polyketide synthase PhlD, is required to produce PG from three molecules of the ubiquitous metabolite malonyl-CoA.[41]

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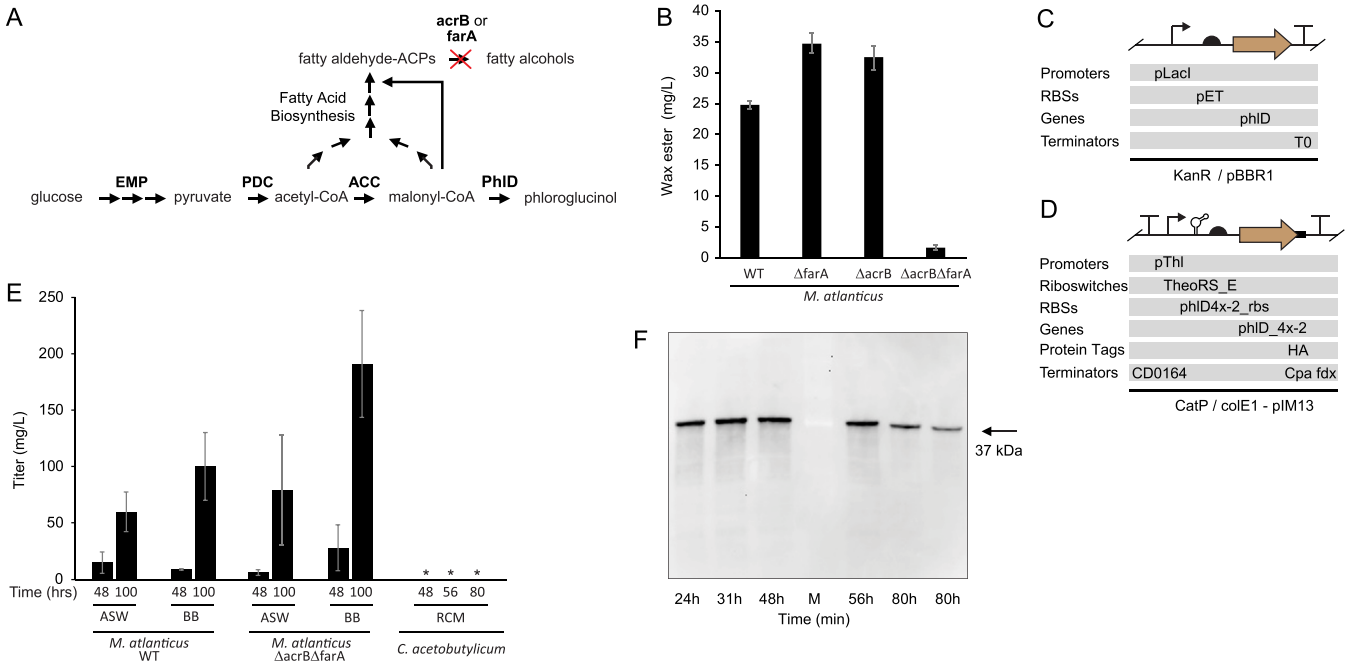

Figure 2. PG production in *Marinobacter* and *Clostridium*. (A) Metabolism of acetyl-CoA and malonyl-CoA to fatty alcohols. Knockout of *acrB* and *farA* prevents formation of fatty alcohols, and is theorized to promote accumulation of upstream metabolites, including acyl-CoA's. (B) Elimination of wax ester production in *Marinobacter atlanticus* CP1 double knockout (data reproduced from ref 51). (C) Design of *phlD* expression vector for *phlD* in *Marinobacter*, using the gene from *P. aeruginosa* P79. (D) Shuttle-vector design for PhlD expression in *C. acetobutylicum* ATCC 824 employing thiolase promoter and a theophylline-inducible riboswitch. This design uses a thermostable variant of PhlD ("4x-2").[43] Some variants contain a hemagglutinin tag as indicated or an alternate Gram-positive replication origin (Supplementary Table S4). (E) PG titers measured from *Marinobacter* and *Clostridium*. In *Marinobacter*, PG production was tested using the *phlD* gene from Pa−P79, in the wild-type (left) and wax-ester mutant (right) grown in artificial seawater medium (ASW) and rich-media (BB) with fermentation times of 48 and 100 h. In *Clostridium*, no PG was detected (see text for details). (F) Despite no observable PG production, full-length protein was detected in *C. acetobutylicum* via Western blot (Supplementary Methods). For *Marinobacter* data, error bars represent the standard deviation of measured titers from three technical replicates. For *Clostridium*, two independent experiments were performed on different days.

Directed evolution has been applied to optimize the enzyme for higher thermostability, and metabolic engineering has been used to improve titers by increasing tolerance via introduction of heat-shock proteins and by increasing intracellular malonyl-CoA concentrations by enabling acetate to be used as a feedstock.[39,42−44]

To satisfy the pressure test requirements, we quickly reconstructed a strain of *E. coli* that expresses PhlD and confirmed its production of PG. We then reached out to United States Department of Defense (DoD) service laboratories to coordinate the application of their unique internal resources to this problem (Figure 1a). We were able to evaluate chassis from the ocean (*Marinobacter atlanticus* CP1) and soil (*Clostridium acetobutylicum*) that were predicted to produce higher titers or be tolerant to the product, but are difficult to engineer. A strain bank of pseudomonads isolated from aircraft was screened for improved PhlD variants and a new variant was identified. The best PG producer was a *Marinobacter* variant whose genome was edited to increase malonyl-coA and carry the PhlD variant. This strain is able to produce PG from succinate in an artificial seawater medium. One of the challenges of screening variants at high-throughput is having an assay for PG production. To this end, we created low-cost cell-based and *in vitro* paper-based systems for rapid PG detection. While the data and materials exchanges underlying this project were largely *ad hoc*, they point to a need for infrastructure, computational resources, and standards to facilitate the application of knowledgebases, strain banks, and genetic parts so they can be applied to emerging challenges.

RESULTS

Construction of *E. coli* Strains for PG Production. Previous efforts for heterologous PG production relied on the *phlD* enzyme found in *P. protegens* Pf-5 (formerly classified as *P. fluorescens* Pf-5).[41] We used this gene to establish a baseline production system in *E. coli*. The protein sequence was codon optimized for *E. coli* expression, cloned into a pET-based vector under inducible control, and sequence-verified. Following the previously reported growth conditions, clones were cultured for 24 and 48 h. After extraction (1:1) with methanol, PG was not detected via LC−MS under these conditions (Supplementary Methods). However, yields of 38 mg/L were observed after substituting EZ Rich defined medium for LB medium (Figure 1c).

