HHS Public Access

Author manuscript

Curr Opin Chem Biol. Author manuscript; available in PMC 2016 July 26.

Published in final edited form as:

Curr Opin Chem Biol. 2015 October ; 28: 20–28. doi:10.1016/j.cbpa.2015.05.012.

Synthetic biology expands chemical control of microorganisms

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Abstract

The tools of synthetic biology allow researchers to change the ways engineered organisms respond to chemical stimuli. Decades of basic biology research and new efforts in computational protein and RNA design have led to the development of small molecule sensors that can be used to alter organism function. These new functions leap beyond the natural propensities of the engineered organisms. They can range from simple fluorescence or growth reporting to pathogen killing, and can involve metabolic coordination among multiple cells or organisms. Herein, we discuss how synthetic biology alters microorganisms' responses to chemical stimuli resulting in the development of microbes as toxicity sensors, disease treatments, and chemical factories.

Introduction

Synthetic biology allows scientists to re-program interactions between genes, proteins, and small molecules. One of the goals of synthetic biology is to produce organisms that predictably carry out desired functions and thereby perform as well-controlled so-called biological devices. Together, synthetic and chemical biology can provide increased control over biological systems by changing the ways these systems respond to and produce chemical stimuli. Sensors, which detect small molecules and direct later cellular function, provide the basis for chemical control over biological systems. The techniques of synthetic biology and metabolic engineering can link sensors to metabolic processes and proteins with many different activities. In this review we stratify the activities affected by sensors to three different levels: sensor-reporters that provide a simple read-out of small molecule levels, sensor-effectors that alter the behavior of single organisms in response to small molecules, and sensor effectors that coordinate the activities of multiple organisms in response to small molecules (Figure 1).

1. Sensor-Reporters

Small molecule sensors used in synthetic biology are often based on RNAs [28-34] or transcription factors [35-42] that bind to specific chemicals and influence the expression of downstream effectors. Sensors with different specificities can be mined from the literature, discovered through screens for small molecule responsive promoters [16,43], or computationally designed [36,44,45**]. Once a suitable sensor is defined, its function can be

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tested by placing the expression of a reporter gene under the sensor's control (Figure 1A). Protein transcription factors are commonly used as sensors and are primarily discussed in this review. RNA based sensors that bind to a variety of ligands can be used to alter transcription, translation initiation, and ribozyme activity [46,47]. Using evolution based methods [48,49], RNAs that bind to specific ligands can be selected; however, it is often unclear how to link ligand binding function to reporter read out, and RNA based sensors for a wide array of compounds largely await development.

Simple sensor-reporter devices can be very powerful detectors of toxins or valuable chemicals. For instance, Trang and co-workers [35] show that a luciferase based bio-reporter for arsenic is capable of measuring arsenic levels in ground water from Vietnam at an accuracy better than established chemical methods. They propose to use this bio-assay in regions where expensive methods like atomic absorption spectroscopy cannot be easily performed. Similar reporters can measure levels of heavy metals, organic pollutants, and methylating compounds [37-39,42].

Sensor-reporter systems also allow one to screen for strains that produce a compound of interest in high yield (Figure 2). For such screening, strains need both the ability to produce a particular compound and the ability to report the amount they produce. This allows one to mutate or engineer these strains and directly measure the amount of reporter produced. High-producing strains can then be separated and used in further rounds of engineering and screening to continue enhancing yields.

The reporter-screening strategy described above has been used to enhance bacterial production of mevalonate [36], lysine [41], butanol [50], dicarboxylic acids [50], and triacetic acid lactone [51]; it is compatible with a wide variety of mutagenesis techniques. In a recent iteration of this technique, Raman *et al* 2014 [52**] used sensors controlling the expression of antibiotic resistance genes to select for *E. coli* strains with enhanced production of naringenin or glucaric acid. In their system, increased production of these compounds enhanced antibiotic resistance and provided a selective advantage over strains with lower production. Here the reporter readout was growth. The authors designed and implemented mutations enhancing production using multiplex automated genome engineering [53] and negative selection strategies to kill off any strains with mutations that simply led to high basal levels of antibiotic resistance. Iterative rounds of mutation, negative selection, and positive selection successfully improved the yields of both naringenin and glucaric acid.

