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Establishing a High-Yielding Cell-Free Protein Synthesis Platform Derived from *Vibrio natriegens*

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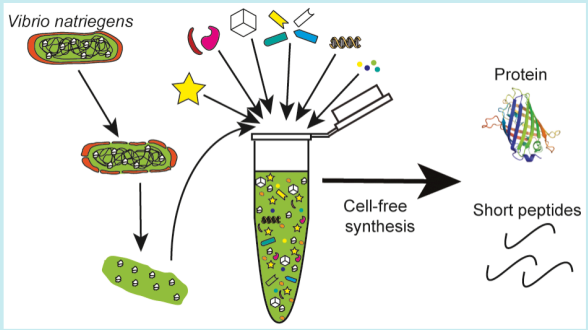
Supporting Information

ABSTRACT: A new wave of interest in cell-free protein synthesis (CFPS) systems has shown their utility for producing proteins at high titers, establishing genetic regulatory element libraries (*e.g.*, promoters, ribosome binding sites) in nonmodel organisms, optimizing biosynthetic pathways before implementation in cells, and sensing biomarkers for diagnostic applications. Unfortunately, most previous efforts have focused on a select few model systems, such as *Escherichia coli*. Broadening the spectrum of organisms used for CFPS promises to better mimic host cell processes in prototyping applications and open up new areas of research. Here, we describe the development and characterization of a facile CFPS platform based on lysates derived from the fast-growing bacterium *Vibrio natriegens*, which is an emerging host organism for biotechnology. We demonstrate robust preparation of highly active extracts using sonication, without specialized and costly equipment. After optimizing the extract preparation procedure and cell-free reaction conditions, we show synthesis of 1.6 ± 0.05 g/L of superfolder green fluorescent protein in batch mode CFPS, making it competitive with existing *E. coli* CFPS platforms. To showcase the flexibility of the system, we demonstrate that it can be lyophilized and retain biosynthesis capability, that it is capable of producing antimicrobial peptides, and that our extract preparation procedure can be coupled with the recently described Vmax Express strain in a one-pot system. Finally, to further increase system productivity, we explore a knockout library in which putative negative effectors of CFPS are genetically removed from the source strain. Our *V. natriegens*-derived CFPS platform is versatile and simple to prepare and use. We expect it will facilitate expansion of CFPS systems into new laboratories and fields for compelling applications in synthetic biology.



KEYWORDS: *cell-free protein synthesis, Vibrio natriegens, genome engineering, protein production, TX-TL, synthetic biology*

Cell-free systems have recently enjoyed a technical renaissance that has transformed them into robust platforms for the synthesis of a wide variety of useful and interesting products.[1−4] Such platforms combine crude cell lysates or purified components with substrates in a test tube, enabling the activation and use of cellular processes *in vitro* (Figure 1). Cell-free protein synthesis (CFPS) systems in particular have made significant advances in increasing reaction volume, duration, and productivity, now reaching g/L quantities of protein.[3,5−12] These systems provide several unique advantages for understanding, harnessing, and expanding the capabilities of natural biological systems. Reactions are open, and are therefore easily accessible for sample extraction and substrate feeding. Dilute reaction environments facilitate the folding of complex eukaryotic protein products which may otherwise express poorly in bacterial systems.[4] Importantly, the removal of genomic material from the chassis organism directs reaction substrates and machinery toward the desired synthesis reaction at high rates. Exploiting these features, CFPS platforms enjoy increasingly widespread use as a complement to *in vivo* expression for applications including biomolecular breadboarding,[13−16] expression of toxic products,[17−20] production of complex protein products that are poorly soluble *in vivo*,[7,21−23] manufacture of glycoproteins,[24−27] detection of disease,[28−30] on demand biomanufacturing,[21,31−35] and education.[36,37]

Despite the emergence of cell-free systems as a prominent research tool for fundamental and applied biology, the vast majority of previous efforts have focused on a select few model

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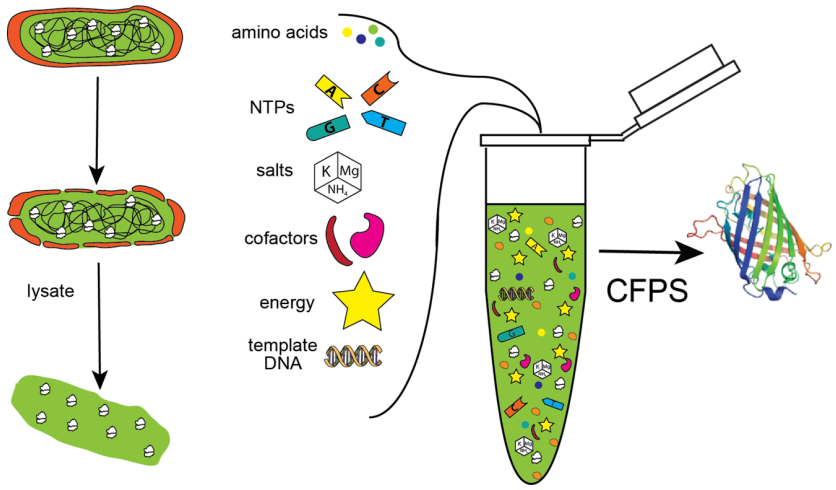

Figure 1. Simplified schematic of the production and utilization of crude lysates from bacterial chassis cells to catalyze cell-free protein synthesis (CFPS). Reactions are supplemented with enzymatic cofactors, energy, and other substrates required for protein synthesis, as well as plasmid DNA template directing the system toward the production of a product of interest.

systems such as *Escherichia coli*, *Saccharomyces cerevisiae*, and Chinese Hamster Ovary cells, among others.[2,5,11,12,38,39] However, we and others hypothesize that developing cell-free systems composed of extracts derived from relevant chassis organisms that better mimic the natural physicochemical environment might enhance predictive power for synthetic biology applications. This idea motivates the development of new cell-free systems. In this context, several new CFPS systems have been developed, including some from *Streptomyces* species and *Bacillus*.[40−44] For example, an elegant study by Freemont and colleagues characterized new DNA parts from the nonmodel bacterium *Bacillus megaterium* by combining automated CFPS and Bayesian models.[44]

