

A Versatile Transcription Factor Biosensor System Responsive to Multiple Aromatic and Indole Inducers

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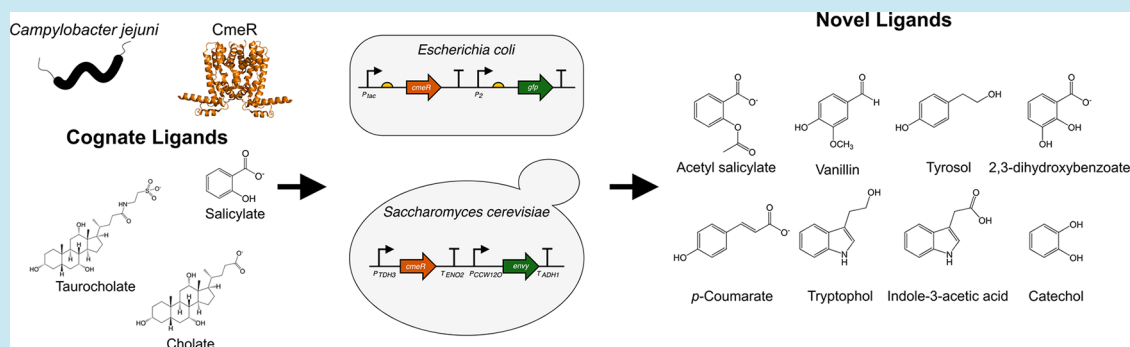
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ABSTRACT: Allosteric transcription factor (aTF) biosensors are valuable tools for engineering microbes toward a multitude of applications in metabolic engineering, biotechnology, and synthetic biology. One of the challenges toward constructing functional and diverse biosensors in engineered microbes is the limited toolbox of identified and characterized aTFs. To overcome this, extensive bioprospecting of aTFs from sequencing databases, as well as aTF ligand-specificity engineering are essential in order to realize their full potential as biosensors for novel applications. In this work, using the TetR-family repressor CmeR from *Campylobacter jejuni*, we construct aTF genetic circuits that function as salicylate biosensors in the model organisms *Escherichia coli* and *Saccharomyces cerevisiae*. In addition to salicylate, we demonstrate the responsiveness of CmeR-regulated promoters to multiple aromatic and indole inducers. This relaxed ligand specificity of CmeR makes it a useful tool for detecting molecules in many metabolic engineering applications, as well as a good target for directed evolution to engineer proteins that are able to detect new and diverse chemistries.

KEYWORDS: CmeR, aTF, biosensor, aromatics, genetic circuits, salicylic acid, indoles

Bacteria have evolved single and multicomponent transcriptional regulatory networks in order to respond to numerous chemical cues. One example of these are allosteric transcription factors (aTFs), which are single-component regulators that play diverse roles in controlling various metabolic processes.² For biosensing, aTFs have been used to alter transcription in response to small molecules in many applications, such as screening large strain or enzyme libraries,^{3,4} disease diagnostics,^{5,6} screening for environmental contaminants,^{7,8} among many others. One major constraint toward the use of aTFs as biosensors is their limited diversity with respect to the repertoire of molecules that they can sense,^{9,10} which makes identifying or engineering aTFs with novel ligand specificities important in order to provide the tools for next-generation biological engineering. This could be achieved by continually bioprospecting to identify and characterize previously unknown aTFs,^{11,12} or by investigating the ligand promiscuity of known aTFs.^{13–16} Other solutions to this limitation include directed evolution of aTFs toward novel ligands,^{17–20} construction and screening of novel fusion proteins as engineered aTFs,²¹ or by *de novo* protein design.²²

Synthetic biology is in constant need for characterized and robust aTF biosensors to implement in new applications. Toward this goal, we investigated the TetR-family repressor CmeR from the gastroenteric pathogen *Campylobacter jejuni*,^{23,24} which regulates the multidrug resistance efflux pump CmeABC in response to intestinal bile salts.²³ In addition to cholate and taurocholate, CmeR was also shown to bind salicylate and glycerol.^{25,26} This diversity in ligands, which includes large aliphatic bile salts as well as small aliphatic and aromatic molecules, has inspired us to explore the binding specificity of CmeR, and further examine its potential application in synthetic biology as a part in genetic circuit biosensors. In this work, we constructed CmeR genetic circuits