2. Whole Cell Effectors

Synthetic gene circuits can lead to many more complex functions (cellular motility, memory, fuel and drug production, etc.) in response to small molecules. Here we focus on effectors with functions designed to improve health and enhance production by microbial chemical factories (Figure 1B). Some of these examples represent the first instance of transfer of synthetic circuits into real animal disease models.

Microbial Sensor-Effectors in Disease Prevention and Treatment

Pseudomonas aeruginosa, a ubiquitous bacterium capable of causing both mild and severe infections in humans, has recently been targeted using engineered *E. coli*. Exploiting the fact that *Pseudomonas aeruginosa* produce the quorum sensing molecule $3OC_{12}HSL$, researchers have generated *E. coli* strains that produce proteins that kill *P. aeruginosa* upon sensing $3OC_{12}HSL$ [6,11], and other *E. coli* strains that respond to $3OC_{12}HSL$ by migrating towards its source, releasing a protein that kills *P. aeruginosa*, and breaking down *P. aeruginosa* biofilms [5*]. The efficacy of these pathogen-killing bacteria has yet to be shown *in vivo*, but they provide a solid groundwork for future bacterial-based therapies.

We recently demonstrated the *in vivo* use of an *E. coli* memory device based on the lambda phage operon to record the presence of a chemical in the mouse gut [54**]. *E. coli* engineered with this memory circuit sustain production of beta-galactosidase after detecting anhydrotetracycline. When populating the mouse gut, these *E. coli* can detect anhydrotetracycline in mouse drinking water and remember its presence (i.e. continue to produce beta-galactosidase) up to 8 days after it is removed from the drinking water. This work is the first demonstration of an *in vivo* gut memory device and provides a platform upon which a variety of engineered gut microbiota responses to various chemicals (e.g. those indicative of infection) can be designed.

Many bacteria thrive in the hypoxic and acidic conditions of the tumor-micro environment with bacterial growth resulting in many anti-tumor effects. This phenomenon has a long history dating back to the 1800s and the use of bacterial extract (Coley's toxin) to boost the immune system in cancer treatment. There has also been extensive work using attenuated and engineered strains of bacteria to combat cancer (Reviewed in [55]). Although therapies using *S. typhimurium* have shown limited efficacy [56-58], an on-going phase I clinical trial using intratumoral injections of attenuated *Clostridium novyi* has shown promising anti-tumor activity [59**]. In addition, the natural anti-tumor activity of *Salmonella typhimurium* has been expanded by giving it the ability to invade cancer cells or to secrete toxins when in the vicinity of tumors [8,9,18,43]. These strategies take advantage of promoters that induce gene expression under conditions indicative of the tumor microenvironment. For example, Flentie *et al* 2012 [43] discovered a *Salmonella typhimurium* promoter responsive to low pH and placed Shiga Toxin 2 under its control in *S. typhimurium*. When injected into mouse xenograph tumors, this strain decreased tumor cell viability within 5 days of injection [43].

The engineered ability to sense and respond to the chemical nature of the tumor microenvironment holds promise in increasing the specificity of bacterial anti-cancer therapies, but this specificity can also be achieved through other means. For example, *Salmonella* selectively kill tumor cells in a mouse xenograph model when they express antibodies to cause their attachment to the tumor cells and thymidine kinase to locally convert the prodrug Ganciclovir into an apoptosis inducer [60*]. Furthermore, *E. coli* have been used as a means of delivering shRNA's silencing genes important for cancer cell viability to mammalian cells and live mice [10,61,62]. Further pairing of sensors with these types of selective treatments will likely lead to therapies with exquisite specificity.