A particularly exciting chassis organism for developing a new cell-free system is the fast-growing halophilic marine bacterium *Vibrio natriegens*. First discovered in a Georgia saltmarsh in 1958, *V. natriegens* (originally classified as *Pseudomonas natriegens*) was identified as the fastest-growing bacterium known to date when it was discovered that cell populations in liquid culture double approximately once every 10 min.[45,46] Despite this noteworthy trait, *V. natriegens* went largely unstudied for decades. Recently, interest in this organism has been renewed, largely out of a desire to leverage its rapid generational time to accelerate molecular biology efforts and improve recombinant protein production.[47,48]

V. natriegens' rapid doubling time is particularly interesting for potential CFPS system development, as in the context of CFPS it is generally accepted that lysate productivity loosely correlates to chassis organism growth rate.[49,50] After all, proper cell division relies on the coordinated activities of a large suite of proteins, so it is reasonable to infer that rapidly dividing cells require high protein synthesis rates, and by extension possess highly active protein translation machinery. This is very likely the case for *V. natriegens*—the species features 12 rRNA operons as compared to the 7 found in *E. coli* strain MG1655.[47] Furthermore, it has been suggested that exponentially growing *V. natriegens* cells contain ∼115 000 ribosomes/cell, significantly higher than the ∼70 000 ribosomes/cell observed in *E. coli*.[51] Considering these advantages, we hypothesized that lysates derived from *V. natriegens* would be enriched in active protein translation machinery and thus compose a highly productive CFPS platform.

In this study, we describe the development of a facile CFPS platform derived from *V. natriegens*. Key design criteria were to make the system robust, easy to use, and accessible to all. Therefore, since cell lysis procedures using homogenization or French press can be expensive, time and labor intensive, and hard to standardize, we focused on developing an extract preparation procedure using standard sonication equipment.[52] Previously, we have shown that sonication offers a simple strategy to reduce cost and variability in crude *E. coli* extract preparation, while eliminating the need for specialized and expensive growth and lysis equipment.[52] We first showed the ability to create a protein synthesis competent cell-free system. We then optimized the extract preparation process by modifying growth media, cultivation time, cell disruption conditions, and lysate clarification conditions. This led to a 400% increase from the nonoptimized case, resulting in a cell-free system capable of synthesizing ∼1 mg/mL of superfolder green fluorescent protein (sfGFP) using a typical *E. coli* CFPS reagent mix. Further optimization of key reagent concentrations increased the productivity of the system to ∼1.6 mg/mL. We lyophilized fully assembled *V. natriegens* CFPS reactions and found that, if prepared in the presence of trehalose, reactions retain 100% productivity after 1 week of storage at room temperature. Next, we applied homologous recombination-based genome engineering to prepare a small library of knockout strains in which the genes encoding putative negative effectors of CFPS have been removed. Lysates were prepared from each of these knockout strains in an attempt to identify a strain background with improved productivity *in vitro*. The *V. natriegens* CFPS system described here is productive, robust, and facile to prepare. We expect it will lower the barrier for entry into the use of CFPS systems.

RESULTS AND DISCUSSION

Identifying Extract Preparation Conditions for *V. natriegens* CFPS. We began our study by trying to develop extract preparation procedures. Because CFPS exploits an ensemble of catalytic proteins prepared from the crude lysate of cells, the cell extract (whose composition is sensitive to

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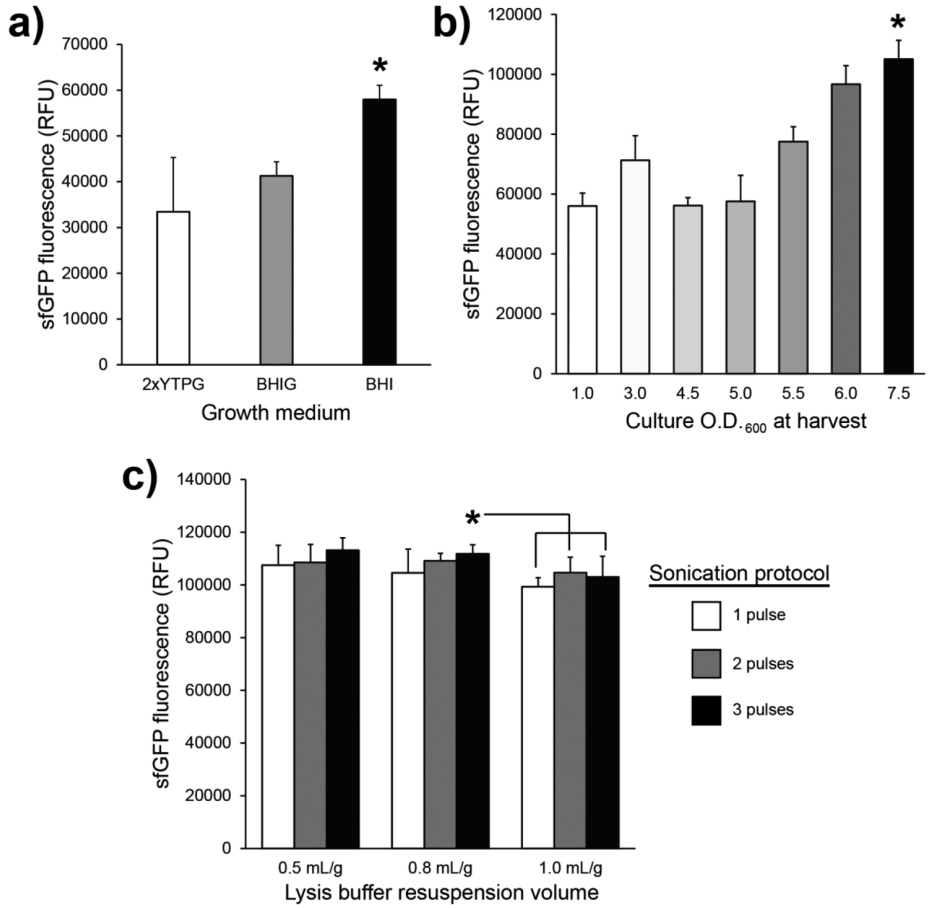