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in the model organisms *Escherichia coli* and *Saccharomyces cerevisiae* using the identified aTF/operator pair and the inducer salicylate,²⁵ which is a precursor to industrially relevant molecules such as muconate²⁷ and acetyl salicylate.^{28,29} Using our circuits, we probed the ligand specificity of CmeR, and identified novel aromatic and indole molecules that induce expression from CmeR-regulated promoters in both prokaryotic and eukaryotic model systems. While many of these molecules have already been identified as inducers for other regulated promoters,³⁰ tyrosol, tryptophol, and tyramine are, to our knowledge, novel inducers of aTF-regulated promoters. This work shows that CmeR is a broadly useful aTF candidate for directed evolution toward novel ligand specificities that belong to aromatics and indoles, and possibly other diverse functional groups.

MATERIALS AND METHODS

Strains, Growth Conditions, and Transformations.

E. coli DH5 α (Thermo Fisher Scientific) was used for all genetic circuit construction and characterization purposes. Unless otherwise stated, *E. coli* cells were cultured at 37 °C for ~16 h either in liquid or solid Luria–Bertani (LB) medium with or without kanamycin (50 μ g mL⁻¹) for plasmid selection. Chemically competent *E. coli* DH5 α ³¹ were transformed by heat shock for 50 s, and recovered in LB medium for 1 h without antibiotics, followed by plating on LB plates containing 50 μ g mL⁻¹ kanamycin.

S. cerevisiae manipulations were conducted in the prototroph CEN.PK113–7D. Strains were grown at 30 °C for ~16 or 48 h in liquid or solid yeast extract-peptone-dextrose (YPD) medium, respectively. Hygromycin (200 μ g mL⁻¹) and G418 (200 μ g mL⁻¹) were added for plasmid selection as needed. Yeast cells were transformed by heat shock following the standard Gietz PEG/LiOAc protocol scaled down to a 25 μ L volume.³² Cells were incubated for 15 min at 30 °C, heat-shocked for 30 min at 42 °C, followed by a recovery step in YPD for 16 h, after which cells were plated on YPD plates containing the appropriate antibiotics for selection.

Chemicals for Genetic Circuit Induction. Chemicals for CmeR circuit characterization were purchased from Sigma-Aldrich and dissolved in water or 25% ethanol as specified in parentheses to obtain 25 mM stock solutions: resorcinol (water), catechol (water), adipic acid (water), epinephrine (water), dopamine (water), protocatechuic acid (water), salicylate (water), 2,3-dihydroxybenzoate (water), acetyl salicylate (ethanol), tyrosol (water), tryptophol (water), tyramine (ethanol), tryptamine (ethanol), caffeic acid (ethanol), phenyl acetate (ethanol), 4-hydroxyphenylacetate (ethanol), phloretic acid (ethanol), *p*-coumarate (ethanol), 1-phenyl ethanol (water), and homovanillic acid (water). IPTG 1 M stock solutions were prepared by dissolving in water.

Synthetic Genes, Oligonucleotides, and Plasmids. *cmeR* (*cj0368c*) from *C. jejuni* was codon-optimized for yeast expression using the IDT tool and purchased from Thermo Scientific. Oligonucleotides were purchased from Thermo Scientific and suspended in nuclease-free water to a concentration of 100 μ M. Sequences of *cmeR* and the oligonucleotides used are listed in [Supplementary Table S1](#).

