Letter

Developing a genetically-encoded, cross-species ammonium biosensor for detecting ammonium and regulating biosynthesis of cyanophycin

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Developing a genetically-encoded, cross-species biosensor for detecting ammonium and regulating biosynthesis of cyanophycin

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Abstract

Responding to nitrogen status is essential for all living organisms. Bacteria have evolved various complex and exquisite regulatory systems to control nitrogen metabolism. However, natural nitrogen regulatory systems, owing to their complexity, often function only in their original hosts and do not respond properly when transferred to another species. By harnessing the *Lactococcus* GlnRA system, we developed a genetically-encoded, cross-species ammonium biosensor that displays a dynamic range up to 9-fold upon detection of ammonium ion. We demonstrated applications of this ammonium biosensor in three different species (*Escherichia coli*, *Pseudomonas putida*, and *Synechocystis* sp.) to detect different nitrogen sources. This ammonium sensor was further used to regulate the biosynthesis of a nitrogen-rich polymer, cyanophycin, based on ammonium concentration. Given the importance of nitrogen responses, the developed biosensor should be broadly applicable to synthetic biology and bioengineering.

Key words: Nitrogen response, ammonium biosensor, GlnRA, biosynthesis, bioremediation

Introduction

Biosensors have broad applications in biodetection, bioproduction, bioremediation, and therapeutic treatment, providing powerful tools to program biological behaviors and to study basic biology [1-9]. Because nitrogen is an essential element for cell survival and growth, response to nitrogen status is important for all living organisms. Nature has evolved a wide variety of sensory systems to monitor nitrogen status and to regulate the expression of nitrogen metabolic genes for cell growth [10-14]. Because natural nitrogen systems are often heavily regulated, they often function only in their natural microbial hosts [10, 15-17]. Rewiring these natural biosensors may disturb the native system and impair cell growth, limiting their broad use in synthetic biology and bioengineering applications. Moreover, native nitrogen regulatory systems have fixed sensitivities and dynamic ranges [18-20], thus further limiting their applications. Therefore, it is desirable to develop a synthetic nitrogen sensor that does not interfere with native nitrogen regulatory systems and can be tuned to meet broad applications in multiple species and under a broad range of conditions. Such a nitrogen biosensor could be used to detect environmental nitrogen signals and consequently regulate gene expressions for biosynthesis, bioremediation, and therapeutic treatment. Unfortunately, a highly tunable, cross-species nitrogen biosensor has not been reported.

In bacteria, several natural nitrogen sensory systems are documented, including the NtrBC system in enteric bacteria [10], the NtcA system in cyanobacteria [12], and the GlnRA system in low GC-content, Gram-positive bacteria [11, 13]. The NtrBC system is a two-component system comprising a sensory histidine kinase NtrB, a cognate transcriptional factor NtrC, and a few ancillary proteins (such as GlnBDEFK) [10, 11, 15, 17, 21]. Under nitrogen-limiting conditions (i.e., a high ratio of 2-oxoglutarate to glutamine in the cell), NtrB becomes phosphorylated and the

phosphorylated NtrB subsequently phosphorylates NtrC, allowing it to bind to its cognate DNA sequences and to activate transcriptions of nitrogen-regulated genes [22]. In the NtcA system, a global transcription factor NtcA (belonging to the cAMP receptor protein superfamily), together with P_{II} (a nitrogen and carbon signal transduction protein) and PipX (a cyanobacterial P_{II}-interacting protein X), modulates expressions of multiple nitrogen-regulated genes [12, 23, 24]. When nitrogen is limited, the intracellular concentration of 2-oxoglutarate (2OG) increases and stimulates formation of the 2OG-NtcA-PipX complex, which activates NtcA-dependent gene expressions. Under nitrogen abundant conditions, 2OG concentration is low and PipX is sequestered by P_{II}, leading to inactivation of the NtcA-dependent genes. In contrast to the NtrBC and NtcA systems, the GlnRA system is relatively simple. The GlnRA system consists of a glutamate-ammonium ligase GlnA **(**also named glutamine synthetase) and a transcription factor GlnR (binding to its cognate DNA sites) [11, 13]. When ammonium concentration is high, GlnA converts ammonium and glutamate to glutamine. GlnA can be inhibited by its own enzymatic product glutamine at high concentration, forming glutamine-bound, feedback-inhibited GlnA (FBI-GlnA). The FBI-GlnA serves as a chaperon that stabilizes the GlnR-DNA complex and prevents RNA polymerase binding at a nearby site, thus blocking transcription initiation [21, 25]. In *Bacillus* and *Lactococcus*, the *glnR* and *glnA* genes are often organized in a single operon, suggesting their co-regulation [11].

Ammonium, as the most readily assimilated nitrogen format, is a limiting nutrient for microorganisms and plants [26]. Thus, it is an important nitrogen source in both the environment and microbial media. Here, we harnessed the GlnRA system from *Lactococcus lactis* [13] and developed a genetically-encoded ammonium biosensor. By engineering synthetic GlnR-controlled promoters and tuning the expression levels of GlnRA, the sensor was optimized to

display a 9-fold response change from high to low nitrogen conditions. We further demonstrated that the ammonium sensor functioned in three different Gram-negative bacterial species (*Escherichia coli*, *Pseudomonas putida*, and *Synechocystis* sp. strain), indicating potential applications in a broad host range. Moreover, we used the sensor to dynamically regulate gene expression for the biosynthesis of a nitrogen-rich polymer, cyanophycin [27], allowing cyanophycin production to be controlled according to ammonium availability.

Results and Discussion

Construction of a genetically-encoded ammonium biosensor using GlnRA from *Lactococcus lactis*

The *glnRA* coding sequences from *Lactococcus lactis* ATCC 19435 (Supplementary Table S1) were amplified from genomic DNA. To tune the expression level of *glnRA*, a small synthetic promoter library P_{lib} was generated by introducing two degenerate nucleotides into the -10 box (TATANN, totally 16 different promoters, Figure 1) of a synthetic constitutive *E. coli* promoter P_{cons} (Supplementary Table S1). This library was then placed at 5' of the *glnRA* coding sequences. Meanwhile, an IPTG-inducible promoter, $P_{trc\text{-}1O}$ [28], was also used to control the expression of *glnRA*, providing an easy method to tune the expression level of *glnRA*. To create a sensor-reporter, three GlnR-regulated synthetic promoters-P_{glnR1}, P_{glnR2}, and P_{glnR3}-were designed to control the expression of a reporter, either an enhanced yellow fluorescent protein (eYFP) or a red fluorescent protein (RFP) (Figure 1A, Supplementary Figure S1). P_{glnR1} contains a 18-bp GlnR-binding site [13] upstream of the -35 box of the constitutive promoter P_{cons}. This GlnR-binding site was moved to the middle of the -35 and -10 boxes in P_{glnR2} [19, 29]. P_{glnR3} contains two GlnR-binding sites flanking the -10 region (Supplementary Figure S2). Such a design often

provides a greater dynamic range in synthetic biosensors [29]. For cross-species applications, these sensor operons were cloned into a broad-host-range IncQ plasmid [30, 31] that contains a replicative origin, *OriV*, and genes encoding for replicative proteins (*repABC*, Supplementary Figure S1). The constructed single plasmid can be easily transferred to target host cells. As negative controls, strains carrying the reporter but without *glnA* or *glnRA* operon were constructed (Supplementary Table S5).