Figure 2. Optimization of *V. natriegens* harvest and lysis procedures. (a) sfGFP fluorescence *in vitro* from cell extracts derived from *V. natriegens* cultured in the specified liquid medium supplemented with v2 salts (204 mM NaCl, 4.2 mM KCl, 23.14 mM $MgCl_2$). Cells were harvested at OD_{600} = 4.5 (b) sfGFP fluorescence from cell extracts derived from *V. natriegens* harvested at the specified OD_{600}. Cell culture was performed using BHI medium plus v2 salts. For both (a) and (b), established *E. coli* protocols were used for lysate preparation. (c) Lysis optimization. *V. natriegens* cell pellets were suspended in the specified volume of S30 lysis buffer and subjected to the indicated number of sonication pulses to achieve lysis. sfGFP fluorescence *in vitro* from the resulting lysates is shown. All CFPS reactions used an existing *E. coli* reagent mix. For all conditions, three independent reactions were performed and one standard deviation is shown. * = statistically significant for $p < 0.05$.

growth media, lysis method, and processing conditions) is the most critical component of extract-based CFPS reactions. In recent years, systematic optimization of each step in extract preparation for *E. coli* CFPS has improved extract robustness and productivity.[1,52] Similar advances have been made in *S. cerevisiae*, *Streptomyces*, and *Pseudomonas* CFPS systems.[38,41,53] Based on these successes, we chose to vary extract preparation conditions in search of parameters that might improve reproducibility between extract preps, increase the level of protein synthesized, and allow for potential downstream scalability. A key focus was to generate a large volumetric yield of lysate even when chassis cells are cultured at volumes that can be accommodated by shake flasks.

Generally, the extract preparation process includes the following major steps: cell cultivation, cell disruption, lysate clarification, and some optional steps like runoff reaction and dialysis. We decided to explore each of these steps. First, we wanted to confirm the previously reported doubling times for *V. natriegens* (*Vnat*). To accomplish this, we prepared liquid cultures of wild type *Vnat* cells in several different liquid growth media. Each growth medium was tested with and without v2 salt supplementation,[47] and culture growth was monitored *via* plate reader over a 20 h incubation (Figure S1A). Under the conditions tested, *Vnat* was found to double approximately every 12−14 min in salt-supplemented growth medium. This range aligns with previously reported data and outpaces standard *E. coli* laboratory strains.[47,48] Unsurprisingly, in most of the media investigated, the growth of *Vnat* is severely inhibited in the absence of salt. This held true at culturing volumes of 1 L incubated in shake flasks, which are typical volumes and conditions used to culture cells for lysate preparation (Figure S1B).

We next set out to establish the growth medium that is optimal for culturing cells for *Vnat* CFPS lysate preparation. 1 L cultures of *Vnat* were grown in 2xYTPG (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 7 g/L K_2HPO_4, 3 g/L KH_2PO_4, 18 g/L glucose), BHI (brain-heart infusion), and a BHI variant supplemented with 1.8% (m/v) glucose (BHIG), in all cases supplemented with v2 salts. Cell pellets were collected at OD_{600} = 4.5 to mimic the best practice of harvesting *E. coli* cultures during midexponential phase, and protocols previously established for *E. coli* were used for lysate preparation and CFPS synthesis of sfGFP. Specifically, we assembled CFPS reactions with the sfGFP template and carried out 15 μL batch reactions for 20 h at 30 °C. Lysates derived from cells cultured in BHI were significantly more

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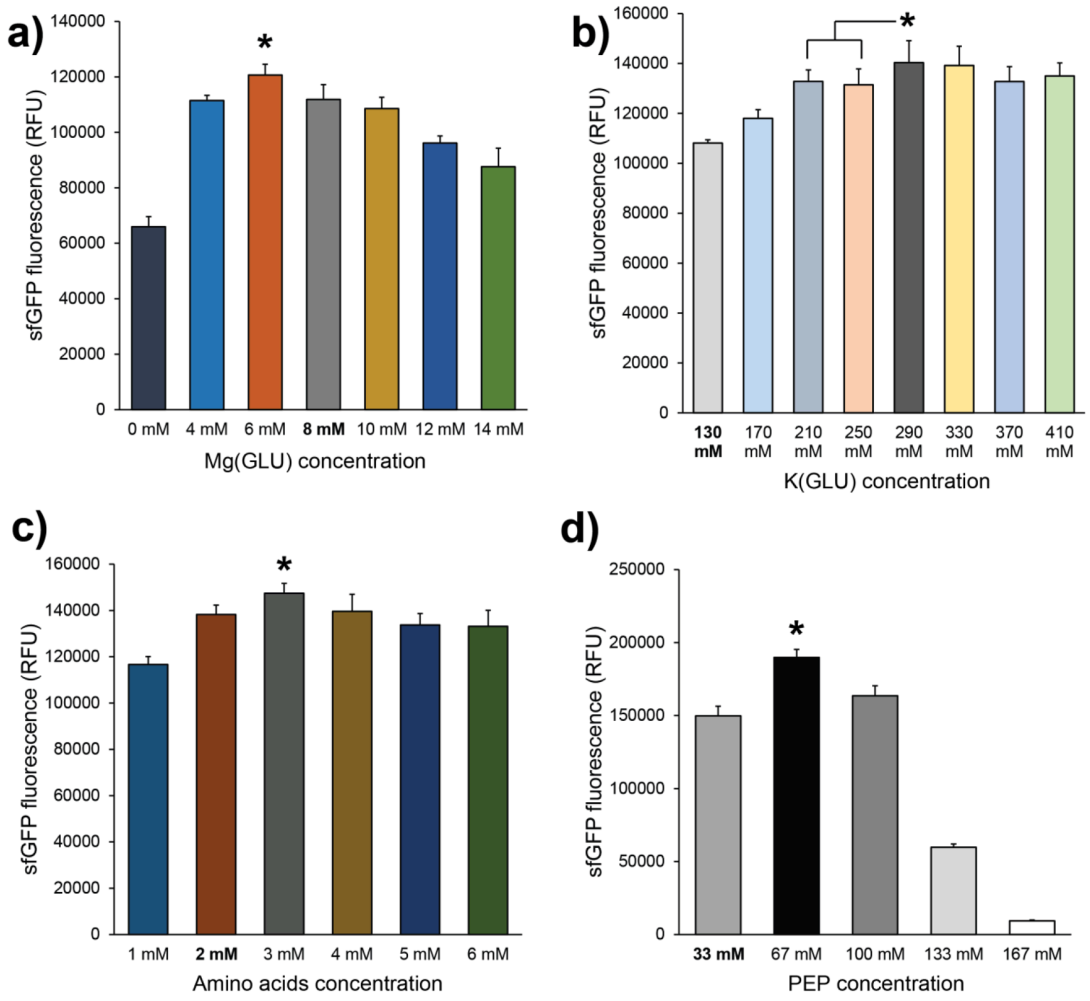

Figure 3. Optimization of CFPS reagent mix. A single *V. natriegens* lysate was prepared using optimized parameters identified in Figure 2. Shown are sfGFP fluorescence values obtained using the lysates *in vitro* when the specified concentration of each of the following reagents is used: (a) Mg(GLU), (b) K(GLU), (c) amino acids, and (d) PEP. As a reference, for each reagent the concentration typically used in *E. coli* CFPS is indicated in bold. For all conditions, three independent reactions were performed and one standard deviation is shown. * = statistically significant for $p < 0.05$.

productive than those from cells grown in either of the other two media types (Figure 2A). On the basis of these cumulative results, we selected BHI for use in *Vnat* cultures going forward.

