ACS SyntheticBiology

Research Article

pubs.acs.org/synthbio

Development of a Transcription Factor-Based Lactam Biosensor

Jingwei Zhang,[#,†,‡,§] Jesus F. Barajas,[#,‡] Mehmet Burdu,[‡] Thomas L. Ruegg,[‡] Bryton Dias,[‡] and Jay D. Keasling*[,†,‡,§,∥,⊥,○,▽]

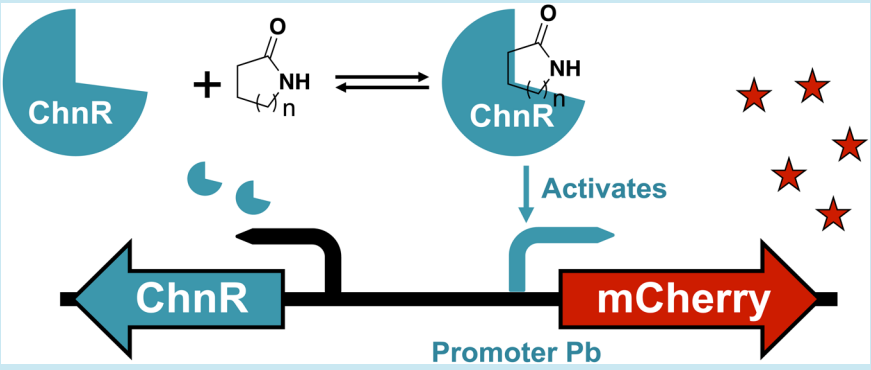
[†]UCSF-UCB Joint Graduate Group in Bioengineering, [§]Synthetic Biology Engineering Research Center, [∥]Department of Chemical & Biomolecular Engineering, [⊥]California Institute for Quantitative BiosciencesUniversity of California, Berkeley, California United States

[‡]Joint BioEnergy Institute, Emeryville, California United States

[○]Biological Systems & Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, California United States

[▽]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Lyngby, Denmark

Supporting Information



ABSTRACT: Lactams are an important class of commodity chemicals used in the manufacture of nylons, with millions of tons produced every year. Biological production of lactams could be greatly improved by high-throughput sensors for lactam biosynthesis. To identify biosensors of lactams, we applied a chemoinformatic approach inspired by small molecule drug discovery. We define this approach as analogue generation toward catabolizable chemicals or AGTC. We discovered a lactam biosensor based on the ChnR/Pb transcription factor-promoter pair. The microbial biosensor is capable of sensing ε-caprolactam, δ-valerolactam, and butyrolactam in a dose-dependent manner. The biosensor has sufficient specificity to discriminate against lactam biosynthetic intermediates and therefore could potentially be applied for high-throughput metabolic engineering for industrially important high titer lactam biosynthesis.

Because of the environmental concerns of traditional chemical synthesis routes, there is a great interest in producing materials from renewable biomass through biorefineries.[1−9] Lactams are important compounds used in the manufacture of polyamides.[10] With an annual production of four million metric tons, ε-caprolactam (caprolactam) is used for the production of nylon-6 found in fabrics, coatings, plastics, lubricants, and other products.[11] δ-Valerolactam (valerolactam) is used as a monomer for nylon-5 and nylon-6,5 synthesis, the addition of which tunes the properties of the resulting polymers.[2,3,12] Butyrolactam was identified by the US Department of Energy as an important C4 "Top Value-Added Chemical from Biomass".[13] It is currently used as the precursor for the production of *N*-vinylpyrrolidone,[14] and is also proposed to be a monomer for nylon-4, a fiber material with the highest hydrophilicity in the nylon family of materials.[15] With recent efforts toward the renewable production of lactams in *E. coli*,[16] pathway yields and productivities need to be significantly improved. Directed evolution has been applied successfully to evolve enzymes and engineer strains.[17−26] Moreover, our ability to generate genotypic diversity is enabled by a set of technologies, including introducing either random or targeted mutations during DNA synthesis, incorporating combinatorial promoter and ribosomal binding site (RBS) libraries during multigene pathway assembly,[27,28] genome engineering techniques, such as multiplex genome engineering (MAGE)[25] and CRISPR/Cas9,[29−42] global transcription machinery engineering (gTEM),[43] multiscale analysis of library enrichment (SCALEs),[44] and random mutagenesis by chemical mutagens or UV radiation. However, our ability to screen through the resulting library lags behind.[19] Although gas and liquid chromatography techniques allow lactam product confirmation and quantification, these analytical methods have limited throughput (10^2−10^3 variants per machine per day), which constrains the number of strains and conditions