***E. coli* Biosensor Genetic Circuit Construction, Characterization, and Data Analyses.** Genetic circuits were constructed using the template plasmid pQacR-Q2, a gift from Christopher Voigt (Addgene plasmid #74690; <http://n2t.net/addgene:74690>; RRID:Addgene_74690).³³ *qacR* was

replaced with the codon-optimized *cmeR* gene using primer sets 1–4 by polymerase incomplete primer extension (PIPE) cloning.³⁴ Promoter P_{QacR} was modified by replacing *qaco* with *cmeO* (TGTAATAAATATTACA) using primers 5–6, and further optimizing its –10 sequence to the consensus TATAAT using primers 7–8, creating promoter “P₂” with the final sequence listed in [Supplementary Table S1](#). As well, *eyfp* was mutated to *egfp* using primer sets 9–10 to introduce a Y203T mutation.

For biosensor response characterization, *E. coli* cells carrying genetic circuit plasmids were grown overnight in LB broth at 37 °C. The following day, different volumes of IPTG and small molecules were dispensed using a Labcyte Echo liquid handler or a Thermo Scientific electronic Finipipette, where applicable, into 96-well clear plates (Falcon). *E. coli* cells were diluted 1:100 in M9 minimal media³⁵ with kanamycin, incubated at 37 °C for 4 h, and then diluted 1:200 in the presence of IPTG and putative ligands as needed. For all biosensor response characterization experiments here, M9 minimal media was supplemented with casamino acids (0.2%) thiamine hydrochloride (0.034%), and ascorbic acid (100 μ M) as an antioxidant. Microtiter plates were then incubated in an Infors HT shaker at 30 °C for 20–24 h. The following day, eGFP fluorescence (excitation: 475–10 nm, emission: 510–10 nm) and OD₆₀₀ of cultures were measured using a CLARIOstar microplate reader (BMG Labtech, Germany).

Unless otherwise stated, dose–response data points are averages of triplicates, shown either as three data points, or a single data point with an error bar representing standard deviation. Where applicable, curves were fitted using GraphPad Prism by modeling them to a four-parameter nonlinear regression equation.

***Saccharomyces cerevisiae* Strain Construction.** *S. cerevisiae* strains were constructed using CRISPR-Cas9-mediated genomic integration and *in vivo* DNA homologous recombination.^{36,37} Yeast cells were transformed with a DNA pool that comprised: (1) PCR-amplified promoters, *cmeR*, Envoy GFP and terminators with ~40 bp homology to the preceding and/or following part, mixed in equimolar ratios (total DNA ~1000 ng). (2) Two ~500 bp homology arms to the genomic locus (200 ng each). (3) Linearized pCas-G418 and/or pCas-Hyg vectors encoding Cas9³⁸ (150 ng each). (4) gRNAs obtained by oligo-extension targeting the characterized chromosomal locus FgF16³⁹ (in which all integrations in this work were made) with 40 bp homology to the Cas9 plasmids to allow plasmid recircularization by *in vivo* homologous recombination (2 μ L of unpurified PCR reaction per transformation). For constructing the control strain, the full P_{CCW12} was amplified using primers MN527 and MN516. For the sensor strain with the operator insertion, P_{CCW12} was divided into two parts with the operator acting as the homology arm in between them. Primer sets MN527 and MN664, and sets MN663 and MN516 were used to amplify both promoter parts. Transformations were recovered for 16 h in YPD, after which they were plated on YPD plates with the appropriate selection (Hygromycin and/or G418 at 200 μ g mL⁻¹ each). Successful genomic integrations were confirmed by colony PCR and Sanger sequencing. Finally, the Cas9 plasmid was cured by streaking colonies two successive times on YPD plates with no selection. Sequences of promoters, gene expression cassettes, gRNA(s) and terminators are listed in [Supplementary Table S1](#).