The Pf-5 *phlD* sequence had also been used as the basis for a directed evolution search for improved activity at higher temperatures.[43] We sought to determine whether a broader search of environmental strains could yield a more active starting enzyme. The Air Force Research Lab (AFRL) selected 52 *Pseudomonas* spp. strains it had collected as part of a microbial library of isolates from 17 interior locations of different aircraft (Figure 1b). Because the genomes were not available, primers were designed to target conserved regions of *phlD* for six clades, as determined using sequences available in NCBI (Supplementary Methods). These primers were able to amplify three

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genes, one each from *P. protogens* P772 (flight deck), *P. aeruginosa* P79 (hydraulic fluid), and *P. aeruginosa* WR202 (cargo area). The *P. protogens* gene was found to be identical at the DNA level to the *phlD* gene from *P. protogens* Pf-5. Likewise, the enzyme sequences from the two *P. aeruginosa* strains were identical to each other and to those found in other genomes of this species, but only share 71% amino acid identity with PhlD from *P. protogens* Pf-5. When expressed under identical conditions, this gene produces 3-fold increased titer in *E. coli* (116 mg/L) (Figure 1c) and was thus used in subsequent engineering efforts.

Evaluation of *Marinobacter*, *Bacillus*, and *Clostridium* as Alternative Chassis. We sought to investigate nonmodel organisms with naturally high levels of malonyl-CoA, focusing on *Marinobacter atlanticus* CP1. *M. atlanticus* CP1 was isolated from a biocathode microbial community enriched from sediment and seawater collected from the Rutgers University Marine Field Station (New Jersey, USA) and studied for the community's ability to fix carbon dioxide and store reduced carbon. In general, *Marinobacter* are studied for to their ability to thrive in diverse environments,[45−47] and for production of surfactants, siderophores, and wax esters.[48] Wax ester production is presumed to be used for carbon storage, and it is of particular relevance to PG production because it indicates that the organism maintains a large pool of acyl-CoA's.[48−50] Previously, we assembled *M. atlanticus* CP1 strains in which the wax-producing genes *farA* and *acrB* were removed.[51] In the absence of the wax ester end point, we reasoned that this strain would accumulate high levels of acyl-CoA's, including malonyl-CoA.

To test PG production, the Pa−P79 *phlD* gene was introduced into both wild-type (WT) CP1 and the double-knockout strain (Δ*farA*Δ*acrB*). The gene was constitutively expressed under a *lac* promoter in a plasmid with a pBBR1MCS-2 backbone and a kanamycin selection marker. Strains were transformed by conjugation with the *E. coli* donor strain WM3064 (a diaminopimelic acid auxotroph) and transformation was confirmed by PCR. It has previously been shown that the deletion of *farA* and *acrB* had no effect on growth compared to the WT in either rich medium (BB) or artificial seawater (ASW) medium supplemented with succinate (26 mM), and both strains reached stationary phase within 24 h in both media with a doubling time of approximately 2−2.5 h when grown on organic acids.[51] Wax ester synthesis does not occur in the Δ*farA*Δ*acrB* mutant (Figure 2b).[51]

PG production was evaluated in both WT and double-knockout strains, using succinate (26 mM)-supplemented artificial seawater (ASW) medium with wax ester promoting levels of nitrogen[49,50] or rich medium composed of 50% lysogeny broth and 50% marine broth (BB). Compared to wild-type *Marinobacter*, the double-knockout exhibited a 41% increase in production in ASW and a 91% increase in production in rich medium at 100 h, ultimately yielding 191.1 mg/L in rich medium at 100 h (Figure 2e).

In parallel, to explore other chassis, the Pf-5 *phlD* gene was codon optimized for expression in *Bacillus subtilis*, assembling plasmids for two different transformation strategies. For electroporation, the gene was cloned into vector pHT01 from MoBiTec, in which expression is driven by the P_{grac01} promoter under inducible control. For inducible conjugation, the gene was also cloned into integrative plasmid pJAB980, which integrates target genes into the starch utilization locus (*amyE*) of the *Bacillus* chromosome.[52] The former was transformed into *B. subtilis* 168 Marburg, and the latter into *B. subtilis* XPORT strain JAB932 (to enable rapid transfer into environmental Gram-positive isolates).[52] Both transformations were verified via colony PCR. Three clones generated by each approach were grown for a 24 and 48 h in 2xYT medium, but no PG was detected via LC−MS analysis.

Clostridium acetobutylicum ATCC 824 is an anaerobic spore-forming bacterium, historically used for fermentation of a broad range of C_6 and C_5 sugars to acetone, butanol, and ethanol.[53] To our knowledge, no synthetic polyketide pathway has ever been implemented in an obligate anaerobe. *C. acetobutylicum* polyketide biosynthesis, important for sporulation and granulose formation, requires malonyl-CoA as a precursor.[54] To explore PG production, shuttle vector variants containing a thermostable *phlD* variant ("4x-2")[43] were constructed (Supplementary Table S2). In all variants, *phlD* expression was driven by the *C. acetobutylicum* thiolase (*CA_C2873*) promoter under inducible regulation of a theophylline riboswitch[55] in most variants (Figure 2d). In order to survey plasmid copy number, plasmids were constructed with the four different Gram-positive replication origins provided with the pMTL80000 modular plasmid system.[56] In addition, to facilitate the detection of the protein, nucleotides encoding a C-terminal hemagglutinin tag were added to $phlD^{4x\text{-}2}$ in one of the plasmids.

Plasmids were transformed into wild-type *C. acetobutylicum* ATCC 824 via electroporation and grown in liquid Reinforced Clostridial Medium containing 2% glucose and 15 μM thiamphenicol in an anaerobic chamber (Supplementary Methods). Cultures were induced with 2 mM theophylline for 48−80 h. Supernatants were analyzed for PG by HPLC (Supplementary Methods). Among the four replication origins tested, pIM13 yielded the highest plasmid copy number in *C. acetobutylicum* as measured by real-time PCR (Supplementary Figure S8). Inducible expression of full length PhlD was confirmed by Western blot (Figure 2f). However, we did not detect PG from any of the *C. acetobutylicum* strains under the conditions described. When *E. coli* harboring these plasmids (with the Gram-negative ColE1 origin) was cultured aerobically in rich medium, up to 200 μM (25 mg/L) of PG was detected (not shown), indicating the plasmids themselves were not defective. Further investigation revealed that *C. acetobutylicum* degrades or consumes up to 10 g/L of externally-supplemented PG (data not shown).