Received: May 3, 2016
Published: December 20, 2016

DOI: 10.1021/acssynbio.6b00136
ACS Synth. Biol. 2017, 6, 439−445

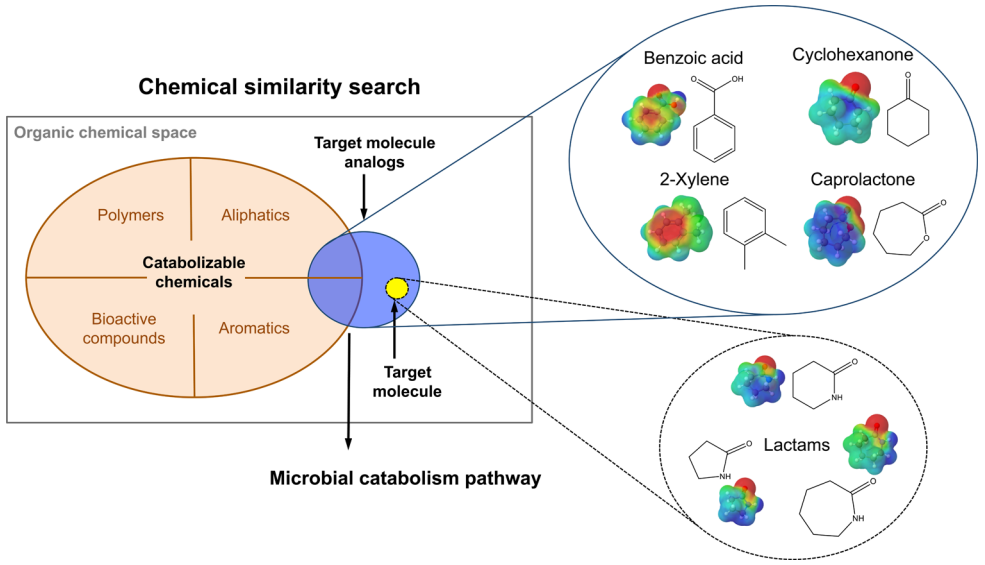


Figure 1. Analogue Generation toward Catabolizable Chemicals (AGTC). Chemical similarity search toward chemicals catabolizable by microbes and lactam analogues in the catabolizable chemical space. The approach follows two-steps principles: (1) identifying catabolizable chemicals with molecular shapes similar to metabolic engineering targets; (2) exploring catabolizing gene clusters of such catabolizable chemicals to find inducible transcription factor candidates.

that can be tested.[19] Until now, no lactam-responsive biosensor has been developed. Therefore, to construct a lactam-responsive biosensor we first sought to identify a naturally occurring transcription factor-promoter pair.

Molecular shape similarity is an important concept in computer-aided drug design and high-throughput virtual screening that has been widely used in drug discovery.[45] However, molecular shape or chemoinformatic similarity algorithms for drug discovery have not been widely used in metabolic engineering for identification of regulatory ligand and cognate transcription factor pair. The similarity assessment for small molecules is often too stringent for molecules of interest in metabolic engineering. For instance, valerolactam comparison with ROCS (Rapid Overlay of Chemical Structures) (OpenEye Scientific),[46] a virtual screening tool that can identify compounds by shape comparison, resulted in molecule analogues of valerolactam containing only amide bonds (Supporting Information, Figure S1). For transcription factor binding, these small differences in chemical structure may not be necessary, since molecule analogues with ester or even α-methylene carbonyl groups can be potentially recognized. Another issue in utilizing molecular shape-based drug screening algorithms is that compound libraries in medicinal chemistry have little relevance to microbial catabolism. Therefore, both the chemical library and the similarity search algorithms need to be adapted in order to apply molecular shape similarity search to metabolic engineering. We employed a targeted strategy to identify appropriate small molecule sensor gene candidates, named "Analogue Generation toward Catabolizable Chemicals" (AGTC). The approach follows two-steps principles: (1) identifying catabolizable chemicals with molecular shapes similar to metabolic engineering targets; (2) exploring catabolizing gene clusters of such catabolizable chemicals to find inducible transcription factor candidates (Figure 1).

RESULTS AND DISCUSSION

Lactam Analogue Search. We used AGTC methodology to search for biosensor(s) that could detect butyrolactam, valerolactam, and caprolactam (Figure 1). First, we generated a list of target chemical analogues of valerolactam using DataWarrior software (see methods for more details) (Figure 1, Figure S2). The valerolactam analogue libraries vary depending on similarity filter cutoff (Figure S2). At 80% similarity based on SkelSpheres Descriptor filter, four compounds were returned, one of which was cyclohexanone, a catabolic intermediate according to the BRENDA enzyme ligand database (Figure S2A). When the similarity cutoff was lowered to 60%, more compound analogue hits, including caprolactone and caprolactam, were observed (Figure S2C). Both caprolactone and caprolactam, which were further filtered by BRENDA, were found to play known or putative roles in microbial metabolism-related enzymatic reactions (Table S1).

The cyclohexanone catabolic gene cluster was identified in more than 45 organisms (Table S2), among which *Acinetobacter sp.* SE19 or NCIMB 9871 has documented genetic evidence that cyclohexanone serves as the inducer for ChnR/Pb transcription factor/promoter pair (Figure S3A).[47−51] Further explanation in lactam analogue search can be found in the Supporting Information.

Design and Confirmation of a Biplasmid Lactam Biosensor System. The ChnR-Pb transcription factor/promoter pair is involved in cyclohexanol oxidation and it activates the expression of both the cyclohexanone 1,2-monooxygenase (ChnB) and the cyclohexanol dehydrogenase (ChnA) in *Acinetobacter* sp. strain NCIMB9871.[47−51] Previous work by Steigedal et al. demonstrated that the ChnR/Pb activity is much higher in *P. fluorescens* than in *E. coli*.[47−51] Given our aim of generating a lactam biosensor for the detection of lactams in *E. coli*, we wanted to explore if the lack of activity of the ChnR/Pb in *E. coli* was due to toxicity of ChnR protein expressed in *E. coli*. To test ChnR protein toxicity in *E. coli*, we first tested the ChnR-Pb as a two-plasmid system in *E. coli* JZ-359. One plasmid, pBbA8a-ChnR, contained *chnR* under control of the arabinose-inducible *araBAD* promoter (P_{BAD}), and the second plasmid, JZ-BCS-2, harbors the *mCherry* gene encoding for mCherry fluorescent

DOI: 10.1021/acssynbio.6b00136
ACS Synth. Biol. 2017, 6, 439−445

protein (mCherry) under control of the *Acinetobacter sp. chnB* promoter (Pb) (Table 1). Biosensor constructs were