Next, we investigated the ideal time point at which to collect *Vnat* cell pellets for CFPS lysate production. As previously mentioned, in *E. coli*-based systems, the most productive lysates are derived from cells gathered during midexponential phase growth.[3,52,54] It is generally accepted that this is because the pool of active ribosomes is most enriched during this phase of the growth cycle. We therefore hypothesized that the most productive *Vnat* lysates would similarly be derived from cells harvested in midexponential phase. To test this, we harvested *Vnat* cultures at a range of optical densities ranging from lag phase, through exponential phase, and even into stationary phase. Lysates were prepared from each cell pellet and directed to synthesize sfGFP in CFPS (Figure 2B). We were surprised to observe that *Vnat* lysate productivity increases with increasing OD_{600} past the exponential phase of growth—indeed, the most productive lysate identified was prepared from cells in early stationary phase. Lysates retain ~85% productivity when prepared from cultures at stationary phase for several hours (Figure S2A) and ~75% productivity when prepared from overnight cultures (Figure S2B). These surprising results run counter to what is typically observed with other CFPS chassis organisms. In *E. coli*, for instance, it is believed that stationary phase cells experience a reduction in active ribosomes in response to the reduced demand for protein synthesis; this effect propagates to lysates, resulting in a severe reduction in CFPS productivity.[55] Going forward, *Vnat* cultures were harvested for lysate preparation immediately upon entry into stationary phase (OD_{600} ~ 7.5).

Identification of Optimal Procedures for Preparation of *V. natriegens* Lysates. Having established *Vnat*-specific cell culture and harvest parameters, we proceeded to identify conditions for preparing *Vnat* lysates *via* sonication that maximized CFPS yields. We focused our investigation on two key factors pertaining to lysate preparation. The first of these, cell pellet resuspension volume, describes the volume of lysis buffer used to resuspend a cell pellet prior to lysis. Modulating this volume has a direct influence on the concentration of cellular components in the final lysate, which in turn affects lysate productivity. The second factor considered was energy delivery to the cells during sonication. During lysis, enough energy must be delivered to ensure adequate rupturing of

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cellular compartments but must then be constrained to prevent denaturation of ribosomes, enzymes, and other fragile cellular components required for robust protein synthesis. Both of these parameters were simultaneously varied for lysis optimization, as we have done before when developing a similar protocol for *E. coli*.[52] *Vnat* cell pellets were resuspended in 0.5, 0.8, or 1.0 mL of S30 buffer per gram of wet cell mass. Each suspension was then sonicated, with lysis achieved using one, two, or three 45-s pulses. Each resulting lysate was directed in CFPS to synthesize sfGFP (Figure 2C).

Collectively, the results reveal that *Vnat* cells are relatively agnostic to both resuspension volume and lysis energy. Productivities of all nine lysates tested were within 10% of one another. The more concentrated suspensions demonstrate a modest (but statistically significant) increase in productivity relative to the samples prepared from cells resuspended in 1.0 mL buffer/g cells, likely a consequence of slightly more concentrated translation components. There is no appreciable difference between samples suspended in 0.5 mL buffer/g cells *vs* 0.8 mL buffer/g cells. Due to the relative difficulty of resuspending cells in 0.5 mL buffer/g as well as the accompanying reduction in the volume of lysate yielded under that condition, we selected 0.8 mL buffer/g cells as our resuspension density going forward. Due to the insignificant differences in productivity when two or three sonication pulses are used for lysis, we settled on the use of three pulses to remain consistent with several recently reported *E. coli* lysis protocols.[3,54]

After we defined a reproducible cell lysis strategy to generate highly active extracts, we decided to investigate a postlysis extract preparation step. Specifically, we evaluated the effect of the runoff reaction. Lysates derived from some strains of *E. coli* benefit tremendously from a runoff reaction, whereby clarified lysate is shaken in an incubator followed by a second clarifying spin to yield the final extract.[3,52,54] It is believed that this incubation allows ribosomes to complete translation of native mRNAs that they were bound to at the moment of lysis and subsequent degradation of those mRNAs by native RNases. In this way, ribosomes are made available for synthesis of a target CFPS product.[56,57] To see if *Vnat* lysate productivity could be improved in this way, we prepared a panel of lysates subjected to runoff reactions at either 30 or 37 °C, both with and without shaking at 250 rpm (Figure S3). This analysis revealed no benefit to performing any sort of runoff reaction. Indeed, overall productivity suffers when *Vnat* lysates are subjected to prolonged agitation or elevated temperatures. Since preincubation is not necessary, we chose to not include the runoff reaction step in our *Vnat* cell extract preparation procedure.

Optimization of Reagent Concentrations and Reaction Conditions in *Vibrio* CFPS. With extraction preparation procedures in hand, we turned our attention to optimizing several reaction conditions which are known to impact CFPS yields. These optimizations were essential, since all reactions performed up until this point had been done using reagent and substrate conditions previously developed for *E. coli* CFPS. Because *Vnat* in nature are found in a different environment than *E. coli*, we reasoned that *Vnat* lysates may have different demands for small molecules and other reagents. Previous studies have demonstrated that CFPS performance can be improved by supplying reagents at concentrations similar to what is encountered by the chassis organism in nature.[5,58] Thus, we hypothesized that yields of the system could be increased by modifying the existing *E. coli* reagent mix to have a more *Vnat*-specific composition. To test this, we varied the concentrations of several key components and observed how each change affected the yields of the system in CFPS.