PG-Responsive Genetic Sensors (*in Vivo* and *in Vitro*). Genetic sensors respond to small molecules by changing the activity of a promoter. When encoded in the DNA in a living cell, genetic sensors can serve as an input to circuits, such as to implement feedback control[57−59] or logic to turn on gene expression in response to specific conditions.[11] *In vitro* or "cell free" systems are increasingly being used to prototype genetic systems[60−62] and serve as stable and nonliving sensors, for example as viral diagnostics,[63,64] identifying plant pathogens,[65] and sensing small molecules.[66−70] Genetic sensors have also been used as part of high-throughput screens, where cells that respond to the product are used to detect improvements in a pathway faster and more cheaply than possible with traditional analytical approaches.[71−73] To these ends, we sought to create a genetic sensor for PG that operates in *E. coli* and then port it to a cell-free system.

A set of putative PG-responsive transcription factors was collected. The *P. fluorescens* PhlF repressor senses diacetylphloroglucinol (DAPG)[74] and *P. protegens* PltR responds to mono- and dichlorinated phloroglucinols.[75] We also included a

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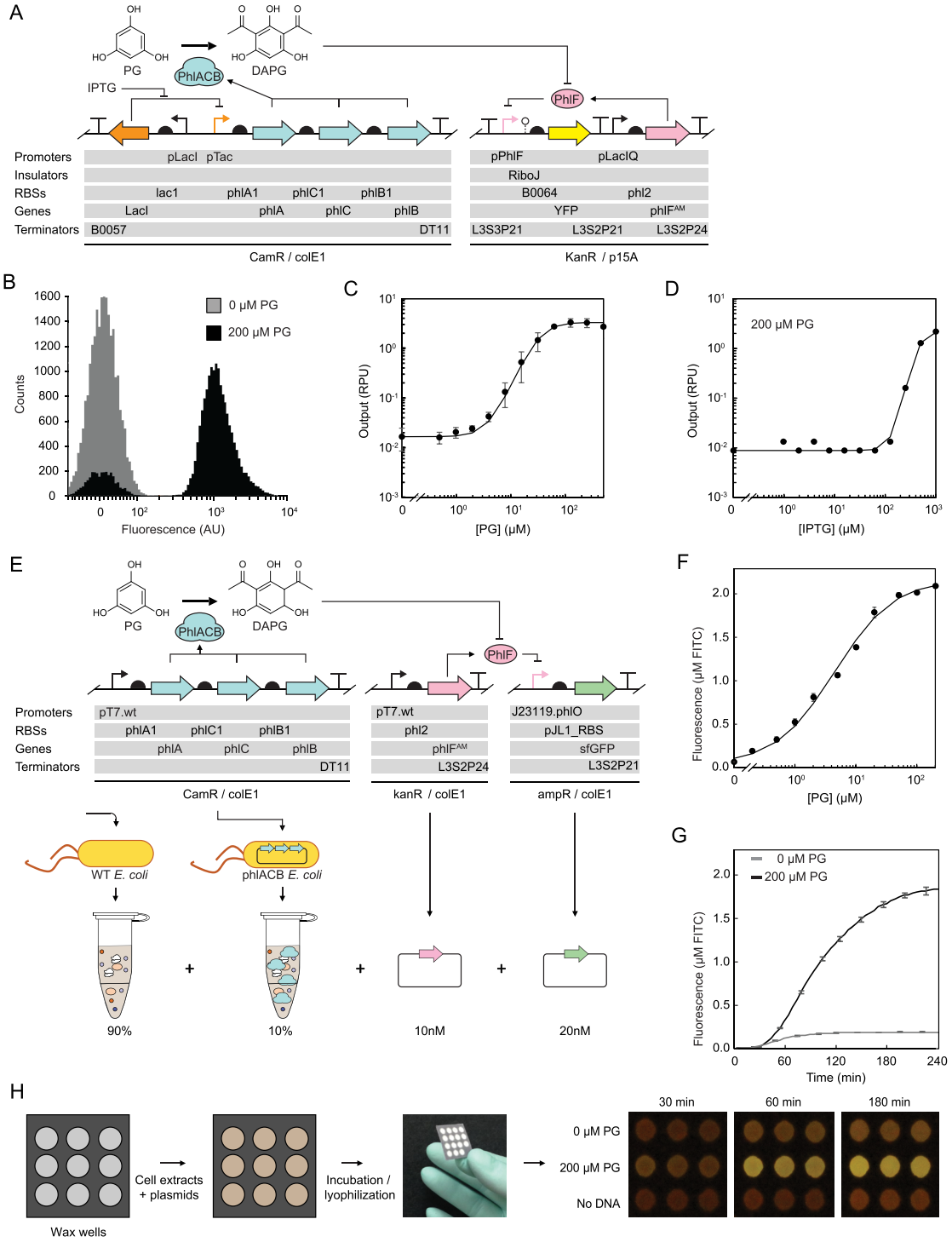

Figure 3. Phloroglucinol-responsive sensors. (A) Genetic diagram for the *in vivo* PG sensor (genetic parts are provided in Supplementary Table S3). (B) Flow cytometry histograms showing the induction of the PG sensor expressed in *E. coli*. Cells were induced for 5 h in LB medium with antibiotics (Supplementary Methods). (C) Response of the cell-based sensing system to PG and Hill function fit (K_d = 7.5 μM) using the evolved sensor protein PhlF^{AM}. The response is shown during exponential growth in medium with 1 mM IPTG (Supplementary Methods). Data for the stationary phase response are provided in Supplementary Figure S5. The fit curve is generated by entering the Hill function into Solver in Microsoft Excel (Supplementary Methods). (D) Response of the *in vivo* sensing system to induction of the *phlACB* operon with IPTG using the evolved regulator protein PhlF^{AM}. (E) Design of the three-plasmid *in vitro* sensor system (genetic parts are provided in Supplementary Table S4). The plasmid with the *phlACB* operon is expressed in *E. coli*, which is then lysed to generate an extract containing active proteins. This extract is combined with an extract from WT *E. coli* (10%/90%), and with plasmids containing the regulator (*phlF*^{AM}) and reporter (*sfGFP*). (F) Response of the *in vitro* sensing system to PG, with Hill function fit (K_d = 13.6 μM) computed as above. (G) Time course of the *in vitro* sensor's response to PG. (H) Deployment of the cell-free sensor in a paper ticket format. Paper tickets were then freeze-dried and exposed to PG (200 μM) or buffer. Images show microscopy of PG-induced fluorescence in wells across three technical replicates. For the responses of the cell-based and *in vitro* systems, error bars represent the standard deviation of fluorescence output from three independent experiments.

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PhlF variant that we have previously identified to have enhanced specificity ($PhlF^{AM}$).[76] Some sensors respond to monoacetylphloroglucinol (MAPG), but none to PG (Supplementary Figure S2b). We also tested repressors that respond to other substituted benzenes.[76] These included sensors of cuminic acid ($CymR^{AM}$), vanillic acid ($VanR^{AM}$), 3,4-dihydroxybenzoic acid (DHBA) ($PcaU^{AM}$), and salicylate ($NahR^{AM}$), none of which responded to PG (Supplementary Figure S2a).