Table 1. *E. coli* Strains

name	relevant genotype	ref
Strains		
DH10B	F⁻ mcrA crmrr-hsdRMS-mcrBC) r-hsdRMS-mcrBC) and oligonucleotide139 Δ(ara, leu) 7697 galU galK alrpsL nupG	Life Technologies (Carlsbad, CA)
DP10	DaraFGH DP-araE and PCP18-araE	
strains	plasmids	Host
JZ-359	JZ-BCS-2 (Kan) + pBbA8a-ChnR	DP10
JZ-439	pBbSLactamC-mCherry	DH10B

cotransformed into *E. coli* DP10 containing a Δ*araE* deletion to enable titratable induction of ChnR. The ChnR protein was induced with arabinose ranging between 0.002 and 0.2% under various concentrations of butyrolactam, valerolactam, and caprolactam when cultured at 30 °C (Figure 2B–D). The optical density (O.D.) was monitored every 15 min. Lactam concentrations ranged from 0 to 100 mM. Fluorescent mCherry response to the various lactams was observed in the low mM range, varying between 3 and 12.5 mM. Although different final O.D. values were observed for *E. coli* grown in higher concentrations of L-arabinose, minimal growth changes were observed (Figure S4E). Overall, no significant ChnR protein toxicity was observed with L-arabinose induction. In the absence of L-arabinose, lactam dose-dependent induction was observed, suggesting that leaky expression of ChnR is sufficient for lactam inducible response.

Characterization of Improved Cyclohexanone Inducible ChnR/Pb Promoter Pair Lactam Biosensor. The initial biosensor system had limited biotechnology usefulness. The two-plasmid system expressing high levels of ChnR was controlled by an external inducer, which would have significant metabolic burden for lactam production strains. The two-plasmid system was not an adequate orthogonal system since multiple constructs for potential lactam biosynthetic pathways would need to be introduced into *E. coli*. Ideally, a low level of ChnR expression that maintain lactam dose dependence behavior is preferred, since the majority of the cellular resources should be devoted to product biosynthesis rather than product sensing. Further improvements were made to create a single plasmid biosensor (JZ-439) for high-throughput metabolic engineering with more desirable features: (1) ChnR was placed under the control of a constitutive promoter cloned from pBbS5C-RFP (Supporting Information and Methods); and (2) a low copy number plasmid was used to minimize the metabolic burden of plasmid replication and protein production (Figure 2A, Tables S3–S4). Using this single plasmid biosensor construct, we aimed to test the biosensor's response to pathway intermediates (Figure S5) and measure the biosensor's dynamic and linear range. A biosensor design (pBbSLactamC-mCherry) was constructed by incorporating a weak RBS 5′ of ChnR, and the SC101 origin of replication (Figure 2A, Table S3). *E. coli* DH10B transformed with pBbSLactamC-mCherry was used for characterization of biosensor response to exogenously added butyrolactam, valerolactam, and caprolactam (Figure 3A). Butyrolactam and valerolactam did not affect growth (Figure S4A,B,D). Because caprolactam was toxic above 50 mM, which results in a decrease in normalized mCherry fluorescence (Figure S4C,D), caprolactam biosensor data were collected only up to the toxicity limit.

Valerolactam and caprolactam had the largest dynamic range, having a 2.38 and 2.08 fold increase in mCherry fluorescence compared to the noninduced mCherry baseline (Table 2), followed by butyrolactam with a 1.47 fold increase. In the case of valerolactam and caprolactam, the linear range of detection was between 1 and 50 mM (Table 2). Butyrolactam displayed the largest linear range, extending from 1 to 100 mM. Furthermore, we examined the K_m or inducer concentration

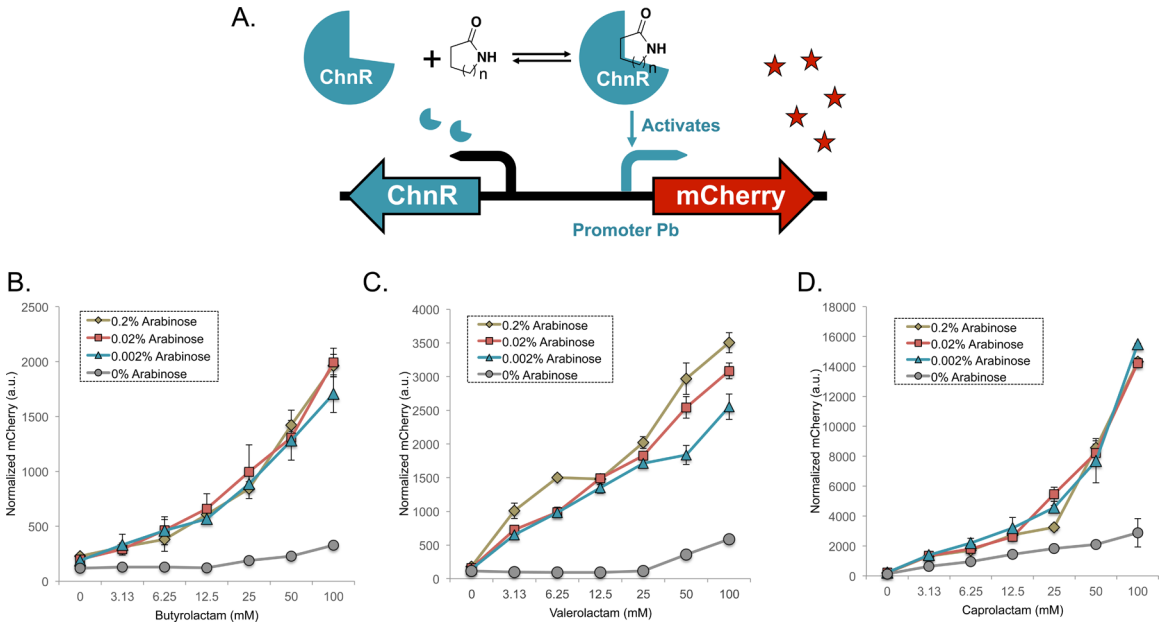


Figure 2. Lactam-inducible ChnR/Pb pair. (A) Proposed lactam-inducible mCherry detection system. Inducible and dose dependent mCherry expression by butyrolactam (B), valerolactam (C), and caprolactam (D). The ChnR protein may be expressed under an inducible promoter, such as P_{bad}, using the two-plasmid system or under control of a constitutive promoter on a single plasmid.