***S. cerevisiae* Biosensor Characterization.** Cells were grown overnight at 30 °C and 300 rpm in 96 deep-well plates

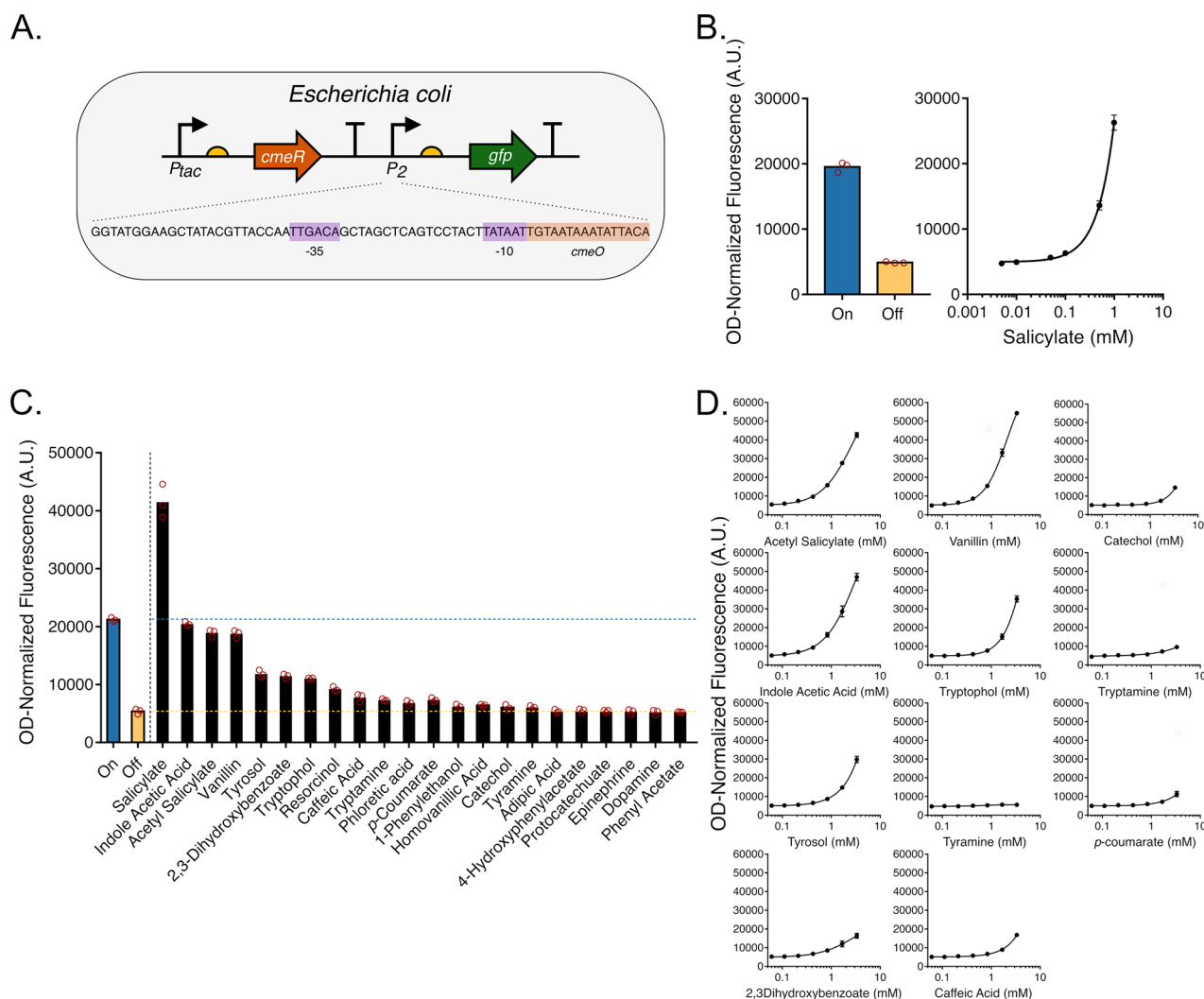


Figure 1. Construction and inducer profile of an *E. coli* salicylate biosensor. (A) One-plasmid genetic circuit structure. The IPTG-inducible *tac* promoter drives CmeR expression, followed by a second engineered salicylate-inducible promoter to drive GFP expression, which contains one copy of the *cmeO* operator sequence inserted immediately downstream of the -10 sequence. (B) Left panel: Fluorescence measurements of cells carrying the CmeR genetic circuit in the on and off states without and with 1 mM IPTG added, respectively. Right panel: Fluorescence measurements of cells in the presence of 1 mM IPTG and increasing concentrations of salicylate. (C) Fluorescence measurements of *E. coli* cultures carrying the CmeR genetic circuit exposed to 1 mM of one of 22 molecules in the presence of 1 mM of IPTG. (D) Dose response curves for 11 candidate inducer molecules in the presence of 1 mM IPTG. All data represents plate reader measurements of GFP fluorescence of *E. coli* cultures performed in triplicate.