We decided to express enzymes that metabolically convert PG to a compound for which we have a sensor, a strategy taken previously to respond to other compounds.[73,77−79] There is a well-characterized pathway from *P. fluorescens* (*phlACB*) that converts PG to 2,4-diacetylphloroglucinol (DAPG), for which we have previously evolved high-performance sensors (Figure 3a). Several designs were tested for the expression of the PhlACB enzymes under IPTG-inducible control (Supplementary Figure S10e-f). The best sensor and its response function in different growth phases are shown in Figure 3b, 3c and Supplementary Figure S5. It produces 97-fold induction with a threshold of 33 μM and sensitivity of 2 μM. The response is also specific to the expression of *phlACB* (Figure 3d).

The sensor was then adapted to a cell-free system. Such systems have been demonstrated to detect quorum-sensing molecules, including those produced by pathogens,[80−82] and for RNAs from gut microbes and Ebola virus,[63,83] among others. Some of these systems are based on a genetic sensor, where a protein binds to the target molecule and up or downregulates a promoter. However, *in vitro* sensing systems that also include enzymatic processing of the molecule to produce a detectable compound have only recently been demonstrated.[67,84]

To develop a cell-free PG sensor, we first tested the ability of the PhlF variants to sense DAPG *in vitro*. The dynamic range of each variant was tested for its ability to repress sfGFP expression from a pTac promoter fused to the phlO operator sequence by adding a plasmid encoding the transcription factor to *E. coli* cell extracts containing the pTac-phlO-sfGFP construct and supplemented with T7 polymerase.[85] $PhlF^{AM}$ worked best, repressing expression by 90% and exhibiting a response to DAPG (Supplementary Figure S3). However, expression was low, with maximal fluorescence of less than 1 μM equivalent of fluorescein isothiocyanate (FITC; Supplementary Methods). To increase expression, the pTac promoter of the reporter was replaced by a σ^{70} promoter based on J23119[85] in which a phlO operator was placed between the −10 and −35 sites (Figure 3e). This design increased the maximum output by 10-fold without reducing the response to DAPG (Supplementary Table S3).

With the ability to sense DAPG at hand, we next tried to add *phlACB* to the *in vitro* system to enable PG detection. The acyltransferase needs to form a complex $Phl(A_2C_2)_2B_4$ heterododecamer for activity.[86] The first approach was to coexpress the genes as an operon under the control of a T7 RNAP promoter from a separate plasmid (Supplementary Figure S10k). This did not lead to a functional sensor despite many rounds of optimization; in fact, even constitutive expression of sfGFP seemed to be inhibited (Supplementary Figure S3c). Radioactive ^{14}C-leucine incorporation studies were performed and these data imply that the proteins may aggregate when expressed in our *in vitro* system (Supplementary Figure S3d). Therefore, an alternative approach was taken to obtain active PhlACB. These proteins were expressed *in vivo* in *E. coli* to make a cell-free extract pre-enriched with the PhlACB enzymes. To ensure sufficient resources were available for transcription and translation of the transcription factor and reporter plasmids, this extract was then combined with unenriched (WT) *E. coli* extract in a 10:90 ratio along with the plasmids (Supplementary Methods). The plasmids containing the transcription factor and the reporter were then added to this mixture (Figure 3e and Supplementary Methods). In this formulation, the "On" signal can be observed above background in approximately 1 h with a 9.8-fold activation over background in the presence of 200 μM PG, with a detection threshold of 13.6 μM and sensitivity of 0.2 μM (Figure 3f).

Paper-based platforms have recently been reported for field-ready deployment in which cell-free systems are freeze-dried and reconstituted at test time. These platforms are cheap to produce, eliminate cold-chain requirements and can have year-long shelf-lives.[63,87] Because PG can be used as a precursor to energetic materials,[88] we explored prospects for adapting the sensing system for environmental surveillance.

The PG-sensing *in vitro* system was then tailored to a paper ticket format, which has been advanced by the U.S. Army Combat Capabilities Development Command Chemical Biological Center.[89] First, compatibility with the paper format was tested. Wax reaction wells were printed onto chromatography paper, and the lysate mixture was added to each well. When 200 μM PG was added to each well, there was a visible signal difference compared to negative controls (Supplementary Figure S7a). Next, performance was optimized by tuning plasmid concentrations, and tickets were lyophilized (Figure 3h). The best performance was observed when extracts and plasmids were deposited into wax wells in slightly different proportions than in tubes, with the sensor plasmid at 5 nM and the reporter plasmid at 10 nM. The resulting devices exhibited 2-fold induction by PG within 30 min and the signal was sustained for >7 h (Supplementary Figure S7d).

DISCUSSION

This manuscript describes a coordinated, interorganizational effort to address an organism engineering problem defined by an external need. There was no formal infrastructure in place to enable coordination of the project. Groups were aware of expertise and capabilities through personal relationships and data, knowledge, and materials were transferred in an *ad hoc* manner. Still, this project led to surprising and unpredictable findings that would not have been possible without distributed expertise. In this case, the best solution was an arcane species of bacteria isolated from the ocean, carrying a gene from an unrelated bacterium from the hydraulics of an aircraft, and whose genome was modified to disrupt alternative routes for carbon flux. In finding these solutions, we were able to screen alternative strains from soil including a difficult-to-modify *Clostridium* and create an *in vitro* system that can be used later for rapid prototyping and further improvement.

As genome engineering becomes more complex, the number of groups participating in the design phase of a project will expand. When exchanging materials and coordinating diverse capabilities across organizations, the greatest challenges involve ensuring compatibility of experimental designs, reagents, and experimental findings. Currently, there are few formalized tools available to facilitate this exchange, so teams must instead develop comprehensive, mutual understanding of each other's capabilities, methods, and results. This requires extensive communication between groups, ranging from high-level design specifications, such as for assays or DNA constructs, down to mundane experimental details, such as selection of time points, standardization of common protocols, sample labeling con-

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ventions, or DNA sequences. Infrastructure is needed to streamline this kind of coordination, including computer aided design software, standards for data and protocol/expertise sharing, parts repositories, and national strain banks.[90−93]

After the design phase, organism construction and testing also require coordination between multiple specialized facilities, including DNA synthesis, genome construction/editing, -omics, high-throughput product screening, purification, and analytical chemistry. The design efforts have to be closely coordinated with the end use, whether it be to enter scale-up in production, field trials, or clinical testing. For example, TATB synthesized from biologically produced PG produces particles with different morphology and performance characteristics than TATB synthesized from 1,3,4-trichlorobenzene.[94] While other engineering fields routinely coordinate specialties for a large design project, this is at an early stage in biotechnology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00393.