DOI: 10.1021/acssynbio.6b00136
ACS Synth. Biol. 2017, 6, 439–445

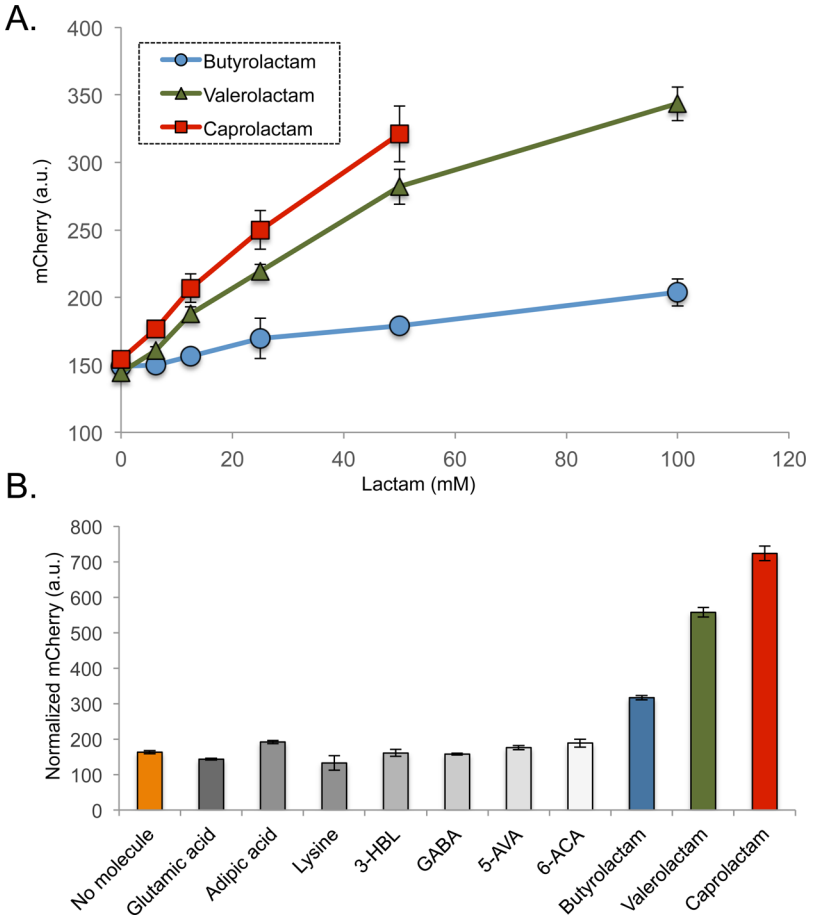


Figure 3. Single plasmid biosensor system. (A) Inducible and dose-dependent mCherry expression by butyrolactam, valerolactam, and caprolactam. (B) Sensitivity of ChnR to lactams and lactam biosynthetic pathway intermediates. ChnR was inducible by 40 mM butyrolactam, valerolactam, and caprolactam. ChnR was not inducible by the same concentration of lactam precursor pathway intermediates (Figure S3.), including glutamic acid, adipic acid, lysine, 3-hydroxy butyrolactone(3-HBL), gamma-aminobutyric acid (GABA), 5 amino-valeric acid (5-AVA), and 6-aminocaproic acid (6-ACA).

Table 2. Biosensor Performance[a]

compound	dynamic range	K_m (mM)	linear range (mM)
butyrolactam	1.47	336.2 ± 73.5	1−100
valerolactam	2.38	48.8 ± 8.4	1−50
caprolactam	2.08	38.2 ± 5.0	1−50

[a]Dose response curves were fitted to the experimental data using Prism GraphPad software. Dynamic range is the ratio of the largest fluorescent signal to the smallest baseline fluorescent signal. K_m is the inducer concentration resulting in half-maximal induction. Linear range is the range over which the output (mCherry signal) varies linearly with the input (lactam concentration).

resulting in half-maximal induction for all three lactams. Data from various concentrations of butyrolactam, valerolactam, and caprolactam (Figure 3A) were fitted into dose response curves using Prism GraphPad software (GraphPad Prism version 7.00 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com). Butyrolactam had the highest half-maximal induction concentration of 336.2 mM ± 73.5 mM, followed by valerolactam (48.8 mM ± 8.4 mM), and caprolactam (38.2 mM ± 5.0 mM) (Table 2).