(Greiner) in an Infors HT shaker. The following day, different volumes of small molecules were dispensed using a Labcyte Echo liquid handler or a Thermo Finipipette, where applicable, into 96-well clear plates (Falcon). Yeast cells were then diluted 1/50 in synthetic complete media that was adjusted to pH 4, supplemented with freshly prepared 5 mM ascorbic acid (Sigma-Aldrich), and dispensed into the plates. Plates were incubated at 30 °C and 300 rpm in the same shaker for 8 h, after which Envoy GFP fluorescence was measured by flow cytometry.

S. cerevisiae Biosensor Experiment and Flow Cytometry. Flow cytometry fluorescence characterization was performed using an Accuri C6 Cytometer (BD Biosciences). Cells were diluted 1:5 in deionized water and measured at an average rate of ~ 2000 events/second for a total of 10 000 events, and the mean fluorescence of the total ungated population was plotted for each molecule and/or concentration tested. Unless otherwise stated, dose–response data

points are averages of triplicates, shown either as 3 data points, or a single data point with an error bar representing standard deviation. When applicable, curves were fitted using GraphPad Prism by modeling them to a four-parameter nonlinear regression equation.

RESULTS AND DISCUSSION

1. Constructing a CmeR Salicylate Biosensor in *E. coli*.

CmeR belongs to the TetR family of repressors, one of the major families of transcription factors in bacteria,⁴⁰ which regulates expression by binding to the promoter region and physically blocking RNA polymerase binding or progression.¹⁰ In order to identify inducers for CmeR-regulated promoters in *E. coli*, we constructed a genetic circuit with CmeR expressed from an IPTG-inducible *tac* promoter and eGFP expressed from a second promoter that was modified for salicylate-responsiveness. In addition to having a consensus -35 (TTGACA) sequence, this promoter was engineered to have