Detailed methods and materials, Supplementary Figures S1−S10, and Supplementary Tables S1−S4 (PDF)

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¶A. Meyer, I. Saaem, A. Silverman, and V. Varaljay contributed equally to this work, and are listed in alphabetical order. C.A.V. conceived of the overarching study. C.A.V. and D.B.G. wrote the manuscript, and I.S. compiled the supplementary methods. C.B., S.L., K.P., and I.S. built plasmids, performed the *E. coli* work, and performed assays on *Marinobacter* samples with oversight by I.S. and D.B.G.; R.M. and E.O. performed the *Marinobacter* work, with oversight by S.G.; V.V. and C.D. performed the *Pseudomonas* work, with oversight by M.G., N.K.-L., and W.C.-G.; R.E. and A.C. performed the linalool work, with oversight by D.B.G.; A.T., N.D.S., and W.M. performed the *Clostridium* work with oversight by K.A.; A.M., A.S., S.B., and F.M. performed the sensor work, with oversight by J.L., M.L., and M.C.J.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Bick, M. J., Greisen, P. J., Morey, K. J., Antunes, M. S., La, D., Sankaran, B., Reymond, L., Johnsson, K., Medford, J. I., and Baker, D. (2017) Computational design of environmental sensors for the potent opioid fentanyl. *eLife 6*, e28909.

(2) Gilbert, C., and Ellis, T. (2019) Biological Engineered Living Materials: Growing Functional Materials with Genetically Programmable Properties. *ACS Synth. Biol. 8*, 1−15.

(3) Glaven, S., Racicot, K., Leary, D. H., Karl, J. P., Arcidiacono, S., Dancy, B. C. R., Chrisey, L. A., and Soares, J. W. (2018) The Current and Future State of Department of Defense (DoD) Microbiome Research: a Summary of the Inaugural DoD Tri-Service Microbiome Consortium Informational Meeting. *mSystems 3*, e00086-18.

(4) Isabella, V. M., Ha, B. N., Castillo, M. J., Lubkowicz, D. J., Rowe, S. E., Millet, Y. A., Anderson, C. L., Li, N., Fisher, A. B., West, K. A., Reeder, P. J., Momin, M. M., Bergeron, C. G., Guilmain, S. E., Miller, P. F., Kurtz, C. B., and Falb, D. (2018) Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nat. Biotechnol. 36*, 857.

(5) Casini, A., Chang, F.-Y., Eluere, R., King, A. M., Young, E. M., Dudley, Q. M., Karim, A., Pratt, K., Bristol, C., Forget, A., Ghodasara, A., Warden-Rothman, R., Gan, R., Cristofaro, A., Borujeni, A. E., Ryu, M.-H., Li, J., Kwon, Y.-C., Wang, H., Tatsis, E., Rodriguez-Lopez, C., O'Connor, S., Medema, M. H., Fischbach, M. A., Jewett, M. C., Voigt, C., and Gordon, D. B. (2018) A Pressure Test to Make 10 Molecules in 90 Days: External Evaluation of Methods to Engineer Biology. *J. Am. Chem. Soc. 140*, 4302−4316.

(6) Lin, G.-M., Warden-Rothman, R., and Voigt, C. A. (2019) Retrosynthetic design of metabolic pathways to chemicals not found in nature. *Curr. Opin. Syst. Biol. 14*, 82−107.

(7) Shemer, B., Palevsky, N., Yagur-Kroll, S., and Belkin, S. (2015) Genetically engineered microorganisms for the detection of explosives' residues. *Front. Microbiol. 6*, 1175.

(8) Antunes, M. S., Morey, K. J., Smith, J. J., Albrecht, K. D., Bowen, T. A., Zdunek, J. K., Troupe, J. F., Cuneo, M. J., Webb, C. T., Hellinga, H. W., and Medford, J. I. (2011) Programmable Ligand Detection System in Plants through a Synthetic Signal Transduction Pathway. *PLoS One 6*, e16292.

(9) Schellenberger, J., Que, R., Fleming, R. M. T., Thiele, I., Orth, J. D., Feist, A. M., Zielinski, D. C., Bordbar, A., Lewis, N. E., Rahmanian, S., Kang, J., Hyduke, D. R., and Palsson, B. Ø. (2011) Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0. *Nat. Protoc. 6*, 1290.

(10) Burgard, A. P., Pharkya, P., and Maranas, C. D. (2003) Optknock: A bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. *Biotechnol. Bioeng. 84*, 647−657.

(11) Nielsen, A. A. K., Der, B. S., Shin, J., Vaidyanathan, P., Paralanov, V., Strychalski, E. A., Ross, D., Densmore, D., and Voigt, C. A. (2016) Genetic circuit design automation. *Science 352*, aac7341.