To further characterize the specificity of the biosensor to the lactams versus various lactam biosynthetic precursor intermediates, the biosensor was tested against known lactam biosynthetic precursors. Previous work in the metabolic engineering of butyrolactam biosynthesis demonstrated the use of glutamic acid and δ-aminobutyric acid (GABA) as precursors for butyrolactam production (Figure S5A).[8] Moreover, work by both Turk et al.[52] and Zhou et al.[53] demonstrated *E. coli* production and optimization of 6-aminocaproic acid, a key precursor for the production of caprolactam, through ketoacid intermediates (Figure S5C). We aimed to test the one-plasmid biosensor against various lactam precursor intermediates (Figure S5A−C). The biosensor displayed selectivity against all lactam precursor intermediates (Figure 3B). Even at significantly high concentrations (40 mM), lactam precursor intermediates did not trigger mCherry expression above the control baseline. As our engineered *E. coli* currently produces butyrolactam at >10 mM titer,[16] the linear range (1−100 mM) of the constructed biosensor strain, JZ-439, is well within a range that can be used to improve titer. In cases where the titer exceeds the linear range of detection, an appropriate dilution of medium could bring the concentration of lactams back to the linear range. From these results, we conclude that the biosensor is well suited for future high-throughput, high-titer lactam screening.

To our knowledge, there has been no reported transcriptional control for butyrolactam, valerolactam and caprolactam, since these chemicals are artificial and do not participate in metabolism. Therefore, we explored chemical analogues with

DOI: 10.1021/acssynbio.6b00136
ACS Synth. Biol. 2017, 6, 439−445

similar chemoinformatic properties as lactams. The AGTC strategy is generally applicable for a number of reasons. First, it assumes that microbes can potentially utilize a wide range of organic compounds for growth given long-term exposure in the environment. There is literature on microbial substrate utilization with well-characterized catabolic pathways and their regulatory mechanisms. Studies on microbial growth in pollutant-contaminated environments greatly expanded the chemical space of catabolizable chemicals, and large-scale metagenomics studies will make more microbial genome information available. In the case where analogues are not documented in the literature, it is still convenient to look for candidates. Second, transcription factor discovery is naturally amenable to high-throughput screening. Even if newly discovered transcription factor candidates displayed low inducibility by the metabolic engineering target molecule, directed evolution of the transcription factor candidate may be implemented to increase compatibility of molecules that are similar to the native ligand.[54]

A number of chemical informatics algorithms have been developed for drug discovery.[55,56] By incorporating features relevant to microbial sensing, curating a chemical space specific for catabolizable chemicals, as well as applying filters in the context of metabolic engineering, future efforts can be made to generate an automated pipeline where chemoinformatic algorithms, such as DataWarrior,[57] communicate directly with enzyme and metabolic pathway databases, such as BRENDA.[58] Such an automated pipeline can expedite transcription factor discovery for small molecule inducibility. Apart from small molecule inducibility, additional filters such as phylogenetic distance from host organism, specificity against metabolic pathway intermediates and relevant dynamic range are also important factors to achieve a functional heterologous expression of the desired protein that can be utilized for high-throughput screening.

METHODS

Analogue Generation toward Catabolizable Chemicals. Analogue generation utilizes the following principles: (1) maintain general molecular shape, either open chain or ring structure; (2) preserve charge distribution and polarity within a molecule, for example, C−N and C−O bonds are interchangeable, and so are nitrile and carbonyl groups; (3) maintain overall hydrophilicity and hydrophobicity within the molecules; and (4) maintain chemical modifications as close to the core functional group as possible (e.g., γ-methyl-caprolactam is more similar to caprolactam than α-methyl-caprolactam).

We used DataWarrior (version 4.4.4), a software with an established "SkelSpheres Descriptor" filter, to perform analogue generation on butyrolactam, valerolactam, and caprolactam with manually set similarity cutoffs.[57] Public compound databases were initially imported from the DataWarrior software due to its large and comprehensive compound library (Under "Database" tab, select "retrieve Wikipedia Molecules"). However, for catabolizable chemicals, other databases such as BRENDA enzyme ligand database, or the MetaCyc's compound database would be more relevant if their ligand data access may be opened.

The chemical analogues were further filtered by whether they were known to be catabolized by microbes. The catabolism reactions are well curated in several databases, including the "BRENDA substructure search" (http://www.brenda-enzymes.org/index.php),[58] and many more are continually being discovered through large-scale metagenomics sequencing efforts.[59,60] We used the BRENDA enzyme/ligand database, filtering with "Enzyme, Ligand" and "exact") to confirm the existence of catabolism pathways and organisms that carry such pathways.

Once the catabolic pathway and its host organisms were identified, the gene cluster encoding the catabolic pathway was explored for regulator candidates and checked for inducibility by either the chemical itself or its downstream catabolic intermediates. When choosing from multiple candidates, higher priority is given to organisms that are closer in phylogeny to the host into which this transcription factor is planned to be engineered.

Biosensing Protocol. *E. coli* JZ-359 and JZ-439 were grown overnight in Luria−Bertani (LB) medium at 30 °C. The overnight culture was inoculated 1:100 into fresh LB medium containing antibiotics (25 μg/mL chloramphenicol, 20 μg/mL kanamycin, and 100 μg/mL ampicillin) to create a culture master stock. Stock chemicals were prepared at various concentrations final concentrations. An aliquot of the master culture stock (142.5 μL) plus 7.5 μL of chemical stock were transferred into polystyrene 96-well Costar Assay Plate (black with clear flat bottom, Corning Inc., New York, NY). Oxygen-permeable adhesive plate seals (Catalog no. AB-0580, Thermo Scientific, Waltham, MA) were used to seal the plate to prevent evaporation. Because of the temperature difference between the culture after plate preparation and the 30 °C incubator, condensation on the inner surface of the seal would interfere with optical signal. Therefore, after the liquid was transferred, the 96-well microplate and the seals were both kept at 30 °C for 5 min before sealing the plate. The plate was shaken in linear mode for 8 min and monitored every 15 min for optical density at a wavelength of 600 nm ($O.D._{600\ nm}$) absorbance and mCherry fluorescence (λ_{ex} = 575 nm, λ_{em} = 620 nm) in Infinite F200 multimode reader (TECAN, San Jose, CA) for 20 h at 30 °C.

