Integrating artificial with natural cells to translate chemical messages that direct E. coli behaviour

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Previous efforts to control cellular behaviour have largely relied upon various forms of genetic engineering. Once the genetic content of a living cell is modified, the behaviour of that cell typically changes as well. However, other methods of cellular control are possible. All cells sense and respond to their environment. Therefore, artificial, non-living cellular mimics could be engineered to activate or repress already existing natural sensory pathways of living cells through chemical communication. Here we describe the construction of such a system. The artificial cells expand the senses of Escherichia coli by translating a chemical message that E. coli cannot sense on its own to a molecule that activates a natural cellular response. This methodology could open new opportunities in engineering cellular behaviour without exploiting genetically modified organisms.
SYNTHETIC BIOLOGY thus far has relied upon the engineering of new cellular function through the insertion and deletion of genetic information in living cells. This genetic engineering based approach has progressed rapidly. There is now available a set of well-characterized biological parts that can be used to build complex genetic circuits within and between the living cells. Further, entire genomes can be edited and synthesized, suggesting that fully designed organisms with heretofore unseen capabilities are likely in the future.

Despite the wide range of technologies and target pathways exploited, the desire to control microorganisms to date has always employed direct genetic intervention. The limitations of these prevalent methods are due to the difficulties of engineering living systems, including evolutionary pressures that may alter engineered pathways over time and the potential long-term consequences of altering ecosystems with engineered organisms. However, it may not be necessary to genetically modify living cells. Extant life is already extremely complex, endowed with numerous sensory and metabolic pathways tuned by billions of years of evolution to be efficiently responsive to changing intracellular and extracellular conditions. A simple change in pH, for example, results in the up and downregulation of nearly 1,000 genes in Escherichia coli. In other words, cells are already capable of sensing many different stimuli and capable of performing many tasks. Therefore, it should be possible to exploit these existing cellular pathways to control cellular behaviour without changing the genetic makeup of the cells.

Here we explore this idea of engineering E. coli through alternative means by targeting the sensory pathways of E. coli. To do so without altering the genetic content of the bacterium, we instead construct artificial cells that could interact with natural cells in order to evoke a behavioural response. The artificial cells in this system function as chemical translators that sense molecules that E. coli alone cannot sense. In response, the artificial cells release a molecule that E. coli can naturally respond to, thereby translating an unrecognized chemical message into a recognized chemical message. In this way, the sensory capabilities of E. coli are expanded without altering the genetic content of the bacterium. The artificial cell is built with a phospholipid vesicle containing isopropyl β-D-1-thiogalactopyranoside (IPTG), DNA, and transcription–translation machinery. The DNA template codes for a previously selected riboswitch that activates translation in response to the presence of theophylline. The theophylline riboswitch controls the synthesis of the pore forming protein α-hemolysin (αHL). Therefore, in the presence, but not the absence, of theophylline a pore that releases IPTG. E. coli alone does not respond to theophylline, and IPTG does not cross the vesicle membrane of the artificial cell in the absence of the pore. The ability of E. coli to receive the chemical message sent by the artificial cells is assessed in two ways. First, the fluorescence of E. coli carrying a plasmid encoding a fluorescent protein behind an IPTG-responsive, lac operator sequence is evaluated. Second, the gene expression of untransformed E. coli is monitored by reverse transcription quantitative PCR (RT–qPCR). To our knowledge, this is the first artificial, cell-like system capable of translating unrecognized signals into a chemical language that natural cells can recognize. The integration of artificial translator cells with natural cells represents a new strategy to introduce synthetic features to a biological system while circumventing the need for direct genetic manipulation.

Results

The theophylline-sensing device is functional in vitro. To build artificial cells that sense theophylline and in response release IPTG (Fig. 1), a theophylline-sensing genetic device was built with a T7 transcriptional promoter, a theophylline riboswitch and a gene encoding a fusion between αHL and superfolder GFP at the carboxy terminus. If functioning properly, this arrangement should result in the expression of protein and thus green fluorescence only in the presence of theophylline. However, cell-free expression in the presence and absence of theophylline showed similar levels of fluorescence (Fig. 2a). Since this same riboswitch was previously shown to function in vitro, the sequence of the αHL-GFP gene was more closely examined. Multiple pairs of potential ribosome binding sites (RBS) and start codons were identified within the αHL portion of the gene that were in-frame with the GFP-encoding region. The theophylline riboswitch controls translation, meaning that sequences behind the theophylline riboswitch are always transcribed. Translation from the RBS within the riboswitch is activated by direct binding of theophylline to the messenger RNA. Therefore, if additional sequences outside of the riboswitch but within the αHL portion of the gene were recognized by the ribosome, then regardless of the theophylline concentration, the expression of truncated peptide products with fluorescently active GFP would have been possible. To test if such internal RBSs were present, the theophylline riboswitch and thus the RBS preceding the αHL-GFP sequence was deleted. In vitro transcription–translation of this construct showed the accumulation of fluorescence over time similar to the riboswitch containing construct (Fig. 2b). Sequence analysis revealed three potential RBS-start codon pairs within the αHL coding portion of the gene. Of these, a putative RBS of