DOI: 10.1021/acssynbio.9b00393
ACS Synth. Biol. 2019, 8, 2746−2755

(12) Xia, B., Bhatia, S., Bubenheim, B., Dadgar, M., Densmore, D., and Anderson, J. C. (2011) Developer's and User's Guide to Clotho v2.0: A Software Platform for the Creation of Synthetic Biological Systems, In *Methods in Enzymology* (Voigt, C., Ed.), Chapter 5, pp 97−135, Academic Press.
(13) de Ridder, D. (2019) Artificial intelligence in the lab: ask not what your computer can do for you. *Microb. Biotechnol. 12*, 38−40.
(14) Richardson, S. M., Mitchell, L. A., Stracquadanio, G., Yang, K., Dymond, J. S., DiCarlo, J. E., Lee, D., Huang, C. L. V., Chandrasegaran, S., Cai, Y., Boeke, J. D., and Bader, J. S. (2017) Design of a synthetic yeast genome. *Science 355*, 1040.
(15) Beal, J., Adler, A., and Yaman, F. (2016) Managing bioengineering complexity with AI techniques. *BioSystems 148*, 40−46.
(16) Wackett, L. P. (2014) Microbial strain collections and information: An annotated selection of World Wide Web sites relevant to the topics in microbial biotechnology. *Microb. Biotechnol. 7*, 371−372.
(17) Lutgring, J. D., Machado, M. J., Benahmed, F. H., Conville, P., Shawar, R. M., Patel, J., and Brown, A. C. (2018) FDA-CDC Antimicrobial Resistance Isolate Bank: a Publicly Available Resource To Support Research, Development, and Regulatory Requirements. *J. Clin. Microbiol.*, DOI: 10.1128/JCM.01415-17.
(18) Wu, L., McCluskey, K., Desmeth, P., Liu, S., Hideaki, S., Yin, Y., Moriya, O., Itoh, T., Kim, C. Y., Lee, J. S., Zhou, Y., Kawasaki, H., Hazbon, M. H., Robert, V., Boekhout, T., Lima, N., Evtushenko, L., Boundy-Mills, K., Bunk, B., Moore, E. R. B., Eurwilaichitr, L., Ingsriswang, S., Shah, H., Yao, S., Jin, T., Huang, J., Shi, W., Sun, Q., Fan, G., Li, W., Li, X., Kurtboke, I., and Ma, J. (2018) The global catalogue of microorganisms 10K type strain sequencing project: closing the genomic gaps for the validly published prokaryotic and fungi species. *GigaScience*, DOI: 10.1093/gigascience/giy026.
(19) Agriculture Collection of China, http://www.accc.org.cn (accessed Sep 5, 2019).
(20) Russian Academy of Sciences All-Russian Collection of Microorganisms, http://www.vkm.ru/ (accessed Sep 5, 2019).
(21) National Centre for Cell Science Repositories, India, https://www.nccs.res.in/index.php/TeamsNCCS/Repositories (accessed Sep 5, 2019).
(22) Berger, K. M., and Schneck, P. A. (2019) National and Transnational Security Implications of Asymmetric Access to and Use of Biological Data. *Front. Bioeng. Biotechnol. 7*, 21.
(23) Herscovitch, M., Perkins, E., Baltus, A., and Fan, M. (2012) Addgene provides an open forum for plasmid sharing. *Nat. Biotechnol. 30*, 316.
(24) Cooling, M. T., Rouilly, V., Misirli, G., Lawson, J., Yu, T., Hallinan, J., and Wipat, A. (2010) Standard virtual biological parts: a repository of modular modeling components for synthetic biology. *Bioinformatics 26*, 925−931.
(25) Kanehisa, M., and Goto, S. (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res. 28*, 27−30.
(26) NCBI Resource Coordinators (2017) Database Resources of the National Center for Biotechnology Information. *Nucleic Acids Res. 45*, D12−D17.
(27) Lin, K., Kools, H., de Groot, P. J., Gavai, A. K., Basnet, R. K., Cheng, F., Wu, J., Wang, X., Lommen, A., Hooiveld, G. J. E. J., Bonnema, G., Visser, R. G. F., Muller, M. R., and Leunissen, J. A. M. (2011) MADMAX − Management and analysis database for multiple ~omics experiments. *J. Integr. Bioinf. 8*, 59.
(28) Dai, X., Wang, G., Yang, D. S., Tang, Y., Broun, P., Marks, M. D., Sumner, L. W., Dixon, R. A., and Zhao, P. X. (2010) TrichOME: A Comparative Omics Database for Plant Trichomes. *Plant Physiol. 152*, 44.
(29) Sakurai, N., Ara, T., Ogata, Y., Sano, R., Ohno, T., Sugiyama, K., Hiruta, A., Yamazaki, K., Yano, K., Aoki, K., Aharoni, A., Hamada, K., Yokoyama, K., Kawamura, S., Otsuka, H., Tokimatsu, T., Kanehisa, M., Suzuki, H., Saito, K., and Shibata, D. (2011) KaPPA-View4: a metabolic pathway database for representation and analysis of correlation networks of gene co-expression and metabolite co-accumulation and omics data. *Nucleic Acids Res. 39*, D677−D684.
(30) Liu, W., Christenson, S. D., Standage, S., and Shen, B. (2002) Biosynthesis of the Enediyne Antitumor Antibiotic C-1027. *Science 297*, 1170−1173.
(31) American Type Culture Collection (ATCC), https://www.atcc.org (accessed Sep 5, 2019).
(32) Amiri, P., Shahpiri, A., Asadollahi, M. A., Momenbeik, F., and Partow, S. (2016) Metabolic engineering of Saccharomyces cerevisiae for linalool production. *Biotechnol. Lett. 38*, 503−508.
(33) Singh, I. P., Sidana, J., Bansal, P., and Foley, W. J. (2009) Phloroglucinol compounds of therapeutic interest: global patent and technology status. *Expert Opin. Ther. Pat. 19*, 847−866.
(34) Abdel-Ghany, S. E., Day, I., Heuberger, A. L., Broeckling, C. D., and Reddy, A. S. (2016) Production of Phloroglucinol, a Platform Chemical, in Arabidopsis using a Bacterial Gene. *Sci. Rep. 6*, 38483.
(35) Janni, J. A., Sylvia, J. M., Clauson, S. L., and Spencer, K. M. (2002) *SERS Detection of the Nuclear Weapons Explosive Triaminotrinitrobenzene*, Vol. *4577*, SPIE.
(36) Albright, D. (1994) South Africa and the Affordable Bomb. *Bull. At. Sci. 50*, 37−47.
(37) Biello, D. (2007) A Need for New Warheads?, In *Scientific American*.
(38) Wang, J., Li, H., Cai, Y., Wang, D., Bian, L., Dong, F., Yu, H., and He, Y. (2019) Direct Blue Light-Induced Autocatalytic Oxidation of o-Phenylenediamine for Highly Sensitive Visual Detection of Triaminotrinitrobenzene. *Anal. Chem. 91*, 6155−6161.
(39) Cao, Y., Jiang, X., Zhang, R., and Xian, M. (2011) Improved phloroglucinol production by metabolically engineered Escherichia coli. *Appl. Microbiol. Biotechnol. 91*, 1545−1552.
(40) Yang, F., and Cao, Y. (2012) Biosynthesis of phloroglucinol compounds in microorganisms–review. *Appl. Microbiol. Biotechnol. 93*, 487−495.
(41) Achkar, J., Xian, M., Zhao, H., and Frost, J. W. (2005) Biosynthesis of phloroglucinol. *J. Am. Chem. Soc. 127*, 5332−5333.
(42) Zhang, R., Cao, Y., Liu, W., Xian, M., and Liu, H. (2017) Improving phloroglucinol tolerance and production in Escherichia coli by GroESL overexpression. *Microb. Cell Fact. 16*, 227.
(43) Rao, G., Lee, J. K., and Zhao, H. (2013) Directed evolution of phloroglucinol synthase PhlD with increased stability for phloroglucinol production. *Appl. Microbiol. Biotechnol. 97*, 5861−5867.
(44) Xu, X., Xian, M., and Liu, H. (2017) Efficient conversion of acetate into phloroglucinol by recombinant Escherichia coli. *RSC Adv. 7*, 50942−50948.
(45) Singer, E., Webb, E. A., Nelson, W. C., Heidelberg, J. F., Ivanova, N., Pati, A., and Edwards, K. J. (2011) Genomic potential of Marinobacter aquaeolei, a biogeochemical "opportunitroph". *Appl. Environ. Microbiol. 77*, 2763−2771.
(46) Rowe, A. R., Chellamuthu, P., Lam, B., Okamoto, A., and Nealson, K. H. (2015) Marine sediments microbes capable of electrode oxidation as a surrogate for lithotrophic insoluble substrate metabolism. *Front. Microbiol. 5*, 784.
(47) Wang, Z., Leary, D. H., Malanoski, A. P., Li, R. W., Hervey, W. J. t., Eddie, B. J., Tender, G. S., Yanosky, S. G., Vora, G. J., Tender, L. M., Lin, B., and Strycharz-Glaven, S. M. (2015) A previously uncharacterized, nonphotosynthetic member of the Chromatiaceae is the primary CO2-fixing constituent in a self-regenerating biocathode. *Appl. Environ. Microbiol. 81*, 699−712.
(48) Knutson, C. M., Lenneman, E. M., and Barney, B. M. (2019) Marinobacter as a Model Organism for Wax Ester Accumulation in Bacteria, In *Biogenesis of Fatty Acids, Lipids and Membranes* (Geiger, O., Ed.), pp 237−258, Springer International Publishing, Cham.
(49) Barney, B. M., Wahlen, B. D., Garner, E., Wei, J., and Seefeldt, L. C. (2012) Differences in substrate specificities of five bacterial wax ester synthases. *Appl. Environ. Microbiol. 78*, 5734−5745.
(50) Lenneman, E. M., Ohlert, J. M., Palani, N. P., and Barney, B. M. (2013) Fatty alcohols for wax esters in Marinobacter aquaeolei VT8: two optional routes in the wax biosynthesis pathway. *Appl. Environ. Microbiol. 79*, 7055−7062.
(51) Bird, L. J., Wang, Z., Malanoski, A. P., Onderko, E. L., Johnson, B. J., Moore, M. H., Phillips, D. A., Chu, B. J., Doyle, J. F., Eddie, B. J., and