Microbial Sensor-Effectors in Microbial Chemical Factories

Often, expressing a single enzyme can be enough to get a microbe to produce a small amount of a desired product, but more complicated metabolic engineering is usually required to divert metabolism away from natural cellular processes and toward production. While sensor-reporters provide a means of detecting high producers, sensor effectors can be used to dynamically modulate metabolism in response to the production of a compound of interest or its precursors providing new means to enhance yields.

Engineered metabolic pathways require coordination to limit the production of toxic intermediates and prevent hindrances on growth. In an early example of dynamic metabolic coordination, Farmer and Liao (2000) [14] enhanced *E. coli* lycopene production by placing lycopene-producing genes under the control of a promoter activated in the presence of acetyl phosphate (indicative of excess flux to acetate production). *E. coli* produce acetyl phosphate and acetate when they have excess glucose, but acetate retards *E. coli* growth. Farmer and Liao [14] hypothesized that high acetyl phosphate concentrations would be indicative of a metabolic state ideal for lycopene production; instead of diverting excess glucose into toxic acetate, acetyl phosphate induction of lycopene genes could re-direct excess glucose into lycopene synthesis. This strategy successfully increased lycopene production by greater than ten-fold compared to strains with lycopene production genes under the control of a lactose inducible promoter.

Our lab has demonstrated the use of an inducible degradation system to modulate *E. coli* fatty acid metabolism for the production of medium chain fatty acids [63] (Figure 3). *E. coli* require long chain fatty acids for normal growth, but medium chain fatty acids are potential precursors to fuel-like compounds. To divert *E. coli* fatty acid synthesis away from the production of long chain fatty acids, we replaced one of *E. coli*'s two fatty acid elongating enzymes with a mutant that can only elongate the fatty acids up to eight carbons. We then made the degradation of the second enzyme inducible by the small molecule isopropyl β-D-1-thiogalactopyranoside (IPTG). Replacing both enzymes with mutants would likely have been lethal, but the inducible degradation system allowed us to push fatty acid synthesis toward medium chain fatty acid production. These efforts, combined with further enzyme deletions designed to shunt cellular resources into fatty acid production, resulted in the production of the medium chain fatty acid octanoate at 12% theoretical yield. Future efforts could use sensors to link similar degradation systems to growth or fatty acid production instead of IPTG.

Similar strategies have been used to improve amorphadiene [16], alpha-santalene [13], fatty acid [15,17*], and fatty acid ethyl-ester [12] production. It is important to note however, that it is unclear whether these strategies can be used at commercial scale.

3. Multi-cell Effectors

While single engineered cells are powerful tools, greater functionality can be achieved by chemically coordinating activities between multiple cells, strains, or species. The use of coordinated consortia can limit the need for researcher intervention, provide means to stabilize compound production by limiting toxicity and/or providing a selective advantage to

high producers, and enhance yields. Applications of chemically coordinated cellular consortia are only just beginning to take shape, but synthetic biology provides us with the tools to chemically coordinate the activities of multiple organisms, as discussed below.

Quorum Sensing

Microorganisms use quorum sensing (QS) to measure population density through sensing the concentration of a small molecule produced by members of the population. QS allows microorganisms to restrict certain behaviors (e.g. virulence, sporulation, and light production) to particular population densities (Reviewed in [64]). Many QS molecules, production enzymes, sensors, and responsive promoters have been discovered [64]. These QS tools allow researchers to coordinate engineered activities within microorganisms. For example, researchers must measure population density when using bacteria to produce recombinant proteins or useful chemicals. If production is turned on too early or too late, yields can be limited. Quorum sensing provides a useful means through which engineered bacteria can measure their own population density and has the added advantage of preventing the use of a potentially expensive inducer molecule [22,23,65] (Figure 1C).