One notable difference between *E. coli* and *Vnat* is that the latter is halophilic.[45−48] Indeed, exponential phase *Vnat* requires significant amounts of cations including Mg^{2+}, K^+, and Na^+.[59] In CFPS, Mg^{2+} is a particularly important reagent as it is a critical cation required for proper ribosome assembly.[60] Thus, we reasoned that the salt content of the extant reagent mix could be improved for use with *Vnat* lysates. Our approach to address this was 2-fold. First, we varied the concentration of magnesium glutamate [Mg(GLU)] in the reaction mix (Figure 3A). The system performed best when supplemented with 6 mM Mg(GLU). We also varied the concentration of potassium glutamate [K(GLU)] in the reaction mix (Figure 3B). System productivity increased steadily with increasing K(GLU) concentration, up to a maximum beginning at 290 mM K(GLU). This is more than double the 130 mM K(GLU) used in the *E. coli* mix, and is in agreement with a previous study documenting the higher demand for K^+ in *Vnat* growth.[59] We also tested various concentrations of sodium glutamate, but observed no significant improvements to the system (Figure S4).

Next, we looked at optimizing the concentration of amino acids in the reaction mix. As the monomeric building blocks of proteins, amino acids are a key CFPS reagent. Besides their central involvement in protein synthesis, some amino acids are also active participants in central metabolic pathways—consequently, CFPS productivity can be impaired by any nonproductive consumption of amino acids by metabolic pathways still active in the lysate.[3,61] We therefore reasoned that due to potential differences in central metabolism, *Vnat* lysates may have different amino acid demands than *E. coli* systems. To test this, we varied the concentrations of all 20 amino acids added to the CFPS reagent mix (Figure 3C). Increasing the concentration of each amino acid from 2 mM to 3 mM yielded a modest, but significant increase in the productivity of the *Vnat* CFPS system.

The final reagent examined was phosphoenol pyruvate (PEP). A secondary metabolite used in the latter half of glycolysis, PEP is the primary source of ATP in the PANOx-SP energy regeneration system.[5] Similar to amino acids, ATP is utilized by a plethora of cellular processes, and in cell-free systems is consumed to an extent by nonproductive side pathways. Thus, as with amino acids, we reasoned that *Vnat* lysates may have different PEP requirements than *E. coli* lysates. To test this, we varied the concentration of PEP added to *Vnat* CFPS reactions (Figure 3D). We found that doubling the PEP concentration from 33 mM (the optimal concentration previously reported for *E. coli*-based CFPS) to 66 mM yielded a 34% increase in the productivity of the system.

Cell-free reaction temperature is another key factor that can be optimized, because it affects enzyme activities and protein folding. We therefore next sought to establish the optimal reaction incubation temperature for *Vnat* CFPS. All prior reactions up to this point had been incubated at 30 °C as per *E. coli* protocols.[3,54] However, as *V. natriegens* evolved in an environment with an ambient temperature of only ∼23 °C, we reasoned that its cellular components may operate more efficiently at lower temperatures. To test this, we incubated *Vnat* CFPS reactions at several temperatures ranging from 16 to 37 °C (Figure S5). We observed that *Vnat* CFPS reactions are most productive at 30 °C, which could also represent the

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optimum for our reporter protein sfGFP to fold into an active conformation. Still, the system only experiences a 10% reduction in productivity when incubated at room temperature (~23 °C), and retains >60% productivity at 16 °C.

The final, optimized *Vnat* CFPS platform described here is capable of synthesizing ~1.6 mg/mL of sfGFP in 20-h batch mode reactions. Figure 4 captures the stepwise yield increases achieved per process optimization. Overall, the yield is comparable to state-of-the-art systems derived from *E. coli*, which have been improved over the last two decades,[1,3,6] and to our knowledge is the highest-yielding CFPS system derived from this relatively understudied nonmodel organism.

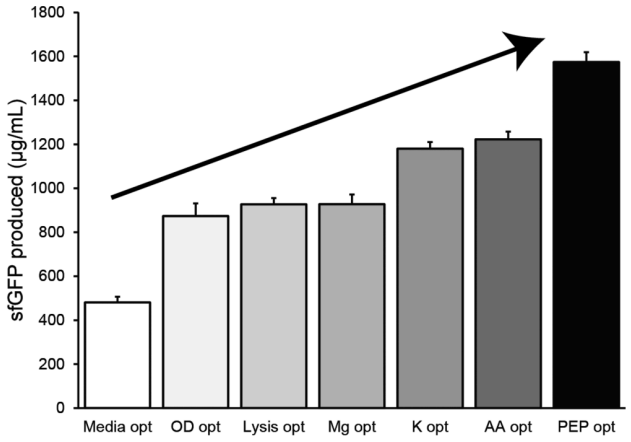

Figure 4. Summary of the development and optimization of *V. natriegens* CFPS. Shown are the stepwise and cumulative improvements to the system following each of the indicated optimizations. sfGFP yield from the best condition identified in each optimization is shown. For each condition, three independent reactions were performed and one standard deviation is shown. Media opt = identification of preferred liquid culture medium; OD opt = identification of optimal OD_{600} at harvest; Lysis opt = identification of optimal sonication procedure; Mg opt = optimization of Mg(GLU) in CFPS reaction mix; K opt = optimization of K(GLU) in CFPS reaction mix; AA opt = optimization of amino acids in CFPS reaction mix; PEP opt = optimization of PEP in CFPS reaction mix.

Assessing the Capabilities of *V. natriegens* CFPS. After a systematic optimization of the *V. natriegens*-based CFPS system, we wanted to assess its capabilities. The ability to lyophilize CFPS reactions for storage at room temperature greatly expands the potential user base for a CFPS platform by removing the requirement of storing the materials in freezers at cold temperatures.[33−35] In *E. coli*-based systems, this flexibility often comes at the expense of productivity, as even in the presence of cryoprotectants the productivity of lyophilized samples can decrease.[33,62] To assess the ability of our *Vnat* CFPS platform to support robust protein synthesis even after lyophilization, we freeze-dried fully assembled reactions both with and without cryoprotectant supplementation (Figure 5A). Samples reconstituted with water immediately following lyophilization performed quite well, experiencing only a small loss of activity likely as a result of the lyophilization process. Addition of 5% trehalose to reactions fully preserves reaction efficacy after a week of room temperature storage, with samples experiencing no detectable loss in productivity after this time. These data suggest freeze-dried strategies developed in other CFPS systems could be applied to our *V. natriegens*-based CFPS system.