Characterization and optimization of the ammonium biosensor in *E. coli*

We first characterized the ammonium biosensor bearing the P_{glnR1}-eYFP reporter in *E. coli* DH10B cells. To search for the variants with proper *glnRA* expression levels and large fold-of-change upon ammonium detection, more than 50 strains from the GlnR1 library were selected. Sequencing showed that many of these selected strains have the same -10 box, indicating these promoter sequences are high favorable for cell growth, thus enriched in the library. From these strains, only 6 non-identical variants were found (Figure 1B). All 6 variants were tested in M9 yeast extract medium containing high (15 mM) or low (0 mM) concentrations of $(NH_4)_2SO_4$. Cell culture fluorescence normalized by cell density was measured after growth in $(NH_4)_2SO_4$ for 36 hours (See Methods). While the control strains, $GlnR_{CeYFP}$ without the *glnRA* and $GlnR_{CeYFP\text{-}A}$ without the *glnA*, showed little difference in cellular fluorescence when grown in the high and low $(NH_4)_2SO_4$ cultures, all 6 GlnR1 strains exhibited expected fluorescence repression under the high $(NH_4)_2SO_4$ concentration. The best P_{glnR1}-eYFP sensor had a 1.9-fold decrease in fluorescence and most of them ranged from 1.1 to 1.5 fold (Figure 1B). Next, more than 50 strains from the GlnR2 library bearing the P_{glnR2}-eYFP reporter were selected and sequenced, resulting in 8 non-identical variants. When screened under the same condition, most sensors bearing the P_{glnR2}-eYFP reporter had dynamic ranges greater than those of P_{glnR1}-eYFP, with the

highest displaying 3.0-fold difference. The enhanced fold-of-repression is consistent with previous observations that placing an operator site between the -35 and -10 regions is more effective than placement upstream of the -35 region [19, 29]. From both the glnRA promoter libraries, some sequences (such as the strong -10 box: TATAAT) were never found, probably owing to the interrupted intracellular glutamate pool[32] caused by GlnRA overexpression, making it toxic to *E. coli*.

The promoter P_{glnR3} was separately tested using plasmid pE1c-GlnRA, where glnRA is under the control of an IPTG-inducible promoter, $P_{trc\text{-}1O}$ (Supplementary Table S1). The GlnRA expression level was varied by adding different amounts of IPTG. When *glnRA* was not induced (i.e., leaky expression), cell culture fluorescence presented a 1.7-fold repression under high $(NH_4)_2SO_4$ concentrations (Supplementary Figure S2). As expected, induction of GlnRA with a low concentration of IPTG (0.01 mM) resulted in greater repression (2.3-fold). However, further increasing the GlnRA expression level by induction with higher IPTG concentration (0.1 mM) led to impaired cell growth, probably because GlnRA overexpression is toxic to *E. coli* as discussed above[32]. Additionally, the promoter P_{glnR3} contains two copies of the same GlnR-binding sites, which often leads to recombination between the two GlnR-binding sequences over a long period of cultivation (Supplementary Table S1). Therefore, we chose the GlnR2 biosensor bearing the P_{glnR2} reporter for the following studies.

To measure the dynamic range of the GlnR2 biosensor, we titrated varying amounts of $(NH_4)_2SO_4$ (from 0.08 to 45 mM) in minimal M9 medium. Reporter expression rates were measured (See Methods) and used to plot response curves at varying ammonium concentrations (Figure 2, Supplementary Figure S3). As expected, the control strain without GlnRA, $GlnR_{CeYFP}$, did not respond to ammonium, whereas all GlnR2 biosensor strains exhibited a decreased signal

with increasing ammonium sulfate concentrations. A sharp response at 5-10 mM ammonium sulfate was observed in all four sensor strains (Figure 2), which was probably caused by the feedback-inhibition of GlnA and/or a sharp change in the intracellular glutamine pool [32, 33]. Previous *in vitro* experiments showed that GlnA switches from an active to a fully-inhibited state within a narrow range of glutamine concentration in the presence of Mg^{2+} and AMP [33]. Additionally, in enteric bacteria, the internal glutamine pool sharply drops by more than 10-fold when cells are switched to an ammonium-limited condition [32]. Among these sensors, GlnR2-1 exhibited the greatest dynamic range, with 9-fold (p value = 0.032) fluorescence repression upon induction with a high ammonium sulfate concentration. Although the GlnR and GlnA expression level can be also tuned by using RBSs with different strengths, here we used their native RBSs in *Lactococcus lactis* to keep their relative expression ratio while tuning their absolute expression levels using a promoter library. Further engineering the substrate binding affinities of GlnR and/or GlnA may allow the fine tuning of the biosensor's detection range and sensitivity.

Unlike most natural nitrogen-sensing systems, which are heavily regulated and difficult to engineer [10, 12, 15, 24], the synthetic ammonium sensor contains just three components: GlnA, GlnR, and a GlnR-binding promoter, making it easy to use. We created a single broad-host-range plasmid bearing all three components, which can be easily transformed to multiple host cells, as demonstrated later in three different species. Moreover, GlnA in our sensor is under the control of a synthetic promoter, preventing any possible interference with natural nitrogen regulatory systems [10, 12, 16, 21, 34].

Responses of the ammonium GlnR2-1 biosensor to various nitrogen sources

Next, we tested whether the GlnR2-1 sensor can respond to other nitrogen-containing chemicals, including two major environmental nitrogen sources (nitrate and urea) and two amino acids

(glutamate and glutamine). Cells with the GlnR2-1 sensor were cultivated in minimal medium (with yeast extract for cell growth, see Methods) containing varying amounts of each nitrogen compound. Because GlnR's DNA binding activity is directly regulated by glutamine, the GlnR2-1 sensor was able to respond to glutamine with a 3.4-fold (p-value = 0.0074) fluorescence repression from low to high glutamine concentrations, consistent with the designed mechanism (Figure 3). Meanwhile, the sensor also showed a 1.4-fold (p-value = 0.020) repression upon addition of glutamate, presumably caused by increased ammonium generated from the glutamate by glutamate dehydrogenase [17]. On the other hand, GlnR2-1 did not respond to nitrate or urea, indicating that these nitrogen sources cannot be converted to ammonium or glutamine under the tested conditions. Indeed, nitrate metabolism in *E. coli* cannot be activated in aerobic conditions [35], and the host cell DH10B lacks ureases [36] to release the ammonium. Therefore, the developed sensor can be used to specifically detect ammonium rather than other environmental nitrogen sources such as nitrate and urea.