QS can also be used to regulate population density and composition. Mixed microbial consortia offer the possibility to partition engineered functions into optimally engineered and cooperating microbial strains or species. QS systems can be used to both activate and repress gene expression [66]. Altered gene expression due to Inter-microbe QS can be used to modify population structures as may be necessary in complicated microbial production schemes [21,24,65,67]. For example, You *et al* 2004 [24] used QS controlled cell lysis to regulate *E. coli* population density. Such a system could be used to maintain an engineered strain at a desired and/or safe level. Hong *et al* 2011 [21] used QS to regulate the bacterial composition of a biofilm. Such biofilm tuning could be used to effect dynamic metabolic output. QS systems can also be incorporated into logic gates [68] and have been use to drive bacterial localization to cancer cells [69]. These developments provide us with many tools, but QS systems await the realization of their full potential in synthetic systems that produce commodity chemicals or treat human disease.

Coordinating Metabolic Pathways

It is not always beneficial or possible to engineer all the components of metabolic pathways in a single organism. Different organisms have different natural metabolic propensities and sensitivities to toxic compounds. It can therefore be advantageous to use multiple organisms to carry out a complex task. For example, Minty *et al* 2013 [70*] co-cultured a fungus that naturally breaks down cellulose with *E. coli* engineered to produce isobutanol; they showed that the two can convert cellulosic biomass into isobutanol. In this example, the *E. coli* are essentially parasitic to the fungi because they use fungal resources and produce a compound (isobutanol) that inhibits fungal growth.

Chemicals produced by consortium members can be used to foster mutualisms, in which the members of the consortium are dependent upon one another for growth. One way to establish mutualism is to have one member of a two-component consortium consume a toxic

product produced by the other member (Figure 4). For example, Zhou *et al* 2015 [25**] used a consortium of *S. cerevisiae* and *E. coli* to produce oxygenated taxanes (precursors to anti-cancer therapeutics). A portion of the metabolic route to the oxygenated taxanes was previously engineered in *E. coli*, but further processing required the use of cytochrome P450s that negatively impacted downstream steps in the pathway [71]. To avoid these negative impacts, the authors separated the pathway into components in *E. coli* and *S. cerevisiae*. The *E. coli* were capable of consuming provided xylose and produced oxygenated taxane precursors, but also produced toxic acetate as a byproduct. The yeast used the acetate as their carbon source thereby limiting its toxicity. The yeast then produced the cytochrome P450s and completed the pathway to oxygenated taxanes. This mutualistic consortium nearly doubled its production of oxygenated taxanes compared to a mixed culture where the *E. coli* and the yeast competed for glucose. A similar system was used by Bayer *et al* 2009 [26] to produce methyl halides from cellulosic biomass.

Further examples of metabolic coordination for the production of useful compounds are rare (Reviewed in [30]), but current research provides additional tools to link microbial metabolism. Microbes can be artificially linked through cross feeding essential metabolites [72,73], metabolites can be used for inter-strain and even inter-species signaling [27,74], and co-dependencies fostered by cross feeding can be used to select for the production of a target compound [75]. Equipped with these tools, future efforts should be able to generate many more mutualistic interactions that foster the cooperation of organisms with diverse functionalities.

Conclusion

We have come to the point in synthetic biology where there are many lab-scale or proof-of-concept examples of chemically controlled systems useful to sense small molecules, treat disease, and produce commercially useful compounds. These systems have great potential, but more attention needs to be paid to their stability, efficacy, and safety. Being that the sensor-effectors discussed above function in living, evolving organisms, it is unclear how well they will retain function when distributed in a patient or in a large-scale bioreactor. Future efforts should focus on developing these sensor-effectors for real-world application. Engineered organisms will only be useful if we can prove that their functions are reliable, predictable, and cost effective.