DOI: 10.1021/acssynbio.9b00393
ACS Synth. Biol. 2019, 8, 2746−2755

Glaven, S. M. (2018) Development of a Genetic System for Marinobacter atlanticus CP1 (sp. nov.), a Wax Ester Producing Strain Isolated From an Autotrophic Biocathode. *Front. Microbiol. 9*, 3176.
(52) Brophy, J. A. N., Triassi, A. J., Adams, B. L., Renberg, R. L., Stratis-Cullum, D. N., Grossman, A. D., and Voigt, C. A. (2018) Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. *Nat. Microbiol. 3*, 1043−1053.
(53) Moon, H. G., Jang, Y. S., Cho, C., Lee, J., Binkley, R., and Lee, S. Y. (2016) One hundred years of clostridial butanol fermentation. *FEMS Microbiol. Lett. 363*, fnw001.
(54) Herman, N. A., Kim, S. J., Li, J. S., Cai, W., Koshino, H., and Zhang, W. (2017) The industrial anaerobe Clostridium acetobutylicum uses polyketides to regulate cellular differentiation. *Nat. Commun. 8*, 1514.
(55) Nakahira, Y., Ogawa, A., Asano, H., Oyama, T., and Tozawa, Y. (2013) Theophylline-dependent riboswitch as a novel genetic tool for strict regulation of protein expression in Cyanobacterium Synechococcus elongatus PCC 7942. *Plant Cell Physiol. 54*, 1724−1735.
(56) Heap, J. T., Pennington, O. J., Cartman, S. T., and Minton, N. P. (2009) A modular system for Clostridium shuttle plasmids. *J. Microbiol. Methods 78*, 79−85.
(57) Moser, F., Espah Borujeni, A., Ghodasara, A. N., Cameron, E., Park, Y., and Voigt, C. A. (2018) Dynamic control of endogenous metabolism with combinatorial logic circuits. *Mol. Syst. Biol. 14*, e8605.
(58) Steen, E. J., Chan, R., Prasad, N., Myers, S., Petzold, C. J., Redding, A., Ouellet, M., and Keasling, J. D. (2008) Metabolic engineering of Saccharomyces cerevisiae for the production of n-butanol. *Microb. Cell Fact. 7*, 36.
(59) Gupta, A., Reizman, I. M. B., Reisch, C. R., and Prather, K. L. J. (2017) Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. *Nat. Biotechnol. 35*, 273−279.
(60) Karim, A. S., and Jewett, M. C. (2016) A cell-free framework for rapid biosynthetic pathway prototyping and enzyme discovery. *Metab. Eng. 36*, 116−126.
(61) Marshall, R., and Noireaux, V. (2018) Synthetic Biology with an All *E. coli* TXTL System: Quantitative Characterization of Regulatory Elements and Gene Circuits, In *Synthetic Biology: Methods and Protocols* (Braman, J. C., Ed.), pp 61−93, Springer, New York, NY.
(62) Pandey, A., and Murray, R. M. (2019) An automated model reduction tool to guide the design and analysis of synthetic biological circuits. *bioRxiv*, 640276.
(63) Takahashi, M. K., Tan, X., Dy, A. J., Braff, D., Akana, R. T., Furuta, Y., Donghia, N., Ananthakrishnan, A., and Collins, J. J. (2018) A low-cost paper-based synthetic biology platform for analyzing gut microbiota and host biomarkers. *Nat. Commun.*, DOI: 10.1038/s41467-018-05864-4.
(64) Ma, D., Shen, L., Wu, K., Diehnelt, C. W., and Green, A. A. (2018) Low-cost detection of norovirus using paper-based cell-free systems and synbody-based viral enrichment. *Synth. Biol., 3* DOI: 10.1093/synbio/ysy018.
(65) Verosloff, M., Chappell, J., Perry, K. L., Thompson, J. R., and Lucks, J. B. (2019) PLANT-Dx: A Molecular Diagnostic for Point-of-Use Detection of Plant Pathogens. *ACS Synth. Biol. 8*, 902−905.
(66) Salehi, A. S., Shakalli Tang, M. J., Smith, M. T., Hunt, J. M., Law, R. A., Wood, D. W., and Bundy, B. C. (2017) Cell-Free Protein Synthesis Approach to Biosensing hTRbeta-Specific Endocrine Disruptors. *Anal. Chem. 89*, 3395−3401.
(67) Voyvodic, P. L., Pandi, A., Koch, M., Conejero, I., Valjent, E., Courtet, P., Renard, E., Faulon, J.-L., and Bonnet, J. (2019) Plug-and-play metabolic transducers expand the chemical detection space of cell-free biosensors. *Nat. Commun. 10*, 1697.
(68) Alam, K. K., Jung, J. K., Verosloff, M. S., Clauer, P. R., Lee, J. W., Capdevila, D. A., Pastén, P. A., Giedroc, D. P., Collins, J. J., and Lucks, J. B. (2019) Rapid, Low-Cost Detection of Water Contaminants Using Regulated In Vitro Transcription. *bioRxiv*, 619296.
(69) Thavarajah, W., Silverman, A. D., Verosloff, M. S., Kelley-Loughnane, N., Jewett, M. C., and Lucks, J. B. (2019) Point-of-Use Detection of Environmental Fluoride via a Cell-Free Riboswitch-Based Biosensor. *bioRxiv*, 712844.
(70) Liu, X., Silverman, A. D., Alam, K. K., Iverson, E., Lucks, J. B., Jewett, M. C., and Raman, S. (2019) Design of a transcriptional biosensor for the portable, on-demand detection of cyanuric acid. *bioRxiv*, 736355.
(71) Zhang, F., Carothers, J. M., and Keasling, J. D. (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol. 30*, 354.
(72) Michener, J. K., Thodey, K., Liang, J. C., and Smolke, C. D. (2012) Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways. *Metab. Eng. 14*, 212−222.