Testing the ammonium biosensor in *Pseudomonas* and *Synechocystis*

To confirm the developed ammonium sensor can work in multiple bacterial species, the sensor constructs were introduced into *P. putida* NRRL B14683 and *Synechocystis* sp. PCC 6803. *P. putida* transformants containing the P_{glnR2}-*rfp* plasmid exhibited a light pink color on a rich LB medium plate, while its control strain without the *glnRA* gene showed a deep red color due to the lack of repression by GlnR (Supplementary Figure S3A). When cultivated in liquid culture, the *P. putida* biosensor strain exhibited a decreased fluorescent signal when $(NH_4)_2SO_4$ concentration increased, with a sharp transition at ~2 mM and a dynamic range greater than 10-fold (Figure 4, Supplementary Figure S3B). Unlike in *E. coli*, the ammonium sensor in *P. putida* responded to urea (Figure 4) at 1-10 mM (see Methods). This response is consistent with the presence of an *P.*

putida urease gene [37], whose enzyme product converts urea to ammonium. We further confirmed that the urease is active because *P. putida* can grow on urea as the sole nitrogen sources (Supplementary Figure S5).

The GlnR2-1 sensor bearing the P_{glnR2}-eYFP reporter was further transformed into *Synechocystis* sp. PCC 6803. The auto-fluorescence of *Synechocystis* sp. overlaps with RFP, thus only eYFP was used for *Synechocystis* sp. PCC 6803. Normalized cell culture fluorescence from the sensor strain increased when ammonium concentrations decreased, while fluorescence of the control *Synechocystis* strain (without the GlnRA) did not (Figure 5). We noticed that the fold of fluorescence repression in the presence of ammonium was low (1.6-fold) compared to that in *E. coli* and *P. putida*. This low repression was partially caused by the long lifetime of eYFP in *Synechocystis*, which made it difficult to dilute the eYFP molecules synthesized before ammonium was added.

Altogether, our designed ammonium biosensor functioned as expected in three tested bacterial species. Although all host cells have native nitrogen regulatory networks (the NtcA system in *Synechocystis* [12], and the NtrBC system in *P. putida* [37] and in *E. coli* [17]), the GlnRA-mediated biosensor can perform in parallel, without significantly interfering with the host's native regulatory systems. On the other hand, the detection specificity of cells bearing the biosensor can be changed by selecting host cells that have different nitrogen assimilation metabolism, demonstrating the biosensor is both practicable and versatile.

Using the ammonium biosensor to control cyanophycin biosynthesis

One useful application of biosensors is to dynamically regulate gene expression level and timing according to environmental conditions. Dynamic regulation of gene expression balances cellular energy use and has the potential to enhance overall bioproduction [19, 29]. On the basis of such a concept, we aimed to design a dynamic control system (an ammonium sensor-actuator) to regulate cyanophycin biosynthesis, based on the developed ammonium sensor. Cyanophycin is an amino acid polymer enzymatically synthesized from aspartic acid and arginine. In cyanobacteria, cyanophycin serves as a natural nitrogen/carbon storage compound and a buffer for fixed forms of nitrogen. Cyanophycin can be used as a source of polyaspartic acid or organic nitrogen fertilizer, and can be modified as a biodegradable polymer to replace non-biodegradable polyacrylic acid [27, 38]. Under low ammonium conditions, it is not reasonable to turn on cyanophycin biosynthesis due to the lack of building blocks and the metabolic burdens to the host cell caused by the activated biosynthesis [29, 39]. Therefore, it is necessary to use an ammonium-responsive sensor to regulate cyanophycion biosynthesis according to environmental nitrogen states.

Our ammonium sensor turns off gene expression in the presence of ammonium. To turn on the expression of cyanophycin synthase in the presence of ammonium, a signal converter is needed (Figure 6A). To do so, the *eYFP* reporter gene in the biosensor plasmid pGlnR2-1was replaced by a repressor gene *tetR*, which represses gene expression from a P_{tet} promoter. Next, a cyanophycin synthetase gene (encoded by *cphA*) [27, 38] from *Synechocystis* sp. PCC 6803 was placed under the P_{tet}. Thus, in the absence of ammonium, the engineered *E. coli* strain (named AB_{cphA}) is expected to overexpress TetR, which represses the expression of cyanophycin synthase. In a high concentration of ammonium (16 mM), TetR expression is expected to be repressed and CphA expression will be turned on to produce cyanophycin. To test the biosensor-

actuator, the strain AB_{cphA} and its control strain AB_c (in which the *tetR* gene was replaced by the *eYFP* gene) were cultivated in media with or without ammonium. The produced cyanophycin was then purified and quantified by SDS-PAGE analysis (Supplementary Figure S6). The sensor-actuator strain AB_{cphA} showed significantly enhanced cyanophycin production in the presence of ammonium sulfate, with 66% more cyanophycin produced than that in the absence of ammonium (Figure 6B). The control strain lacking TetR only slightly increased cyanophycin production (20%) from media with or without ammonium sulfate. Overall, our results demonstrated that the designed sensor-actuator could synthesize varying amounts of cyanophycin, depending on the availability of extracellular ammonium, thus avoiding unnecessary expression of cyanophycin synthase in the absence of ammonium.

Nitrogen response is one of the most important bioprocesses for all living organisms, so the developed ammonium biosensor should have broad applications. A long-term goal in agricultural biotechnology is to transfer the ability to fix nitrogen from diazotrophic bacteria (such as rhizobia) to non-diazotrophic species, particularly to plants [26]. Although successful transfer has been achieved to some non-diazotrophic bacterial species [26, 40, 41], practical applications require further improvement in nitrogen-fixing activities. Our developed ammonium sensor could be readily used as a nitrogen fixation indicator, for easy detection of fixed ammonium and for high throughput screening of engineered nitrogen-fixing strains. Furthermore, our genetically-encoded sensor could be used to dynamically tune the nitrogen fixation (i.e., the nitrogen fixation is turned on by the biosensor in a low nitrogen condition, and vice versa), enabling control of the fixed nitrogen level. In addition, our sensor-actuator is potentially valuable for environmental bioremediation applications. Excess agricultural fertilizer use has caused elevated ammonium concentrations in water bodies and subsequently resulted in serious environmental problems and

human health problems. The biosensor-actuator can switch on or off the bioproduction of nitrogen-rich materials (e.g., cyanophycin) based on environmental nitrogen conditions, removing excess nitrogen from water more efficiently.