Acknowledgements

We thank Stephanie Hays and Jessica Polka for critical reading of the manuscript. This work was conducted with support from the National Science Foundation Graduate Research fellowship (to T.J.F.), and the Ruth L. Kirschtein National research Service Award program of Harvard Catalyst, The Harvard Clinical and Translational Science Center Award UL1 RR 025758 and financial contributions from Harvard University and its affiliated academic health care centers (to T.J.F.). The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic health care centers, the National Center for Research Resources or the National Institutes of Health. This material is based upon work supported by the National Science Foundation. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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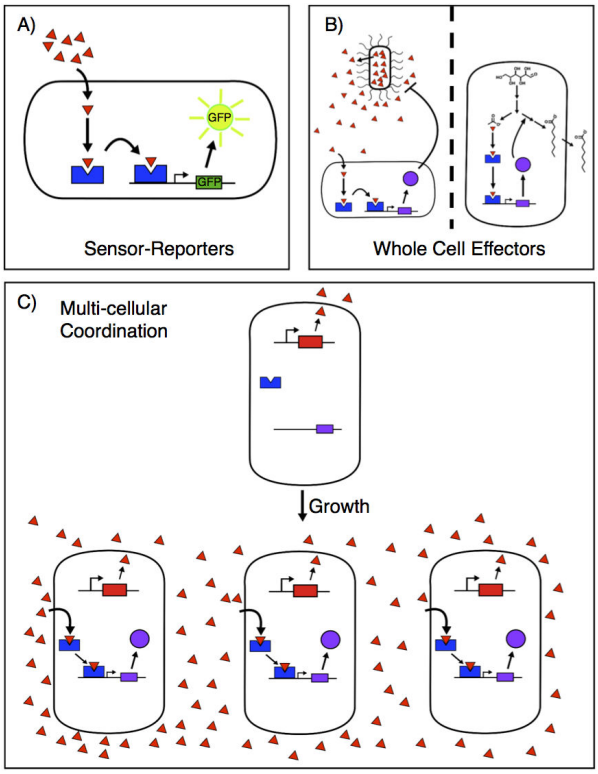

Figure 1.

A) Sensors (blue polygons) are the basic functional units microorganisms use to detect chemical stimuli (red triangles). Sensors act through effectors to achieve a particular function. Reporters (green sun) are simple effectors that provide an easily measured readout (growth, fluorescence) to indicate that a sensor has detected a particular compound. B) Whole cell effectors (purple circle) drastically alter cellular behavior allowing microorganisms to carry out a variety of functions. Effectors can give microorganisms the ability to incorporate signals from multiple sensors [1-4], to fight pathogens (left) [5*-11], and coordinate microbial metabolism to produce useful compounds (right) [12-17*]. C) Chemicals (red triangles), sensors (blue polygons), and effectors (purple circles) can coordinate populations of multiple cells, strains, or species with specialized abilities to detect or produce chemical stimuli resulting in the performance of tasks with labor shared among the population [18-25**,26,27]. In this example, growth leads to increased concentration of a chemical signal (red triangle) that coordinates the production of an effector (purple circle) by the entire population.

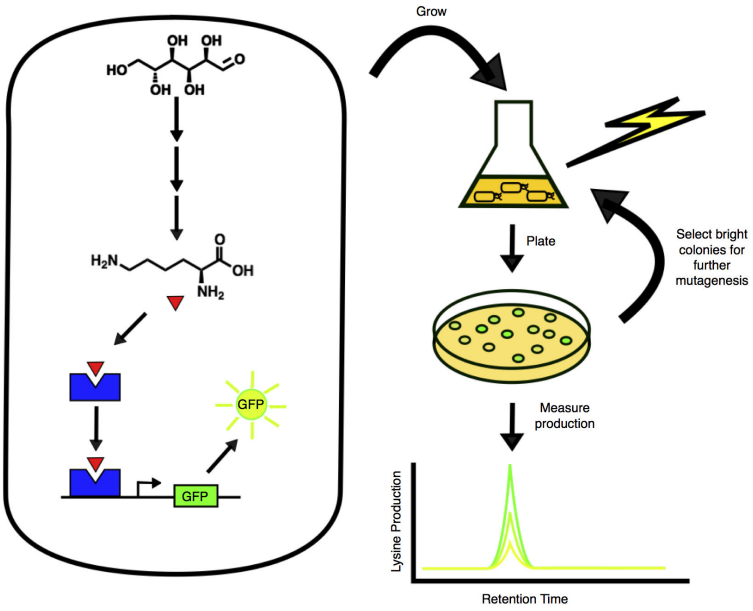

Figure 2.