Biosensor Performance Calculations. The biosensor performance features (such as K_m, the ligand concentration that results in half-maximal induction) were derived from the data using the Prism GraphPad Prism version 7.00 software dose response program (GraphPad Software, La Jolla California USA, www.graphpad.com).

CONCLUSION

The ChnR/Pb transcription factor/promoter pair is a proof of concept demonstration of transcription factor scouting using the analogue generation toward catabolizable chemicals (AGTC) strategy. Plasmid origin of replication, promoter strength, and RBS 5′ of either the transcription factor or reporter protein are parameters that can fine-tune the properties of the biosensors. The ChnR/Pb biosensor system can potentially enable high-throughput screening of lactam titer improvement. Here, we report a one-plasmid ChnR/Pb mCherry reporter system that is selective for lactams. The wide linear range of the modified ChnR/Pb mCherry reporter provides a useful tool for the screening of high-titer *E. coli* producing lactam strains in a high-throughput fashion.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00136.

DOI: 10.1021/acssynbio.6b00136
ACS Synth. Biol. 2017, 6, 439−445

Further experimental details for lactam biosensor search analysis, experiments described in the main text, and additional experiments (PDF)

AUTHOR INFORMATION

Corresponding Author

*Fax: +1 510 495 2630. E-mail: keasling@berkeley.edu.

ORCID

Jay D. Keasling: 0000-0002-4910-2222

Author Contributions

#J.Z. and J.F.B. contributed equally to this work. J.Z. and J.K. conceived the project. J.F.B. and J.Z. designed and executed the experiments. J.Z. and B.D. constructed the plasmids. B.D. and T.L.R. performed NitR related experiments. J.Z. and J.F.B. performed the ChnR biosensor experiments. J.Z. and J.F.B. analyzed the data. J.Z., J.F.B., and J.K. wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): J.D.K. has a financial interest in Amyris and Lygos.

ACKNOWLEDGMENTS

This work was funded by the Joint BioEnergy Institute (http://www.jbei.org/), which is supported by the US Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the US Department of Energy, and by the Synthetic Biology Engineering Research Center (SynBERC) through National Science Foundation Grant NSF EEC 0540879. Plasmid pMS119 was a kind gift from provided by Prof. Svein Valla (Department of biotechnology at the Norwegian University of Science and Technology). We thank Dr. Magnus Steigedal for discussion on transcription factors. Plasmid pNIT-1 was a kind gift provided by Prof. Christopher M. Sassetti (Department of Microbiology & Physiological Systems at University of Massachusetts). We thank Mitchell G. Thompson for his guidance on fluorescence instrumentation and Dr. Heather M. Jensen for her guidance in cell growth analysis using python notebooks.

REFERENCES

(1) Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., Khandurina, J., Trawick, J. D., Osterhout, R. E., Stephen, R., Estadilla, J., Teisan, S., Schreyer, H. B., Andrae, S., Yang, T. H., Lee, S. Y., Burk, M. J., and Van Dien, S. (2011) Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. *Nat. Chem. Biol.* 7 (7), 445−52.

(2) Liu, P., Zhang, H., Lv, M., Hu, M., Li, Z., Gao, C., Xu, P., and Ma, C. (2014) Enzymatic production of 5-aminovalerate from L-lysine using L-lysine monooxygenase and 5-aminovaleramide amidohydrolase. *Sci. Rep. 4*, 5657.

(3) Park, S. J., Kim, E. Y., Noh, W., Park, H. M., Oh, Y. H., Lee, S. H., Song, B. K., Jegal, J., and Lee, S. Y. (2013) Metabolic engineering of Escherichia coli for the production of 5-aminovalerate and glutarate as C5 platform chemicals. *Metab. Eng. 16*, 42−7.

(4) Adkins, J., Pugh, S., McKenna, R., and Nielsen, D. R. (2012) Engineering microbial chemical factories to produce renewable "biomonomers". *Front. Microbiol. 3*, 313.

(5) Beardslee, T., and Picataggio, S. (2012) Bio-based adipic acid from renewable oils. *Lipid Technol. 24* (10), 223−225.

(6) Keasling, J. D. (2010) Manufacturing molecules through metabolic engineering. *Science 330* (6009), 1355−8.

(7) McKenna, R., and Nielsen, D. R. (2011) Styrene biosynthesis from glucose by engineered E. coli. *Metab. Eng. 13* (5), 544−54.

(8) Nikolau, B. J., Perera, M. A., Brachova, L., and Shanks, B. (2008) Platform biochemicals for a biorenewable chemical industry. *Plant J. 54* (4), 536−45.

(9) Qian, Z. G., Xia, X. X., and Lee, S. Y. (2009) Metabolic engineering of Escherichia coli for the production of putrescine: a four carbon diamine. *Biotechnol. Bioeng. 104* (4), 651−62.

(10) Ritz, J., Fuchs, H., Kieczka, H., and Moran, W. C., (2005) Ullmann's Encyclopedia of Industrial Chemistry. In *Caprolactam*, Wiley-VCH [Online], Weinheim.

(11) PCI-Nylon Polyamide 6. http://pcinylon.com/index.php/markets-covered/polyamide-6 (accessed 2015−10−08).

(12) Park, S. J., Oh, Y. H., Noh, W., Kim, H. Y., Shin, J. H., Lee, E. G., Lee, S., David, Y., Baylon, M. G., Song, B. K., Jegal, J., Lee, S. Y., and Lee, S. H. (2014) High-level conversion of L-lysine into 5-aminovalerate that can be used for nylon 6,5 synthesis. *Biotechnol. J. 9* (10), 1322−8.