Methods

Materials, Reagents, and Medium

Enzymes and cloning kits were purchased from Thermo Fisher Scientific and New England BioLabs Inc.. Chemicals were purchased from Sigma Aldrich. *E. coli* and *P. putida* cells were grown in a lysogeny-broth (LB) rich medium or a M9 minimal medium with or without yeast extract (0.04% for *E. coli* or 0.08% for *P. putida* to improve cell growth). The minimal M9 medium contained glucose 4 g/L, Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, $MgSO_4$ 0.12 g/L, and $CaCl_2$ 0.01 g/L. In titration experiments, one of the nitrogen sources (ammonium, glutamine, glutamate, urea, or nitrate) was supplemented into the growth medium in amounts varying from 0.01 up to 45 mM. Leucine (40 mg/L) was supplemented to the minimal medium for the leucine auxotrophic strain *E. coli* DH10B. Cyanobacteria *Synechosystis* sp. PCC 6803 was grown in BG11 medium (pH 8.2), which contains (in one liter) TES 2.29 g, $NaNO_3$ 1.5 g, $CaCl_2{\cdot}2H_2O$ 0.036 g, $MgSO_4{\cdot}7H_2O$ 0.075 g, citric acid 0.006 g, K_2HPO_4 0.03 g, ferric ammonium citrate 0.006 g, EDTA (disodium salt) 0.84g, Na_2CO_3 0.02 g, and trace amount of nutrients (H_3BO_3 2.86 mg, $MnCl_2{\cdot}4H_2O$ 1.81 mg, $ZnSO_4{\cdot}7H_2O$ 0.222 mg, $NaMoO_4{\cdot}2H_2O$ 0.39 mg, $CuSO_4{\cdot}5H_2O$ 0.079 mg, and $Co(NO_3)_2{\cdot}6H_2O$ 0.0494 mg). BG11(0) is a nitrogen-free medium with a similar composition to BG11 medium, except that the sodium nitrate in BG11 is removed and the ferric ammonium citrate is replaced by ferric citrate.

Plasmid construction

The *glnRA* operon was cloned from *Lactococcus lactis* ATCC 19435 using a pair of primers, ggLlGlnRs and ggLlGlnRAa, (see Supplementary Table S4). All plasmids were constructed by a one-step Golden-Gate DNA assembly method [42]. To generate the ammonium biosensor plasmid (pGlnR1), the plasmids pPMQAK1[30], and pDeg and pTrc1O-eYFP [31] were used as backbones, and the GlnR DNA binding site was introduced into the promoter P_{glnR1} via the primer ggpglnReYFPs1. The promoter P_{lib} was constructed by inserting two degenerate nucleotides into the -10 region of the promoter P_{cons}, using the primers ggPmtrcs/ggPmtrca. Details for pGlnR1construction are presented in Supplementary Figure S1 and Supplementary Tables S4 and S5. Using the pGlnR1 as a template, its derivatives were also constructed by the Golden-Gate assembly method using different primers. To create cyanophycin producing constructs, a *cphA* gene was amplified from *Synechosystis* sp. PCC 6803 and cloned under a P_{tet} promoter in a BglBrick plasmid [28]. All genes were sequenced, and are listed in Supplementary Table S1. The strains used in this study are listed in Table 1. All primers, and plasmids are listed in Supplementary Tables S4 and S5, respectively.

Cell culture and fluorescence assay

E. coli (DH10B or BL21) and *P. putida* NRRL B14683 were transformed by electroporation [43]. *Synechocystis* sp. PCC 6803 strain was naturally transformed. In detail, *Synechocystis* sp. PCC 6803 cells were grown in BG11 medium at a light intensity of 40 µmol of photons m^{-2} s^{-1} for 2-3 days (OD_{730} ~0.5) and condensed by 100-fold. Then, 100 µl cells were mixed with 1 µg DNA for 5 hrs and plated on selective agars. After two weeks, positive colonies were selected and confirmed by colony PCR using KlenTaq LA (DNA Polymerase Technology). *E. coli* cultures were cultivated at 37°C, while *Synechosystis* and *P. putida* were cultivated at 30°C. Antibiotics

were added to proper amounts (kanamycin 25 mg/L for *E. coli* and *P. putida*, 10 mg/L for *Synechosystis*, and chloramphenicol 30 mg/L for *E. coli*).

The ammonium biosensor libraries (including GlnR1, 2, 3) were screened in 96-deep-well plates in M9 yeast extract medium. Specifically, an overnight LB cell culture (4 µl) was inoculated and grown in 1000 µl of M9 yeast extract medium with either 0 or 1.5 mM ammonium sulfate for low N conditions or 15 mM ammonium sulfate for high N condition. After 36 hrs, cell density (OD_{600}) and cell culture fluorescence were both recorded using an Infinite F200PRO plate reader (TECAN) using method as previously described[19, 20]. To measure the reporter expression rate, cells (*E. coli* or *P. putida*) were first grown in M9 medium with excess ammonium sulfate (15 mM) for over 15 hrs and washed in nitrogen-free medium three times to remove residual ammonium. Then, 150 µl of cells (initial OD 0.4-0.8) in M9 medium with various amounts of ammonium (or other nitrogen sources) were transferred into a 96-well plate and incubated in an Infinite F200PRO plate reader at 218 rpm. Cell culture fluorescence and cell density were recorded every 1000 seconds. An excitation wavelength of 535 nm and an emission wavelength of 620 nm were used for RFP fluorescence, while an excitation wavelength of 485 nm and an emission wavelength of 532 nm were used for eYFP. The reporter expression rate (n=3) was measured as the change of cell-density-normalized fluorescence per unit time, [fluorescence/OD]/time, in a stable phase (always 6-10 hrs post inoculation), during which the expression rate was constant (Supplementary Figure S3). Data were fitted to a Hill equation. Hill coefficients in Figures 2 and 3 are presented in Supplementary Table S3.

Synechocystis strains were cultivated only in test-tubes, owing to its slow growth and light requirement. The cells were first cultivated in BG11 liquid medium at a light intensity of 50 µmol of photons m^{-2} s^{-1} for five days, then harvested and washed using BG11(0). The cells were

then incubated into BG11(0) medium with an initial OD_{730} = 3.0 with glucose (1 g/L) and various amounts of ammonium sulfate (0, 0.3, or 3 mM) under weak light (20 μmol of photons m^{-2} s^{-1}). After two days, cell culture fluorescence was measured (n=3) as described above.