Figure 1 | Artificial cells translate chemical signals for E. coli. (a) In the absence of artificial cells (circles), E. coli (oblong) cannot sense theophylline. (b) Artificial cells can be engineered to detect theophylline and in response release IPTG, a chemical signal that induces a response in E. coli.
AAAGAA was selected as the most likely candidate for giving fluorescent protein expression based on sequence composition and spacing. The putative internal RBS was removed by mutation to TCTACC, resulting in a carboxy-terminal GFP tagged K30S E31T zHL construct. Fluorescence from this mutated construct was reduced threefold, consistent with the removal of an internal RBS (Fig. 2b). Finally, K30S E31T zHL-GFP was placed behind the theophylline riboswitch to test the activity of the cell-free sensing device. A clear difference was observed between protein expression in the presence and absence of theophylline (Fig. 2c), and the fluorescence arising in the absence of theophylline was within 20% of the construct lacking an RBS upstream of the full gene. The data were consistent with a functioning riboswitch sensor with background fluorescent protein expression arising from internal RBS within zHL. Therefore, the final artificial cellular mimic described below was built with zHL lacking a GFP tag to avoid complications arising from the expression of truncated fluorescent protein product.

**Active zHL is produced in response to theophylline in vitro.** To ensure that the cell-free expressed zHL was active as a pore, the ability of zHL to degrade red blood cells was assessed through a standard haemolysis assay. Each construct was expressed in vitro at 37°C for 6h after which, an aliquot was removed and added to red blood cells. Haemolysis was quantified by measuring attenuation at 650 nm. In the presence of theophylline, 90% haemolysis was observed when the genetic construct containing a riboswitch-controlled zHL was expressed. The cell-free expression of the same construct in the absence of theophylline gave haemolysis levels similar to the negative control reactions (Fig. 2d), as was expected for a functioning theophylline riboswitch that controls the production of zHL. Control reactions with commercial zHL-purified protein and in vitro-expressed zHL and zHL-GFP were all active (Fig. 2d, Supplementary Table 2), whereas aliquots from in vitro-expressed GFP alone and zHL with a carboxy-terminal His-tag were inactive (Supplementary Table 2). zHL with a carboxy-terminal His-tag was previously shown to have reduced activity. Also, comparison of the riboswitch activity fluorescence data with the haemolysis assay data was consistent with the production of GFP containing protein fragments from an internal RBS without an active zHL domain. For example, the zHL–GFP construct lacking one of the putative internal RBSs failed to produce protein with haemolysis activity (Supplementary Table 2), despite giving rise to fluorescence during in vitro transcription–translation (Fig. 2b).

**Artificial cells can translate chemical messages for E. coli.** After demonstrating that the riboswitch was able to control the in vitro expression of zHL in response to theophylline and that the expressed zHL molecules formed functional pores, the component parts were next assembled inside of phospholipid vesicles to build artificial cells. Theophylline is capable of passing through the membrane of vesicles. Phospholipid vesicles were generated in the presence of IPTG, transcription–translation machinery and DNA encoding zHL under the control of the theophylline riboswitch. The vesicles were then purified by dialysis at 4°C to remove unencapsulated molecules. The receiver bacterial cells were mid-exponential phase E. coli BL21(DE3) pLysS carrying a...
Theo plasmid encoding GFP behind a T7 promoter and a lac operator sequence. In this commonly exploited system, IPTG induces the expression of a chromosomal copy of T7 RNA polymerase in E. coli BL21(DE3) and derepresses the expression of GFP from the plasmid. Background expression is typically low with such an arrangement because of the presence of constitutively expressed lysozyme from pLysS, a natural inhibitor of T7 RNA polymerase.