To further demonstrate the ease-of-use of our system and to demonstrate possible applications, we tested CFPS reactions using lysates derived from a strain of *Vnat* recently developed and commercialized by Synthetic Genomics, Inc. that includes a genomic insert encoding the T7 RNA polymerase under the control of an IPTG-inducible promoter (Vmax Express).[47] Such a chassis strain circumvents the need to supply this polymerase to CFPS reactions in purified form, partially addressing limitations imposed on reaction volume scale-up related to high costs of reaction substrates.[61] To test the ability of this strain to compose a one-pot CFPS platform, we prepared lysates from cells in which polymerase expression was induced and performed synthesis reactions both with and without supplementation with purified polymerase (Figure 5B). This analysis revealed that, while overall productivity of

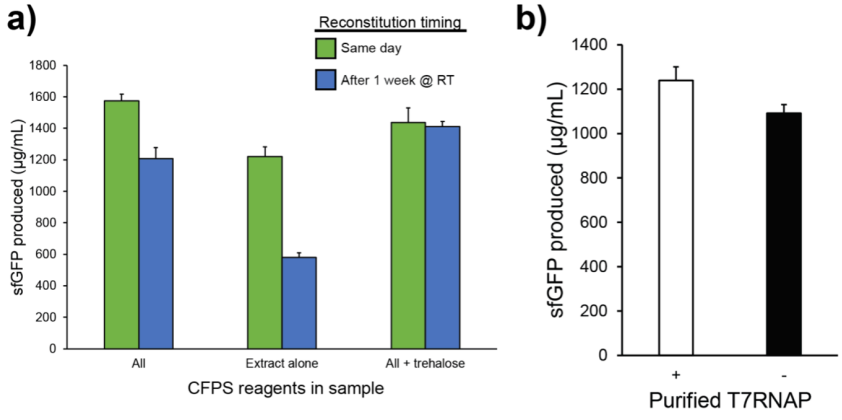

Figure 5. Demonstration of the capabilities of *V. natriegens* CFPS. (a) Yields of sfGFP from lyophilized *V. natriegens* CFPS reactions. Reactions were assembled both without (All) and with supplementation with 2.5% trehalose (All + trehalose). These reactions, along with samples of just *V. natriegens* lysate (Extract alone) were lyophilized overnight. Lyophilized samples were reconstituted either immediately (Same day) or after incubation at room temperature (~23 °C) for 1 week. For each condition 3 independent reactions were performed, and one standard deviation is shown. (b) One-pot CFPS using *V. natriegens* lysates. A lysate was prepared from engineered *V. natriegens* strain Vmax Express (Synthetic Genomics, Inc.), which features an IPTG-inducible genomic insert expressing T7 RNA polymerase. sfGFP yields from this lysate in CFPS performed both with and without supplementation with purified polymerase is shown. For each condition, 3 independent reactions were performed, and one standard deviation is shown.

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the engineered strain is reduced relative to the wild type strain (~21% reduction), the lysates were enriched with enough T7 polymerase to catalyze more than 1 mg/mL of sfGFP synthesis.

Next, we aimed to expand the targets of our CFPS reactions beyond our reporter protein (sfGFP). Short peptides (<10 kDa) have emerged as important agents in biological engineering and synthetic biology. These small biomolecules are widely used as protein mimics for interrogating protein−protein interactions and assessing enzyme substrate preferences,[24,63−65] and bacteria-killing antimicrobial peptides (AMPs) are increasingly being considered for use as next-generation antibiotics as we rapidly approach a postantibiotic era.[31,66,67] The use and study of peptides has historically been limited by our ability to synthesize usable amounts of these molecules—solid phase peptide synthesis is generally applicable only to peptides shorter than 30 amino acids,[68] and recombinant expression in bacterial hosts is opposed by the degradation of peptide products by host proteases.[31,67] We reasoned that protease activity in *Vnat* may be reduced since unwanted proteins could simply be diluted out by rapid cell divisions, which in turn may make this organism well suited for peptide synthesis. We thus set out to see if our *Vnat* CFPS platform could catalyze robust expression of peptides. To test this, *Vnat* CFPS was applied toward the synthesis of the AMPs cecropin A,[69] cecropin P1,[31] and opistoporin I[31] (Table S1). Peptide products were quantified using ^{14}C-leucine radioactive incorporation (Table 1). Opistoporin I expression in particular surpassed 250 μg/mL, which was higher than results in *E. coli*, suggesting that our *Vnat* CFPS platform might have utility for the recombinant expression of peptides.

Table 1. Yields of Antimicrobial Peptides Using *V. natriegens* CFPS[a]

peptide name	length (AAs)	mass (Da)	yield (μg/mL)
cecropin A	38	4136	22.4 ± 1.4
cecropin P1	32	3470	96.8 ± 1.2.8
opistoporin 1	45	4968	278.4 ± 9.1

[a]Shown are yields of the indicated antimicrobial peptides using *V. natriegens* CFPS, quantified *via* ^{14}C-leucine incorporation followed by scintillation counting of 15% TCA-precipitated peptides. For each peptide 3 independent reactions were performed, and one standard deviation is indicated.

Increasing System Productivity *via* Genome Engineering. Finally, in an effort to further enhance protein synthesis yields, we pursued increases in *Vnat* CFPS productivity *via* genome engineering. Recent efforts in *E. coli* have seen tremendous success in increasing *in vitro* productivity by genetically inactivating or removing genes whose products may destabilize key substrates in CFPS.[3,54,61] These so-called "negative effectors" have broadly included targets such as DNases, RNases, proteases, and central metabolic enzymes that might nonproductively consume ATP and/or amino acids throughout the CFPS reaction. Here, we prepared a short list of genes that were previously identified in *E. coli* as being beneficial knockout targets for increasing CFPS yields (Table S2).[3] We performed a bioinformatics search to identify close homologues of these genes in the genome of *Vnat*. Then, we applied an approach to leverage *Vnat*'s natural propensity for homologous recombination to replace each gene individually with a selectable marker to generate a library of *Vnat* knockout strains.[47,70] Lysates were prepared from each strain using optimal practices identified here, and assessed in CFPS (Figure S6).

Unfortunately, we were unable to recapitulate the beneficial effects of any of these knockouts in our *Vnat* system. This could be a result of the pronounced difference in strain backgrounds—*Vnat* is significantly diverged from *E. coli* such that knockout targets which work well for one species may not necessarily inform engineering efforts in the other. In this case, a more comprehensive screen of knockouts in *Vnat* may be required to identify effectual knockouts. It is also possible that system productivity is being limited by something more fundamental, such as ribosome or energy availability, which could mask any observable benefit of these knockouts to overall protein yield. Further studies are needed.