Cyanophycin production and measurement

Strain AB_{cphA} (containing the ammonium sensor–actuator) and its control strain AB_c were used for cyanophycin production. Overnight LB cultures were used to inoculate M9 yeast extract medium with or without ammonium sulfate (8 mM) and cultivated for 22 hrs. Then, each culture was harvested and transferred to the same fresh medium (with initial OD_{600} 0.2), supplemented with 100 mg/L arginine and 100 mg/L aspirate and incubated for 2 hrs. The cells were collected by centrifuge and kept at -20°C until use. For each sample, the biomass was normalized by OD_{600}.

A modified method [27, 44] was used to purify cyanophycin. Briefly, cell pellets were suspended in 0.3 ml 50 mM Tris-HCl (pH 7.5), lysed by sonication, then centrifuged at 15,000 g for 10 mins. The pellets were then washed with 50 mM Tris-HCl buffer (pH 7.5) to remove soluble proteins. The cyanophycin-enriched pellets were re-suspended in 0.5 ml 0.1 M HCl by shaking for 15 mins, and the centrifuged for 10 mins at 15,000 g. Next, 0.5 ml supernatant was transferred to a new tube and mixed with 0.5 ml 0.1M Tris-HCl (pH 12) to precipitate cyanophycin. To completely harvest the cyanophycin, the mixture was kept on ice for 10 min and centrifuged at 15,000 g and 4 °C for 10 min. The final cyanophycin was re-suspended in 150 μl of protein SDS-PAGE loading buffer (62.5 mM Tris-HCl pH 6.8, 2.5 % sodium dodecyl sulfate, 0.002 % bromophenol blue, 5% β-mercaptoethanol, 10 % glycerol). SDS-PAGE was performed using 12% gel in standard protocols, and the gel was stained by Coomassie Brilliant Blue R-250. Gel image analysis and quantitative analysis were carried out using imageJ (National Institutes of Health).

ASSOCIATED CONTENT

Supporting Information

Figures S1-S6 and Tables S1-S5

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Notes

The authors declare no competing financial interest.

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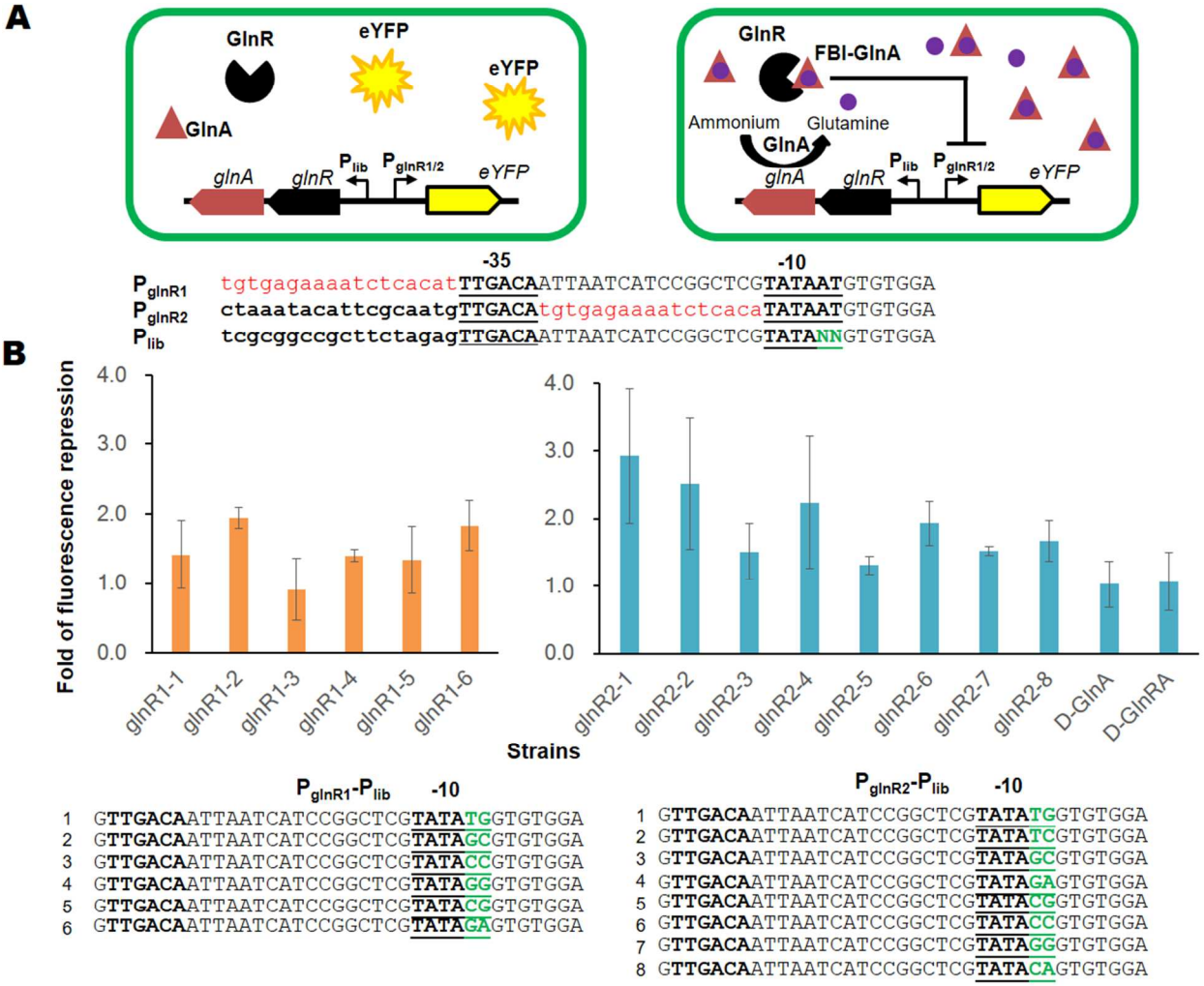

Figure 1. Construction of the ammonium biosensor. A. Design principle of the ammonium biosensor. A transcription factor, GlnR, and a glutamate-ammonium ligase, GlnA, are encoded by the *glnRA* genes. In the absence of ammonium, the reporter (an eYFP or any other fluorescent protein), can be normally expressed from a GlnR-regulated synthetic promoter, P_{glnR}. When ammonium is present, GlnA converts ammonium and glutamate to glutamine. The produced glutamine binds to GlnA and stabilizes the GlnR-DNA complex. The GlnR-DNA complex represses the reporter expression, leading to deceased fluorescence signals. The sequences of two synthetic GlnR-regulated promoters, P_{glnR1} and P_{glnR2}, and the promoter library, P_{lib}, are listed. The GlnR-binding sites are colored in red. The -35 and -10 regions are bold and underlined. Two degenerated nucleotides in P_{lib} are colored in green. **B**. Screening of ammonium sensors. Each set of data is from strains using the same GlnR-regulated promoter (left-$P_{glnR1;}$ right-P_{glnR2}). Within each data set, each strain contains a different promoter, $P_{lib\#}$, to control the expression of glnA-glnR. Cell culture fluorescence was measured in the presence of high (15 mM) and low (0 mM) concentrations of ammonium sulfate and was normalized by cell density (n=3). The fold of fluorescence repression (cell-density-normalized fluorescence in low vs high nitrogen conditions) are presented. Sequences of the glnA-glnR promoters ($P_{lib\#}$) are shown at the bottom. The strains $GlnR_{CeYFP}$ and $GlnR_{CeYFP}$-$_A$ bearing biosensor plasmids without *glnRA* (D-GlnRA) and *glnA* (D-GlnA) were used as controls.