(13) Werpy, P. (2004) Top Value Added chemicals from Biomass. *Volume I - Results of Screening for Potential Candidates from Sugars and Synthesis Gas*, U.S. Department of Energy.

(14) BASF 2-Pyrrolidone. http://www.basf.ca/group/corporate/ca/en/brand/2_PYRROLIDONE (accessed 03.05).

(15) Park, Kim, E. Y., Noh, W., Oh, Y. H., Kim, H. Y., Song, B. K., Cho, K. M., Hong, S. H., Lee, S. H., and Jegal, J. (2013) Synthesis of nylon 4 from gamma-aminobutyrate (GABA) produced by recombinant Escherichia coli. *Bioprocess Biosyst. Eng. 36* (7), 885−892.

(16) Zhang, J., Kao, E., Wang, G., Baidoo, E. E.K., Chen, M., and Keasling, J. (2016) Metabolic Engineering of E. coli for the biosynthesis of 2-pyrrolidone. *Met. Eng. Com. 3*, 1.

(17) Amin, Erdin, S., Ward, R. M., Lua, R. C., and Lichtarge, O. (2013) Prediction and experimental validation of enzyme substrate specificity in protein structures. *Proc. Natl. Acad. Sci. U. S. A. 110* (45), E4195−202.

(18) Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C., and Robins, K. (2012) Engineering the third wave of biocatalysis. *Nature 485* (7397), 185−94.

(19) Dietrich, J. A., Mckee, A. E, and Keasling, J. D. (2010) High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu. Rev. Biochem. 79*, 563−90.

(20) Dougherty, and Arnold, F. H. (2009) Directed evolution: new parts and optimized function. *Curr. Opin. Biotechnol. 20* (4), 486−91.

(21) Esvelt, Carlson, J. C., and Liu, D. R. (2011) A system for the continuous directed evolution of biomolecules. *Nature 472* (7344), 499−503.

(22) Evans, B. S., Chen, Y., Metcalf, W. W., Zhao, H., and Kelleher, N. L. (2011) Directed evolution of the nonribosomal peptide synthetase AdmK generates new andrimid derivatives in vivo. *Chem. Biol. 18* (5), 601−7.

(23) Shiue, and Prather, K. L. J. (2014) Improving D-glucaric acid production from myo-inositol in E. coli by increasing MIOX stability and myo-inositol transport. *Metab. Eng. 22*, 22−31.

(24) Tyo, K. E., Nevoigt, E., and Stephanopoulos, G. (2011) Directed evolution of promoters and tandem gene arrays for customizing RNA synthesis rates and regulation. *Methods Enzymol. 497*, 135−55.

(25) Wang, H. H., and Church, G. M. (2011) Multiplexed genome engineering and genotyping methods applications for synthetic biology and metabolic engineering. *Methods Enzymol. 498*, 409−26.

(26) Gilliam, E. M. J., Copp, J. N., and Ackerley, D. F. (2014) *Directed Evolution Library Creation*, Springer.

(27) Tsvetanova, B., Peng, L., Liang, X., Li, K., Yang, J. P., Ho, T., Shirley, J., Xu, L., Potter, J., Kudlicki, W., Peterson, T., and Katzen, F. (2011) Genetic assembly tools for synthetic biology. *Methods Enzymol. 498*, 327−48.

(28) Xu, P., and Koffas, M. A. G. (2013) Assembly of Multi-gene Pathways and Combinatorial Pathway Libraries Through ePathBrick Vectors. *Methods Mol. Biol. 1073*, 107−129.

(29) Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L. A. (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol. 31* (3), 233−9.