■ CONCLUSIONS

In this study, we present the development of a novel CFPS platform composed of crude lysates derived from the nonmodel bacterium *V. natriegens* (Figure 6). We identified optimal culture harvest conditions for this organism, demonstrating that culturing in BHI media supplemented with v2 salts and harvesting at stationary phase is optimal for the generation of productive lysates. This feature is convenient, as it eliminates the need to collect cell pellets within a tightly specific OD_{600} window in order to preserve lysate quality. Researchers can "set and forget" *V. natriegens* cultures and

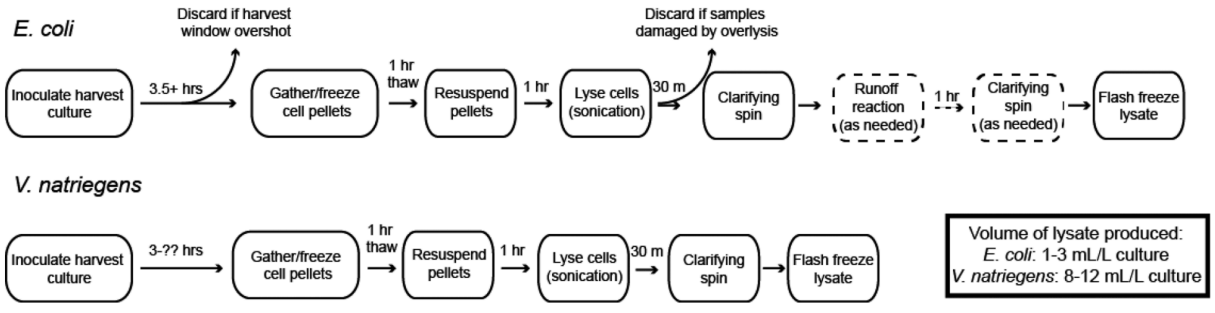

Figure 6. Comparison of *E. coli* and *V. natriegens* lysate preparation workflows. As compared to *E. coli*, *V. natriegens* cells are easier to handle both in the context of culture harvest and lysis. It is difficult to spoil a *V. natriegens* extract preparation. *V. natriegens*' overall workflow is faster due to the organism's rapid growth rate and omission of any runoff reaction step. Finally, because *V. natriegens* pellets are gathered at stationary phase, they are significantly larger than those gathered for *E. coli*—consequently, the volume of *V. natriegens* lysate derived from a single 1 L harvest is significantly higher.

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harvest whenever it is convenient without worrying about spoiling the resulting extracts. Attempts to optimize lysis parameters revealed that *V. natriegens* lysates are highly resistant to damage *via* overlysis, and overall the system is relatively agnostic to both lysis buffer resuspension volume and lysis energy delivery. Surprisingly, the system is much less sensitive to overlysis during lysate preparation as compared to *E. coli*-based systems, a huge benefit for first-time or inexperienced CFPS users.

Optimization of the CFPS components specifically for use with *V. natriegens* lysates significantly increased the overall productivity of the system to ∼1.6 mg/mL sfGFP, comparable to popular platforms based on lysates derived from *E. coli* and the highest-yielding CFPS system derived from a nonmodel organism, to our knowledge. This is also higher than a recent study published during the preparation of this manuscript which demonstrated expression titers of 0.4 mg/mL of green fluorescent protein in a *V. natriegens* CFPS system.[71] Further, our system is stable at room temperature if lyophilized in the presence of trehalose, is capable of synthesizing small peptide products, and can be carried out in a one-pot system with the use of the Vmax Express chassis strain.

Looking forward, we anticipate that the CFPS system described here will find use in the identification and characterization of *V. natriegens* genetic parts. Recent publications have developed a suite of tools for using *V. natriegens* for cloning and recombinant protein expression,[47,48] but the knowledge base for this organism still lags behind the more established *E. coli* with regards to regulatory sequences such as promoters, terminators, and ribosome binding sites (RBSs). Due to its open and easily accessible nature, the CFPS platform developed here could be used to interrogate many such parts in parallel for rapid characterization. The resulting data could in turn be used to inform construct design for use *in vivo*, supporting the increasing interest in using this organism as an alternative to *E. coli* for molecular biological applications. This idea has already been briefly explored using a *V. natriegens*-based CFPS platform.[71]

Perhaps the most surprising finding to arise from this effort was that the most productive *V. natriegens* extracts are derived from stationary phase cells. This not only contradicts what is usually found in other bacterial CFPS systems, but is also is overall difficult to rationalize alongside the generally accepted notion that ribosomes are downregulated in stationary phase.[55] It is possible that sequestration of ribosomes by native mRNAs in lysates derived from exponential phase cells accounts for the relatively low productivity of these lysates, despite the presence of a larger ribosome pool. In other systems this issue is alleviated by subjecting the lysate to a runoff reaction,[3,52,54,56,57] but this approach was not successful here.

Going forward, we expect the efficacy of *V. natriegens* CFPS to improve. Indeed, the system development and characterization described here accomplished in a very short amount of time what took decades of research and development in *E. coli*. Exploration of the use of alternative energy regeneration systems is one obvious future direction. Indeed, development of an entirely novel energy regeneration system (perhaps using sucrose as the starting substrate[48]) might ultimately be required to fully optimize *V. natriegens* CFPS. Another fruitful direction is in the screening of a larger, more comprehensive library of negative effector knockout strains. This could perhaps be informed by a time course analysis of small molecule concentrations in CFPS reactions, with knockouts targeted toward metabolic pathways that might be siphoning away critical substrates.

In conclusion, the *Vnat* cell-free platform is excellent for early forays into the use of CFPS systems, as the cells are fast and easy to grow, easy to lyse, and a high volume of active lysate is generated from as little as 1 L of cell culture. Collectively, these features reduce the need for specialized knowledge and equipment that have limited the use of CFPS. We expect that this reduced entry barrier will facilitate the spread of these systems into new areas for use on exciting, novel applications in synthetic biology.