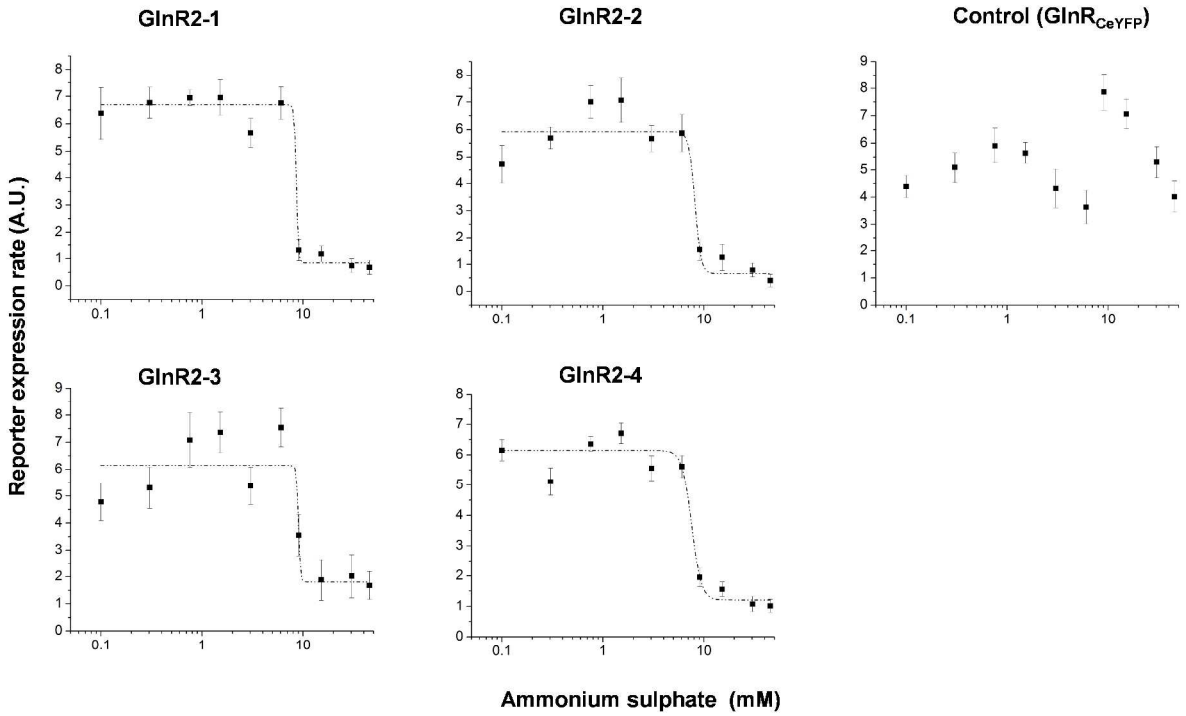

Figure 2. Responses of ammonium biosensors to various amounts of ammonium sulfate. Four *E. coli* strains bearing different ammonium biosensor constructs (GlnR2-1, GlnR2-2, GlnR2-3, and GlnR2-4) and the control strain GlnR$_{CeYFP}$, which lacks the *glnRA* genes, were selected and cultured in minimal M9 medium containing varying amounts of ammonium sulfate. Reporter expression rate was measured (n=3). P-values are presented in Supplementary Table S2.