To test if the artificial cells could function as chemical translators for E. coli, the artificial cells were incubated with E. coli BL21(DE3) pLysS carrying the GFP-encoding plasmid at 37 °C, and the fluorescence of E. coli was evaluated by flow cytometry. A control reaction in which theophylline was directly added to E. coli in the absence of artificial cells failed to show green fluorescence after 3 h (Fig. 3a). Similarly, IPTG loaded vesicles that did not contain the machinery necessary to form pores did not induce fluorescence in E. coli. Therefore, theophylline was not able to induce a detectable response in E. coli, and IPTG could not cross the vesicle membrane in the absence of zHL, which was consistent with permeability measurements (Supplementary Fig. 1). However, when E. coli was incubated with artificial cells and theophylline, 17 ± 10% and 69 ± 3% of the bacteria fluoresced green after 0.5 and 3 h, respectively. When the same experiment was repeated in the absence of theophylline, 3 ± 1% and 24 ± 5% of the bacteria were fluorescent after 0.5 and 3 h, respectively (Fig. 3a,b). Longer incubations resulted in diminishing differences between the two samples suggesting the presence of low levels of zHL expression in the absence of theophylline. Also, the GFP response was encoded within a medium copy number plasmid. Therefore, higher background levels of GFP were to be expected in comparison with gene expression from the chromosome. The flow cytometry experiments were consistent with the ability of artificial cells to translate an unrecognized chemical signal (theophylline) into a signal (IPTG) that E. coli could respond to.

Although the artificial cells were capable of communicating with E. coli, the induction of GFP synthesis, as observed above, exploited an engineered response. To assess whether artificial cells could elicit a natural, chromosomally encoded response, RT–qPCR was used to measure gene expression from the lac operon of E. coli. The lac operon is one of the most thoroughly characterized sensory pathways15. The presence of allolactose (or the non-hydrolyzable analogue IPTG) induces the expression of lacZ, lacY and lacA. To facilitate detection of E. coli responding to the chemical message sent from the artificial cells, E. coli BL21 (DE3) pLysS were grown in LB supplemented with glucose to decrease the background expression of the lac operon and then transferred to M9 minimal media prior to incubation with artificial cells. The artificial cells were prepared as described for the GFP induction experiments above. After incubating together artificial cells with E. coli in the presence and absence of theophylline for 4 h, aliquots were collected for RNA isolation. The RNA was then reverse transcribed and lacZ, lacY, and lacA expression quantified by qPCR. The RNA isolated from bacteria incubated with artificial cells plus theophylline showed on average over 20-fold higher lacZYA expression than samples incubated with artificial cells alone (calculated from AC/(AC + theo) as shown in Fig. 3c). Taken together, the data are consistent with the ability of artificial cells to translate chemical messages and induce both engineered and natural pathways in E. coli.

Discussion

Direct genetic engineering of living cells is not needed to control cellular behaviour. It is possible, instead, to coerce desired activity through communication with artificial cells. The foundation for such technologies has already been laid by both cell-free and in vivo studies. Engineered communication paths between living cells have been constructed to coordinate cellular activities in response to external stimuli6,16 and are being developed for therapeutic purposes17. In these systems, sender cells often can process information and in response release molecules that affect other cells. What has been shown herein builds on these past efforts but does so by integrating reconstituted, non-living systems with living cells. This allows for the genetic engineering component of the system to be moved from the living, evolving, replicating cells to the more controllable, ephemeral artificial

![Figure 3](image-url)
cells. When the artificial cells degrade, the natural cells go back to their original state, thereby diminishing the possibility of unintended long-term consequences. For example, rather than engineering bacteria to search for and clean up environmental contaminants, artificial cells could be built to sense the contaminant molecules and in response release chemoattractants that bring natural bacteria capable of feeding on the contaminants35-37 to the affected site.

Several recent reports have described the engineering of seek-and-destroy bacteria for the eradication of tumours or bacterial infections19-22. However, these methods ultimately rely on administering living bacteria to the patient. Artificial cells could be built to carry out similar tasks if the sensor module of the artificial cell was designed to detect the chemical conditions associated with the ailment. For instance, rather than spraying engineered bacteria into the lungs of cystic fibrosis patients, artificial cells could be built to detect the presence of Pseudomonas aeruginosa biofilms through the quorum signalling molecules that are naturally secreted by the organism, such as N-(3-oxodecanoyl)-l-homoserine lactone, a molecule capable of crossing membranes without the aid of transporters. Subsequently, the artificial cells could release small molecules, for example, D-amino acids23 to disperse the biofilm and thus clear the infection. Moreover, the use of dispersion rather than killing would decrease the probability of the bacteria developing resistance. Similar strategies with artificial cells could be developed to substitute for engineered probiotics that integrate with gut microbiota and prevent disease25,26.