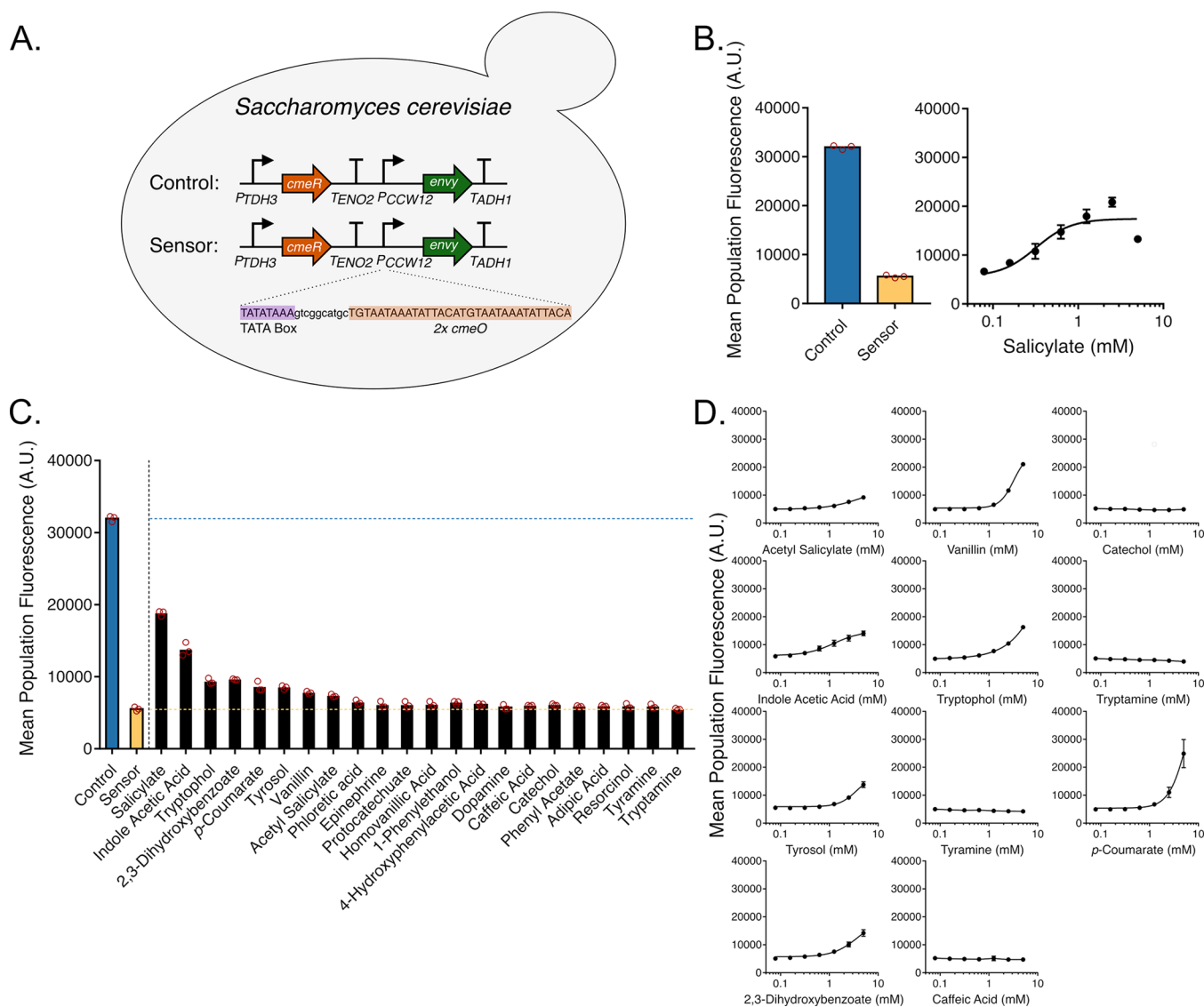


Figure 2. Construction and inducer profile of a *S. cerevisiae* salicylate biosensor. (A) Chromosomally integrated genetic circuit design in *S. cerevisiae*. CmeR is expressed from the strong constitutive promoter P_{TDH3} . P_{CCW12} is used for expressing Envy GFP in the control strain, which was subsequently engineered in the sensor strain for salicylate responsiveness by inserting two copies of *cmeO* downstream of the TATA box. (B) Left panel: Fluorescence measurements of cells carrying the control and sensor circuit. Right panel: Fluorescence measurements of the sensor strain in the presence of increasing concentrations of salicylate. (C) Fluorescence measurements of the sensor strain exposed to 1 mM of one of 22 molecules. (D) Dose response curves for 11 candidate inducer molecules. All data represents flow cytometry measurements of GFP fluorescence of *S. cerevisiae* cultures performed in triplicate.

a consensus -10 (TATAAT) sequence followed by *cmeO* (Figure 1A). Adding IPTG to cultures carrying this plasmid repressed fluorescence by inducing CmeR expression (in turn repressing eGFP expression), and adding increasing concentrations of one of the known inducers, salicylate, resulted in a maximal induction of eGFP expression by >5 fold due to the reduction in CmeR affinity to its operator site²⁵ (Figure 1B). Titrating IPTG and salicylate concentrations allowed us to further tune the level of expression of eGFP from this engineered promoter (Supplementary Figure S1).