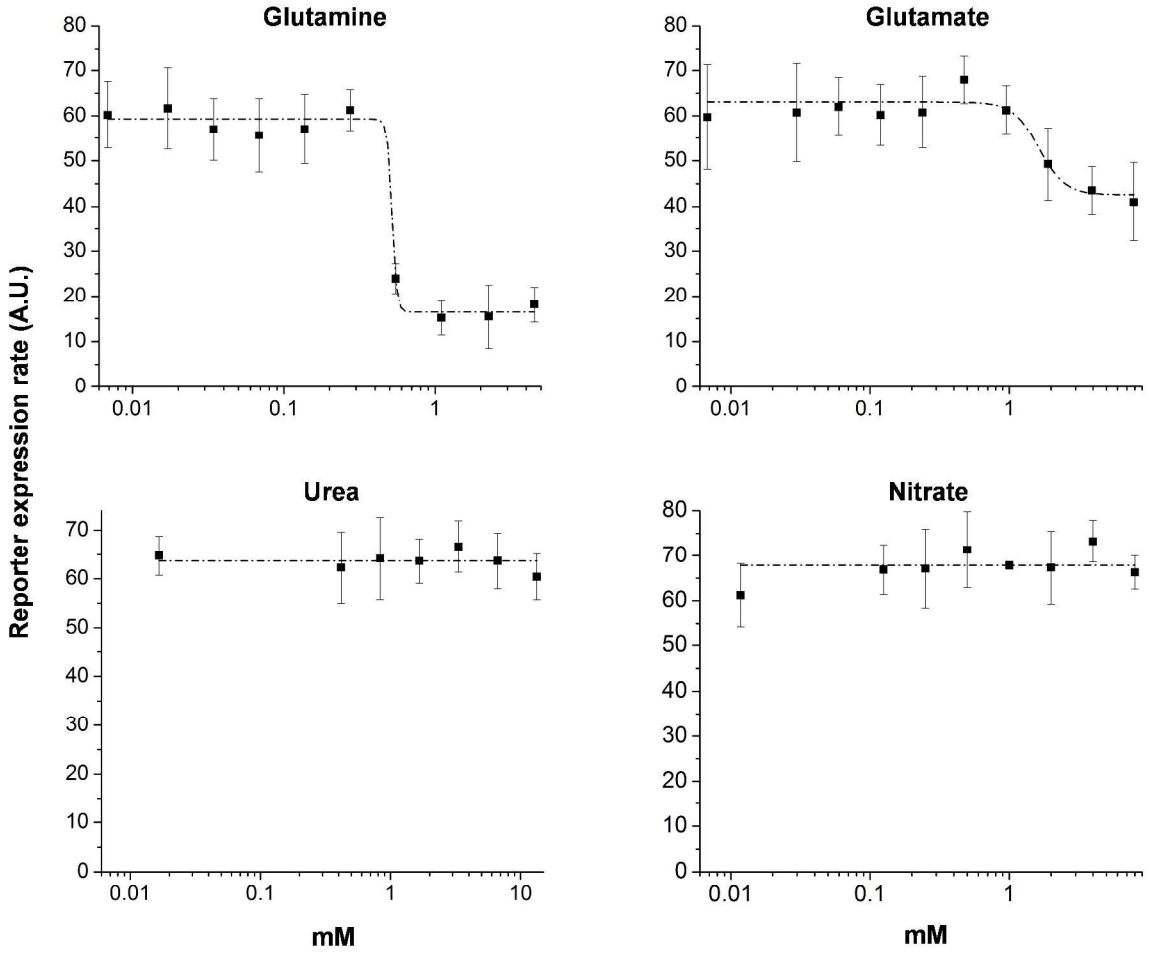

Figure 3. Responses of the ammonium-biosensor in *E. coli* to various nitrogen-containing chemicals. Ammonium sensor strain GlnR2-1(rfp) was titrated with varying amounts of glutamate, glutamine, nitrate, or urea. Cells were cultured in M9 medium supplemented with a small amount of yeast extract to facilitate cell growth. Reporter expression rate was measured (n=3). P-values are presented in Supplementary Table S2.

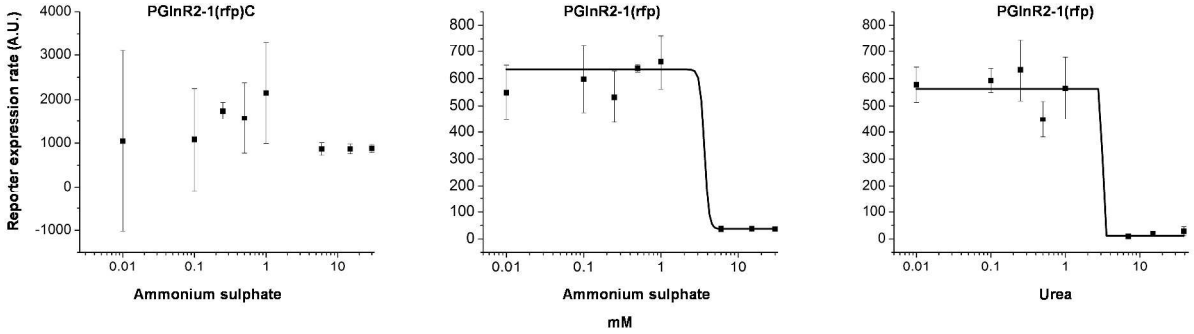

Figure 4. Response of the ammonium-biosensor in *P. putida* to ammonium sulfate and urea. *P. putida* cells with the ammonium sensor construct, PGlnR2-1(rfp), and its control PGlnR2-1(rfp)C, which lacks the *glnA-glnR* genes were cultured in M9 yeast extract medium and titrated with varying amounts of ammonium sulfate or urea. Reporter expression rate was recorded (n=3). P-values are presented in Supplementary Table S2.

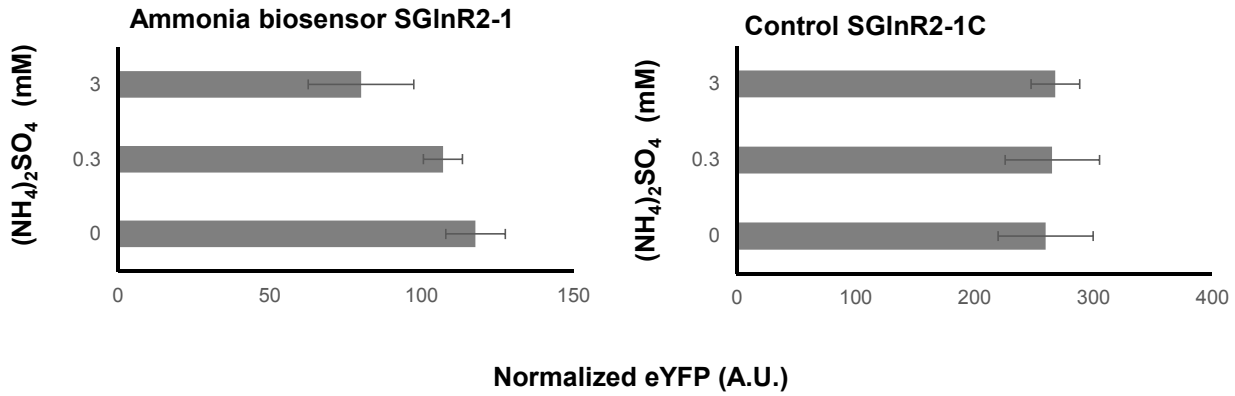

Figure 5. Responses of the ammonium biosensor to ammonium sulphate in *Synechocystis* sp. PCC 6803. *Synechocystis* sp. PCC 6803 cells with the ammonium sensor construct (SGlnR2-1) or the control strain SGlnR2-1C without GlnRA were first grown in BG11 medium for five days. Cells were then washed to remove residual nitrogen. The washed cells were incubated into a BG11(0) medium with 0, 0.3, or 3 mM ammonium sulfate for 44 hrs. Cell-density-normalized fluorescence was measured (n=3).