DOI: 10.1021/acssynbio.6b00136
ACS Synth. Biol. 2017, 6, 439−445

(30) Auer, T. O., Duroure, K., De Cian, A., Concordet, J. P., and Del Bene, F. (2014) Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res. 24* (1), 142−53.
(31) Baena-Lopez, L. A., Alexandre, C., Mitchell, A., Pasakarnis, L., and Vincent, J. P. (2013) Accelerated homologous recombination and subsequent genome modification in Drosophila. *Development 140* (23), 4818−25.
(32) Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., and Marraffini, L. A. (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res. 41* (15), 7429−37.
(33) Garneau, J. E., Dupuis, M. E., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadan, A. H., and Moineau, S. (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature 468* (7320), 67−71.
(34) Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S., and Qi, L. S. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell 154* (2), 442−51.
(35) Haurwitz, R. E., Jinek, M., Wiedenheft, B., Zhou, K., and Doudna, J. A. (2010) Sequence- and structure-specific RNA processing by a CRISPR endonuclease. *Science 329* (5997), 1355−8.
(36) Heigwer, F., Kerr, G., and Boutros, M. (2014) E-CRISP: fast CRISPR target site identification. *Nat. Methods 11* (2), 122−3.
(37) LaFlamme, B. (2014) A CRISPR method for genome-wide screening. *Nat. Genet. 46* (2), 99−99.
(38) Marraffini, L. A., and Sontheimer, E. J. (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science 322* (5909), 1843−5.
(39) Menke, D. B. (2013) Engineering subtle targeted mutations into the mouse genome. *Genesis 51* (9), 605−18.
(40) Pyzocha, N. K., Ran, F. A., Hsu, P. D., and Zhang, F. (2014) RNA-guided genome editing of mammalian cells. *Methods Mol. Biol. 1114*, 269−77.
(41) Ran, F. A., Hsu, P. D., Lin, C. Y., Gootenberg, J. S., Konermann, S., Trevino, A. E., Scott, D. A., Inoue, A., Matoba, S., Zhang, Y., and Zhang, F. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell 154* (6), 1380−9.
(42) Terns, R. M., and Terns, M. P. (2014) CRISPR-based technologies: prokaryotic defense weapons repurposed. *Trends Genet. 30* (3), 111−8.
(43) Alper, H., and Stephanopoulos, G. (2007) Global transcription machinery engineering: a new approach for improving cellular phenotype. *Metab. Eng. 9* (3), 258−67.
(44) Lynch, M. D., Warnecke, T., and Gill, R. T. (2007) SCALEs: multiscale analysis of library enrichment. *Nat. Methods 4* (1), 87−93.
(45) Nicholls, A. M., McGaughey, G. B., Sheridan, R. P., Good, A. C., Warren, G., Mathieu, M., Muchmore, S. W., Brown, S. P., Grant, J. A., Haigh, J. A., Nevins, N., Jain, A. N., and Kelley, B. J. (2010) Molecular Shape and Medical Chemistry: A Perspective. *J. Med. Chem. 53*, 3862−3886.
(46) Pubchem Struture Search. https://pubchem.ncbi.nlm.nih.gov/search/search.cgi (accessed November 10, 2016).
(47) Brautaset, T., Lale, R., and Valla, S. (2009) Positively regulated bacterial expression systems. *Microb. Biotechnol. 2* (1), 15−30.
(48) Hiroaki Iwaki, Y. H., Teraoka, M., Tokuyama, T., Bergeron, H., and Lau, P. C. K. (1999) Identification of a Transcriptional Activator (ChnR) and a 6-Oxohexanoate Dehydrogenase (ChnE) in the Cyclohexanol Catabolic Pathway in Acinetobacter sp. Strain NCIMB 9871 and Localization of the Genes That Encode Them. *Appl. Environ. Microbiol. 65* (11), 5158.
(49) Iwaki, H., Hasegawa, Y., Teraoka, M., Tokuyama, T., Bernard, L., and Lau, P. C. K. (2002) Cyclohexanol Biodegradation Genes. *A Pathway of Opportunities 840*, 80−92.
(50) Qiong Cheng, S. M. T., Kostichka, K., Valentine, J. R., and Nagarajan, V. (2000) Genetic Analysis of a Gene Cluster for Cyclohexanol Oxidation in Acinetobacter sp. Strain SE19 by In Vitro Transposition. *J. Bacteriol. 182* (17), 4744.
(51) Steigedal, M., and Valla, S. (2008) The Acinetobacter sp. chnB promoter together with its cognate positive regulator ChnR is an attractive new candidate for metabolic engineering applications in bacteria. *Metab. Eng. 10* (2), 121−9.
(52) Turk, S. C., Kloosterman, W. P., Ninaber, D. K., Kolen, K. P., Knutova, J., Suir, E., Schurmann, M., Raemakers-Franken, P. C., Muller, M., de Wildeman, S. M., Raamsdonk, L. M., van der Pol, R., Wu, L., Temudo, M. F., van der Hoeven, R. A., Akeroyd, M., van der Stoel, R. E., Noorman, H. J., Bovenberg, R. A., and Trefzer, A. C. (2016) Metabolic Engineering toward Sustainable Production of Nylon-6. *ACS Synth. Biol. 5* (1), 65−73.
(53) Zhou, H., Vonk, B., Roubos, J. A., Bovenberg, R. A., and Voigt, C. A. (2015) Algorithmic co-optimization of genetic constructs and growth conditions: application to 6-ACA, a potential nylon-6 precursor. *Nucleic Acids Res. 43* (21), 10560−70.
(54) Vee Aune, T. E., Bakke, I., Drablos, F., Lale, R., Brautaset, T., and Valla, S. (2010) Directed evolution of the transcription factor XylS for development of improved expression systems. *Microb. Biotechnol. 3* (1), 38−47.
(55) Wale, N., Karypis, G., and Watson, I. A. (2007) Method for effective virtual screening and scaffold-hopping in chemical compounds. *Comput. Syst. Bioinformatics Conf 6*, 403−14.
(56) Wale, N., Watson, I. A., and Karypis, G. (2008) Indirect similarity based methods for effective scaffold-hopping in chemical compounds. *J. Chem. Inf. Model. 48* (4), 730−41.
(57) DataWarrior version 4.4.4. http://www.openmolecules.org/datawarrior/ (accessed 11.10).
(58) Chang, Schomburg, I., Placzek, S., Jeske, L., Ulbrich, M., Xiao, M., Sensen, C. W., and Schomburg, D. (2015) BRENDA in 2015: exciting developments in its 25th year of existence. *Nucleic Acids Res. 43*, D439−46.
(59) McAlpine, J. B., Piraee, M., Tremblay, S., Alarco, A.-M., Zazopoulos, E., Farnet, C. M., and Bachmann, B. O. (2005) Microbial Genomics as a Guide to Drug Discovery and Structural Elucidation: ECO-02301, a Novel Antifungal Agent, as an Example. *J. Nat. Prod. 68* (68), 493−496.
(60) Zazopoulos, Huang, K., Staffa, A., Liu, W., Bachmann, B. O., Nonaka, K., Ahlert, J., Thorson, J. S., Shen, B., and Farnet, C. M. (2003) A genomics-guided approach for discovering and expressing cryptic metabolic pathways. *Nat. Biotechnol. 21* (2), 187−90.

DOI: 10.1021/acssynbio.6b00136
ACS Synth. Biol. 2017, 6, 439−445