We next sought to probe the ligand specificity of CmeR. In order to do this, we exposed a strain (*E. coli* DH5 α) carrying our genetic circuit to 1 mM concentrations of various aromatic, indole, and aliphatic molecules in the presence of IPTG. This concentration range was chosen due to solubility limit and molecule toxicity constraints, and to be able to properly

compare the circuit response to different molecules. We observed an increase in fluorescence with some of these molecules that ranged from ~ 1.1 fold (catechol) to ~ 3.8 fold (the plant auxin indole-3-acetic acid or IAA) (Figure 1C). Next, we characterized the dose–response of a subset of the tested molecules and show that in addition to the identified cognate inducers, expression from the CmeR-regulated promoter was induced by acetylsalicylate, vanillin, IAA, tryptophol, tyrosol, 2,3-dihydroxybenzoate (DHBA), catechol, caffeic acid, and *p*-coumarate. Expression from the CmeR-regulated promoter appeared to be minimally induced by tryptamine (the amine derivative of tryptophol), while remaining uninduced by tyramine (the amine derivative of tyrosol) (Figure 1D).

2. Constructing a CmeR Salicylate Biosensor in *S. cerevisiae*. To expand its use as a biosensor, we

transplanted CmeR into a eukaryotic chassis and tested for repression and subsequent induction activities using GFP as a reporter. Designing genetic circuits with transcription repressors in eukaryotes requires placing the operator sequence between the TATA box and the transcription start site in order to allow for the repressor to sterically interfere with the binding or progression of RNA polymerase.⁴¹ To this end, we designed and constructed a chromosomally integrated CmeR genetic circuit in *S. cerevisiae*. In this circuit, CmeR (without a nuclear localization signal) was expressed from the strong constitutive promoter P_{TDH3} . A second strong constitutive promoter, P_{CCW12} , was simultaneously used to express the Envoy GFP variant,⁴² yielding the control strain (Figure 2A). P_{CCW12} was engineered to be salicylate-responsive by introducing two copies of the *cmeO* operator sequence 11 bp downstream of the TATA box consensus sequence TATA(A/T)A(A/T)(A/G)⁴³ found at −133–125 nt relative to the transcription start site, thereby generating P_{CCW12O} and the sensor strain (Figure 2A). Due to the toxicity of salicylate to *S. cerevisiae*,^{44,45} all experiments were conducted at a lower yeast culture dilution factor and were measured after 8 h. GFP fluorescence outputs of the control and sensor strains measured by flow cytometry showed a marked drop in fluorescence upon inserting *cmeO*, which in turn increased by adding increasing concentrations of salicylate to the culture media, reaching up to ~4.3-fold induction in 2.5 mM salicylate (Figure 2B). This data shows that, in a manner similar to our *E. coli* genetic circuit, CmeR functions as a transcription repressor in *S. cerevisiae*, and is inducible by salicylate.

We then aimed to determine whether the CmeR ligands identified using the *E. coli* circuit would induce GFP expression in *S. cerevisiae* as well using our modified $CCW12$ promoter. Introducing 1 mM solutions of putative inducers to our *S. cerevisiae* sensor strain followed by flow cytometric analyses showed that many of the same ligands that induced GFP expression in *E. coli* successfully induce expression from P_{CCW12O} by ~1.1–2.5 fold (Figure 2C). Further characterization of the circuit dose–response with the same 11 molecules used in the previous section highlights some of the differences between the CmeR induction profile in *E. coli* and *S. cerevisiae*, the latter which exhibited maximal fluorescence inductions ranging from ~1.8 fold (acetyl salicylate) to ~5 fold (*p*-coumarate). Overall, acetylsalicylate, IAA, vanillin, DHBA, tyrosol, tryptophol, and *p*-coumarate, maintained their capacity to induce expression from P_{CCW12O} in *S. cerevisiae*. Catechol, caffeic acid, and tryptamine fail to induce GFP in the tested conditions (Figure 2D). Doubling the maximum concentration of these molecules to 10 mM resulted in a modest increase in fluorescence of ~1.3 fold in 10 mM catechol, but no changes in fluorescence with 10 mM caffeic acid and 10 mM tryptamine were detected (Supplementary Figure S2), suggesting that catechol is indeed a very weak inducer of CmeR in *S. cerevisiae* as it also is in *E. coli*. Furthermore, like in *E. coli*, tyramine failed to induce CmeR in *S. cerevisiae* (Figure 2D). Notably, the response to many of these molecules in our *S. cerevisiae* CmeR circuit, including to the cognate inducer salicylate, appears to be dampened in general relative to the *E. coli* circuit, which has been shown previously with other biosensors in *E. coli* and *S. cerevisiae*.¹⁸ In the case of CmeR, this could be due the many differences between both circuits, such as the *E. coli* one being expressed from a multicopy vector as opposed to a single copy genomic integration in the case of *S. cerevisiae*, or the number of *cmeO*