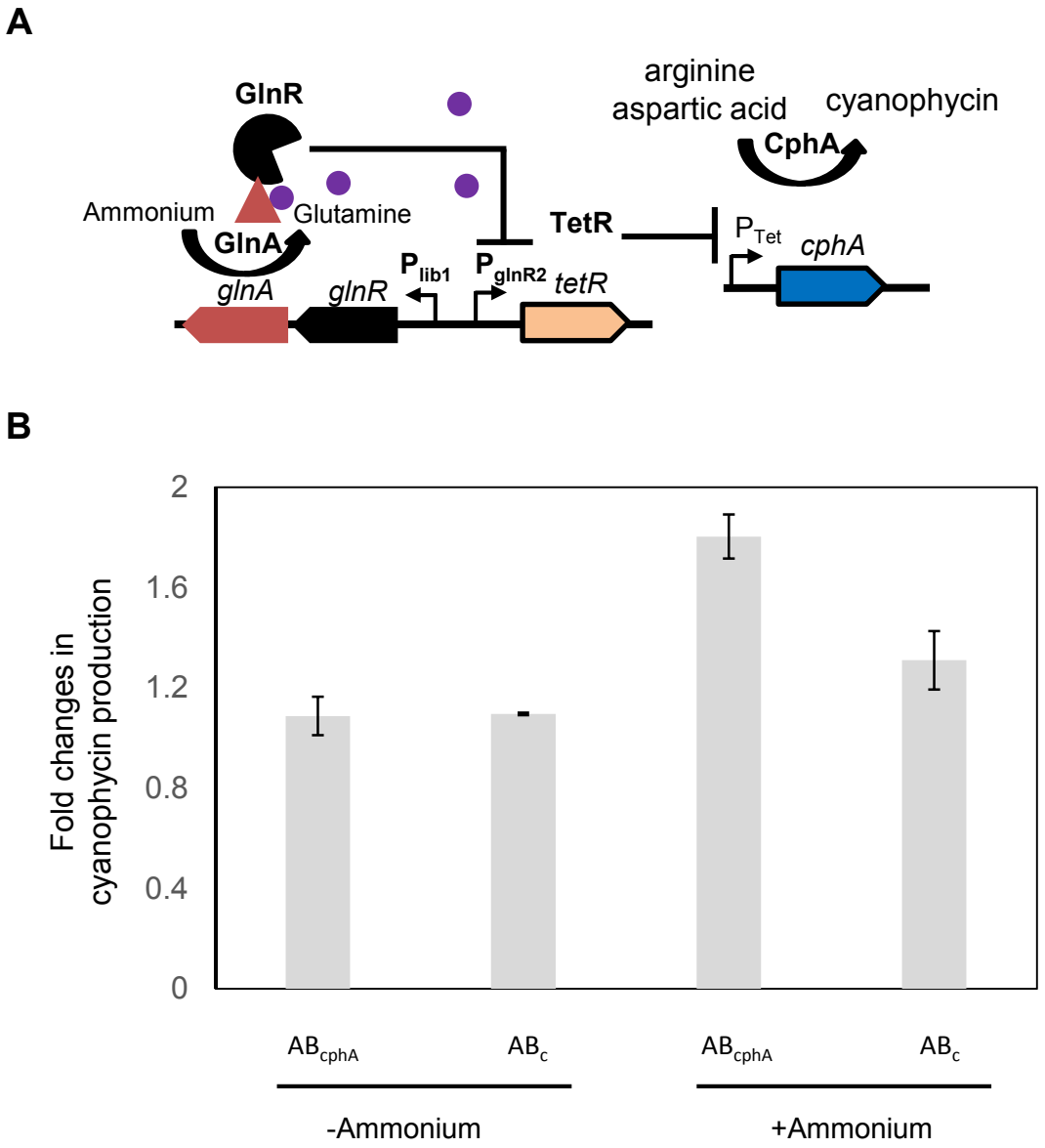

Figure 6. Construction of an ammonium-responsive sensor-actuator for cyanophycin production. A, Design of sensor-actuator to regulate cyanophycin biosynthesis. A *tetR* gene is placed under the control of the ammonium biosensor (via P_{glnR2}). A TetR-repressed promoter, P_{Tet}, was used to control the expression of a cyanophycinase (encoded by *cphA*), whose enzyme product synthesizes cyanophycin from arginine and aspartic acid. In the absence of ammonium, TetR expression is turned on by the ammonium sensor, which in turn blocks the expression of

CphA. The presence of ammonium (16 mM) turns off TetR expression, leading to the expression of CphA due to the lack of repression. **B**, Cyanophycin produced by the sensor-actuator with or without ammonium. Produced cyanophycin was purified, quantified by SDS-PAGE, and normalized by cell density. Fold changes of the cyanophycin production with or without ammonium (16 mM) were calculated (n=3). The producing strain was AB_{cphA} with the sensor-actuator. The control strain was strain AB_c, where *tetR* is replaced by *eYFP*. The production by control strain AB_c in non-ammonium conditions, is set as 1.

Table 1. Strains used in this study.

Strains	Descriptions	Genotypes
GlnR1-1,2,3,4,5,6	The promoter $P_{lib1*1,2,3,4,5,6}$ regulated GlnRA expression, while the promoter P_{glnR1} regulated eYFP expression. Each P_{lib} presents a different promoter sequence.	*E. coli* DH10B[a]/pGlnR1-1,2,3,4,5,6[b]
GlnR2-1,2,3,4,5,6,7,8	The promoter $P_{lib2*1,2,3,4,5,6,7,8}$ regulated GlnRA expression, while the promoter P_{glnR2} regulated eYFP expression. Each P_{lib} presents a different promoter sequence.	*E. coli* DH10B/pGlnR2-1,2,3,4,5,6,7,8
GlnR2-1(rfp)	The promoter P_{lib2*1} regulated GlnRA expression, while the promoter P_{glnR2} regulated RFP expression.	*E. coli* DH10B/pGlnR2-1(rfp)
$GlnR_{CeYFP-A}$	A control stain of GlnR2-1, where the operon *glnA* was omitted.	*E. coli* DH10B/ $pGlnR_{CeYFP-A}$
$GlnR_{CeYFP}$	A control stain of GlnR2-1, where the operon *glnRA* was omitted.	*E. coli* DH10B/ $pGlnR_{CeYFP}$
$GlnR_{Crfp}$	A control stain of GlnR2-1(rfp), where the operon *glnRA* was omitted.	*E. coli* DH10B/ $pGlnR_{Crfp}$
GlnR3	The IPTG-inducible promoter P_{trc-1O}controlled GlnRA expression, while the	*E. coli* BL21[c]/ pE1c-GlnRA+ pBGlnR3k-rfp

	promoter PglnR3 regulated RFP expression.	
AB_{cphA}	A cyanophycin producing strain with the ammonium sensor-actuator.	*E. coli* BL21/ pGlnR2-1-tetR+pA2c-cphA
AB_c	A cyanophycin producing strain without ammonium sensor-actuator.	*E. coli* BL21/ pGlnR2-1+pA2c-cphA
PGlnR2-1(rfp)	A *P. putida* strain with the ammonium biosensor using RFP as an output.	*P. putida* NRRL B14683 /pGlnR2-1(rfp)
PGlnR2-1(rfp)C	A *P. putida* PGlnR2-1(rfp) control strain without the ammonium biosensor.	*P. putida* NRRL B14683 /$pGlnR_{Crfp}$
SGlnR2-1	A *Synechocystis* strain with the ammonium biosensor using eYFP as an output.	*Synechocystis* sp. PCC 6803 /pGlnR2-1
SGlnR2-1C	A *Synechocystis* SGlnR2-1 control strain without the ammonium biosensor.	*Synechocystis* sp. PCC 6803 / $pGlnR_{eYFP}$

[a], *E. coli* strains DH10B (F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-)

[b], Plasmids used in this study are listed in Supplementary Table S5.

[c],BL21 (*E. coli* str. B F^- ompT gal dcm lon hsdSB($r_B^-m_B^-$) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) [$malB^+$]$_{K-12}$(λ^S))

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Developing a genetically-encoded, cross-species biosensor for detecting ammonium and regulating biosynthesis of cyanophycin

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