Using sensors to screen for high yields of a desired compound. The bacterium on the left has been engineered to produce lysine (red triangle) which is sensed by a transcription factor (blue polygon) that activates the expression of GFP. After mutagenizing this strain, lysine production by different mutants can be detected via their level of GFP expression. Mutants that produce high amounts of lysine also produce high amounts of GFP and can be picked for further mutagenesis. Directly measuring lysine quantities produced by different stains using a technique like liquid chromatography mass spectroscopy (LC-MS) confirms the correlation between GFP expression and lysine production.

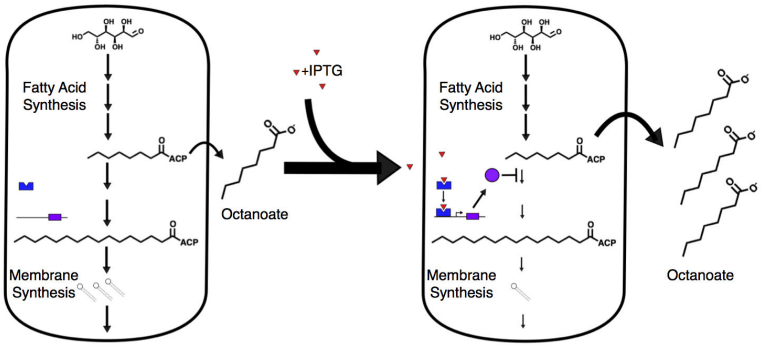

Figure 3.

Inducible inhibition of fatty acid elongation to enhance medium chain fatty acid production. In the system used in [63], fatty acid elongation was inhibited by an effector (purple circle) whose transcription was activated by exogenously added IPTG (red triangle). This slowed fatty acid synthesis and enhanced the production of the medium chain fatty acid, octanoate. Future systems could sense long chain fatty acids and avoid the need to add an exogenous inducer like IPTG.

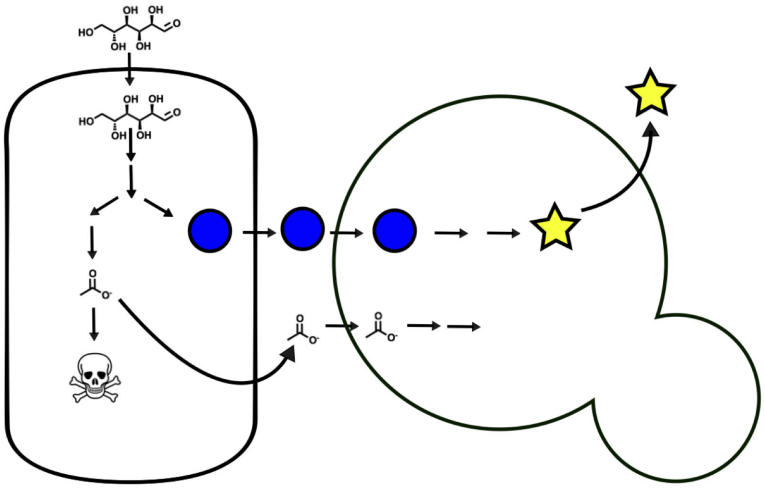

Figure 4.

Coordinating multi-microbe metabolism. Multiple organisms can be coordinated through chemical exchanges and metabolism to produce a desired compound. Here the organism on the left eats glucose and has been engineered to produce the blue circle, but also produces toxic acetate as a byproduct. The organism on the right further converts the blue circle into the end product (yellow star) and also eats the toxic acetate thereby promoting the growth of the organism on the left. Due to their linkage through acetate, the organisms are mutually dependent upon one another for optimal growth.