In vitro characterization of the riboswitch. Plasmids were amplified in E. coli Novabla (Novagen) and purified with Wizard Plus SV Miniprep DNA Purification System (Promega). Plasmid DNA was phenol–chloroform extracted, ethanol precipitated and resuspended in deionized and diethyl pyrocarbonate-treated water. PCR products were purified with Wizard Plus SV Gel and PCR Clean-Up System (Promega). For transcription-translation reactions using PURExpress In Vitro Protein Synthesis Kit (New England Biolabs) supplemented with 20 units of Human Placenta RNase Inhibitor (New England Biolabs), reactions were monitored by fluorescence with a CFX96 Touch real-time PCR (Bio-Rad) using the SYBR green filter set.

Preparation of E. coli receiver cells. Mid-exponential E. coli BL21(DE3) pLYS S transformed with a plasmid encoding superfolder GFP behind a T7 promoter and a lac operon sequence (CD101A) were grown in LB supplemented with 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol to an optical density of 0.5 at 600 nm. A quantity of 200 µl aliquots in 10% (vol/vol) glycerol were flash frozen with liquid nitrogen and later used. Aliquots were rapidly thawed and mixed with 2 ml LB supplemented with 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol and incubated for 2 h at 37°C with 220 r.p.m. shaking. Finally, the cells were gently pelleted and resuspended in 1 ml M9 minimal media.

Preparation of artificial cells. Vesicles were prepared as previously described36,37. Briefly, 12.5 mg cholesterol (Avanti Polar Lipids) in chloroform were mixed in a round bottom flask. A thin lipid film was made through rotary evaporation with a Buchi Rotovapor R-210 equipped with a Buchi Vacuum Pump V-700 for 5 h. A quantity of 2 ml DEPC-treated deionized water was then added to the thin lipid film and vigorously vortexed. The resulting liposome dispersion was homogenized with an IKA T10 basic homogenizer at a power setting of 4 for 1 min. A quantity of 100 µl aliquots were frozen in liquid nitrogen or dry ice and lyophilized overnight in a vacuum concentrator (Centrifrap DNA concentrator, Labconco) at 40°C. The lyophilized empty liposomes were stored at −20°C. A quantity of 100 µl aliquots of freeze-dried liposomes were hydrated with 25 µl of 100 mM IPTG (Sigma) dissolved in 50 mM HEPES pH7.6, 25 µl of the PURE system, 500 ng DNA and 20 units of human placenta RNase inhibitor (final volume of 50 µl), unless otherwise noted. Solutions were gently mixed for 30 s.

To remove extravesicular material, the vesicles were dialyzed following a method previously described by Zhu and Szostak38. The original membranes of 500 µl Slide-a-Lyzer dialysis cassettes (Pierce) were exchanged with 25 mm diameter polycarbonate track-etched membranes with a 1 µm pore size (Whatman). A quantity of 50 µl unpurified vesicles were loaded onto the center of the dialysis system with a 100 µl Hamilton syringe and dialyzed against 250 ml of buffer A (50 mM HEPES, 10 mM MgCl₂, 100 mM KCl, pH 7.6) with stirring. The dialysis continued for 4 h, after which two more rounds of dialysis in which the buffer was changed after 30 min incubations were further performed. All dialysis steps were carried out at 4°C.

Artificial-natural cell communication. Purified vesicles containing DNA, the PURE system, and IPTG were incubated with E. coli BL21(DE3) pLYSS transformed with CD101A in M9 minimal media supplemented with 1 mg ml⁻¹ of Proteinase K and 5 mM theophylline at 37°C in a final volume of 40 µl. Control reactions did not contain theophylline. At different time points, 1 µl was removed and diluted 1:100 in PBS. The sample was then analysed by flow cytometry with a FACS Canto A (BD Biosciences). The FITC filter was used for the detection of positive cells. The incident light was at 488 nm for forward scatter (FSC), side scatter (SSC) and fluorescence. Detection for SSC and fluorescence was at 488 ± 10 nm and 530 ± 30 nm, respectively. The threshold parameters were 200 for both FSC and SSC. The PMT voltage settings were 525 (FSC), 403 (SSC) and 600 (FITC). The flow rate was set to ‘low’. For each sample 30,000 events were
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Author contributions

Design, cloning and maturation of genetic constructs were done by R.L., A.C.S., J.F., S.P.S., M.D.S. R.L., J.L.T., D.C., F.C. and S.P.S. ran the cell flow cytometry experiments, and M.D.S. R.L., J.L.T., D.C., F.C. and S.P.S. analysed and interpreted the data and contributed to the writing of the manuscript.

Additional information

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