(73) Rogers, J. K., and Church, G. M. (2016) Genetically encoded sensors enable real-time observation of metabolite production. *Proc. Natl. Acad. Sci. U. S. A. 113*, 2388−2393.
(74) Delany, I., Sheehan, M. M., Fenton, A., Bardin, S., Aarons, S., and O'Gara, F. (2000) Regulation of production of the antifungal metabolite 2,4-diacetylphloroglucinol in Pseudomonas fluorescens F113: genetic analysis of phlF as a transcriptional repressor. *Microbiology (London, U. K.) 146*, 537−546.
(75) Yan, Q., Philmus, B., Chang, J. H., and Loper, J. E. (2017) Novel mechanism of metabolic co-regulation coordinates the biosynthesis of secondary metabolites in Pseudomonas protegens. *eLife*, DOI: 10.7554/eLife.22835.
(76) Meyer, A. J., Segall-Shapiro, T. H., Glassey, E., Zhang, J., and Voigt, C. A. (2019) Escherichia coli "Marionette" strains with 12 highly optimized small-molecule sensors. *Nat. Chem. Biol. 15*, 196−204.
(77) Libis, V., Delepine, B., and Faulon, J. L. (2016) Expanding Biosensing Abilities through Computer-Aided Design of Metabolic Pathways. *ACS Synth. Biol. 5*, 1076−1085.
(78) Mohn, W. W., Garmendia, J., Galvao, T. C., and de Lorenzo, V. (2006) Surveying biotransformations with a la carte genetic traps: translating dehydrochlorination of lindane (gamma-hexachlorocyclohexane) into lacZ-based phenotypes. *Environ. Microbiol. 8*, 546−555.
(79) Lee, S. K., and Keasling, J. D. (2005) A propionate-inducible expression system for enteric bacteria. *Appl. Environ. Microbiol. 71*, 6856−6862.
(80) Kawaguchi, T., Chen, Y. P., Norman, R. S., and Decho, A. W. (2008) Rapid screening of quorum-sensing signal N-acyl homoserine lactones by an in vitro cell-free assay. *Appl. Environ. Microbiol. 74*, 3667−3671.
(81) Yang, Y. H., Kim, T. W., Park, S. H., Lee, K., Park, H. Y., Song, E., Joo, H. S., Kim, Y. G., Hahn, J. S., and Kim, B. G. (2009) Cell-Free Escherichia coli-Based System To Screen for Quorum-Sensing Molecules Interacting with Quorum Receptor Proteins of Streptomyces coelicolor. *Appl. Environ. Microbiol. 75*, 6367−6372.
(82) Wen, K. Y., Cameron, L., Chappell, J., Jensen, K., Bell, D. J., Kelwick, R., Kopniczky, M., Davies, J. C., Filloux, A., and Freemont, P. S. (2017) A Cell-Free Biosensor for Detecting Quorum Sensing Molecules in P. aeruginosa-Infected Respiratory Samples. *ACS Synth. Biol. 6*, 2293−2301.
(83) Pardee, K., Green, A. A., Takahashi, M. K., Braff, D., Lambert, G., Lee, J. W., Ferrante, T., Ma, D., Donghia, N., Fan, M., Daringer, N. M., Bosch, I., Dudley, D. M., O'Connor, D. H., Gehrke, L., and Collins, J. J. (2016) Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell 165*, 1255−1266.
(84) Pandi, A., Grigoras, I., Borkowski, O., and Faulon, J.-L. (2019) Optimizing Cell-Free Biosensors to Monitor Enzymatic Production. *ACS Synth. Biol. 8*, 1952−1957.
(85) Silverman, A. D., Kelley-Loughnane, N., Lucks, J. B., and Jewett, M. C. (2019) Deconstructing Cell-Free Extract Preparation for in Vitro Activation of Transcriptional Genetic Circuitry. *ACS Synth. Biol. 8*, 403−414.
(86) Pavkov-Keller, T., Schmidt, N. G., Żądło-Dobrowolska, A., Kroutil, W., and Gruber, K. (2019) Structure and Catalytic Mechanism of a Bacterial Friedel−Crafts Acylase. *ChemBioChem 20*, 88−95.

(87) Pardee, K., Green, A. A., Ferrante, T., Cameron, D. E., DaleyKeyser, A., Yin, P., and Collins, J. J. (2014) Paper-Based Synthetic Gene Networks. *Cell 159*, 940−954.
(88) Hoffman, D. M., and Fontes, A. T. (2010) Density Distributions in TATB Prepared by Various Methods, Propellants, Explos. *Pyrotech. 35*, 15−23.
(89) Mgboji, G., Blum, S., Funk, V., and Lux, M. (2018) Assessment of Paper Types for Cell Free Reactions, ECBC-TR-1527, https://apps.dtic.mil/dtic/tr/fulltext/u2/1057327.pdf.
(90) Marchisio, M. A., and Stelling, J. (2009) Computational design tools for synthetic biology. *Curr. Opin. Biotechnol. 20*, 479−485.
(91) Medema, M. H., van Raaphorst, R., Takano, E., and Breitling, R. (2012) Computational tools for the synthetic design of biochemical pathways. *Nat. Rev. Microbiol. 10*, 191.
(92) McLaughlin, J. A., Myers, C. J., Zundel, Z., Mısırlı, G., Zhang, M., Ofiteru, I. D., Goñi-Moreno, A., and Wipat, A. (2018) SynBioHub: A Standards-Enabled Design Repository for Synthetic Biology. *ACS Synth. Biol. 7*, 682−688.
(93) Densmore, D. M., and Bhatia, S. (2014) Bio-design automation: software + biology + robots. *Trends Biotechnol. 32*, 111−113.
(94) Final Report, SERDP WP-1582, http://serdp-estcp.org/content/download/12203/147268/file/WP-1582-FR.pdf (accessed Sep 5, 2019).

DOI: 10.1021/acssynbio.9b00393
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