operator copies, which is 1 in *E. coli*, and 2 copies in *S. cerevisiae*. Furthermore, *p*-coumarate appear to be an exception to this rule, as it exhibits a stronger response in *S. cerevisiae* than in *E. coli*. This variability could be a result of the effects the differences in membrane transport mechanisms and cellular metabolism across both organisms, in addition to other unknown mechanisms. Such processes need to be taken into account when designing and engineering aTF biosensors, especially when transplanting them between different organisms. Possible ways of optimizing the response of the *S. cerevisiae* CmeR biosensor could be by varying the operator copy number and distance from the TATA box,⁴¹ as well as the number of genomically integrated copies of the genetic circuit.

CONCLUSION

One of the aims of synthetic biology is to reprogram new cellular functions, for which aTFs serve as excellent tools. As a result, there is a constant need of new aTFs that are functional and have predictable responses in microbial systems. In this work, we describe the construction and characterization of a CmeR-based salicylate whole cell biosensor in the prokaryotic and eukaryotic model systems *E. coli* and *S. cerevisiae*, respectively. In addition to salicylate and the bile salts cholate and taurocholate, we identified new ligands for CmeR in *E. coli* (acetylsalicylate, vanillin, catechol, IAA, tryptophol, tyrosol, caffeic acid, catechol, *p*-coumarate, DHBA, and to a lesser extent, tryptamine) and in *S. cerevisiae* (acetylsalicylate, vanillin, IAA, tryptophol, tyrosol, *p*-coumarate, DHBA, and to a lesser extent, catechol). In particular, tyrosol, tryptophol, and tyramine, to our knowledge, have never been described before as inducers of aTF-regulated promoters. The ability to detect these molecules would be useful for metabolic engineering applications, since a lot of these molecules are either intermediates or final products of pathways toward many commodity chemicals and value-added products.^{46–48} The caveat of this multiligand specificity however is that direct use of CmeR in metabolic engineering applications would be limited due to the significant crosstalk which might occur between different molecules in the same pathway. Furthermore, this work shows that the ligand promiscuity exhibited by CmeR makes it an excellent candidate for directed evolution and protein engineering efforts to detect novel molecules, as well as eliminate crosstalk between existing inducers. Although the inducer-binding site of TetR-family repressors is spatially conserved in a triangle formed by helices 5–7, exceptions to this rule exist.^{2,49} Therefore, solving the crystal structure of CmeR with salicylate in addition to these novel ligands would greatly facilitate these protein engineering efforts. Finally, this work also offers insights into additional roles of CmeR in *C. jejuni* pathogenesis. Salicylate has been shown to increase the minimum inhibitory concentrations of various antibiotics for *C. jejuni*.^{25,50,51} Given the ligand promiscuity demonstrated here, it would be tempting to investigate whether other aromatic and indole molecules play a role in the induction of antibiotic resistance in *C. jejuni*.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.2c00063>.

Additional response measurements of *E. coli* CmeR sensors; List of DNA and oligonucleotide sequences employed in this study (PDF)

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Author Contributions

M.A.N., D.H.K., and V.J.J.M. designed the research. M.A.N. performed the experiments. L.R.T. assisted in preliminary experiments. D.H.K. and V.J.J.M. supervised the research. M.A.N. wrote the manuscript with editing help from L.R.T., D.H.K., and V.J.J.M.

Notes

The authors declare no competing financial interest.

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