METHODS

Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in Table S3. *V. natriegens* was purchased from the American Type Culture Collection (ATCC 14048). Vmax Express was purchased from SGI-DNA, a subsidiary of Synthetic Genomics, Inc. pJL1 plasmids encoding the antimicrobial peptides were synthesized and assembled by Twist Bioscience. Assembled plasmids were submitted to the NUSeq Core facility along with forward primers, and sequences were confirmed using traditional Sanger sequencing. Kanamycin (50 μg/mL) was used for maintaining pJL1-based plasmids. Chloramphenicol (34 μg/mL) was used to select for all negative effector knockout mutants.

Cell Culture. *V. natriegens* cells were grown in BHI media supplemented with v2 salts (204 mM NaCl, 4.2 mM KCl, 23.14 mM $MgCl_2$) unless noted otherwise. For confirmation of *V. natriegens* growth rate, 100 μL cultures were assembled in a clear 96-well plate (Costar 3370; Corning, Corning, NY) and shaken at 250 rpm at 37 °C for 20 h in a Synergy H1 plate reader (BioTek, Winooski, VT) which continuously monitored the OD_{600} of each sample. To minimize sample evaporation, plates were covered and sealed with Parafilm. Doubling times were calculated using time points corresponding to OD_{600} values between 0.02 and 0.2. For cultures performed at 1 L scale, cells were grown in a 2.5 L Tunair shake flask and incubated at 37 °C at 250 rpm. Except for experiments performed to identify optimal harvest OD_{600}, cultures were grown until the onset of stationary phase (an approximately OD_{600} of 6.5−7.5). 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to cultures of Vmax Express cells between OD_{600} 0.6−0.8 to induce expression of T7 RNA polymerase. In all cases, cells were pelleted by centrifuging for 15 min at 5000*g* at 4 °C, washed three times with cold S30 buffer (10 mM tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 2 mM dithiothreitol),[72] and stored at −80 °C until lysed.

Extract Preparation. Unless otherwise noted, cell pellets were thawed and suspended in 0.8 mL of S30 buffer per gram of wet cell mass. Prior to the optimization of lysis parameters, cell pellets were instead resuspended in 1.0 mL buffer per gram of cells. Following suspension, 1.4 mL of cell slurry was transferred into 1.5 mL microtubes. The cells were lysed using a Q125 Sonicator (Qsonica, Newtown, CT) with 3.175 mm diameter probe at a 20 kHz frequency and 50% amplitude. Sonication was continued for three cycles of 45s ON/59s OFF unless stated otherwise. To minimize heat damage during sonication, samples were placed in an ice−water bath. For each 1.4 mL sample, the input energy was ∼270 Joules/sonication cycle. The lysate was then centrifuged at 12 000*g* at 4 °C for 10 min. The supernatant was flash-frozen using liquid nitrogen

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and stored at −80 °C until use. For preparations including a runoff reaction, following the first clarifying spin supernatant was transferred to a new tube and subjected to a 1 h incubation at 30 or 37 °C, either stationary or with shaking at 250 rpm. Following this incubation, samples were centrifuged at 10 000*g* at 4 °C for 10 min after which supernatant was flash-frozen and stored at −80 °C until use.

CFPS Reaction. A modified PANOx-SP system was utilized for CFPS reactions. Briefly, a 15 μL CFPS reaction in a 2.0 mL microtube was prepared by mixing the following components: 1.2 mM ATP; 0.85 mM each of GTP, UTP, and CTP; 34 μg/mL folinic acid; 170 μg/mL of *E. coli* tRNA mixture; 13.3 μg/mL plasmid; 16 μg/mL T7 RNA polymerase; 3 mM for each of the 20 standard amino acids; 0.33 mM nicotinamide adenine dinucleotide (NAD); 0.27 mM coenzyme-A (CoA); 1.5 mM spermidine; 1 mM putrescine; 4 mM sodium oxalate; 290 mM potassium glutamate; 10 mM ammonium glutamate; 6 mM magnesium glutamate; 57 mM HEPES, pH 7.2; 67 mM phosphoenolpyruvate (PEP), and 4 μL (27% v/v) of cell extract. Each CFPS reaction was incubated for 20 h at 30 °C unless noted otherwise. Experiments performed prior to determining an optimal reagent mix for *V. natriegens* lysates used the above mix with the following changes: 2 mM for each of the 20 standard amino acids, 130 mM potassium glutamate, 10 mM magnesium glutamate, and 33 mM PEP were used instead. As individual reagent concentrations were optimized, their optimal value listed above were used for all reactions from that point onward. *E. coli* total tRNA mixture (from strain MRE600) and phosphoenolpyruvate was purchased from Roche Applied Science (Indianapolis, IN). ATP, GTP, CTP, UTP, 20 amino acids and other materials were purchased from Sigma (St. Louis, MO) without further purification. T7RNAP was purified in house as described previously.[3] To direct synthesis of a specific product, 200 ng of pJL1 template plasmid encoding the product was added to each reaction.

Quantification of Active sfGFP. CFPS reactions were diluted 1:25 in nanopure water and active full-length sfGFP protein yields were quantified by measuring fluorescence using a Synergy 2 plate reader (BioTek, Winooski, VT) with excitation at 485 nm, emission at 528 nm, and cutoff at 510 nm in 96-well half area black plates (Costar 3694; Corning, Corning, NY). sfGFP fluorescence units were converted to concentration using a standard curve established with ^{14}C-Leu quantified sfGFP as described previously.[54]

CFPS Lyophilization. Samples were assembled in 2 mL microtubes and lyophilized overnight using a VirTis BenchTop Pro Freeze-Dryer (SP Scientific, Warminster, PA). Lyophilized samples were stored at room temperature under vacuum in a desiccator with Drierite desiccant. For reconstitution of fully assembled reactions, template plasmid DNA plus nuclease-free water were added to each sample. Samples consisting of only lyophilized lysate were reconstituted with the complete CFPS reagent mix. Where indicated, lyophilized reactions were supplemented with 2.5% (m/v) trehalose.

Quantification of Antimicrobial Peptide Yield in CFPS. Radioactive ^{14}C-Leucine was added into 15 μL CFPS reactions to a final concentration of 25 μM. After incubation, yields were quantified by determining radioactive ^{14}C-Leu incorporation into peptides precipitated in 15% (m/v) trichloroacetic acid (TCA).[72] Radioactivity of TCA-precipitated samples was measured using liquid scintillation counting (MicroBeta2, PerkinElmer, Waltham, MA).

Generation of Negative Effector Knockout Library. The *V. natriegens* ATCC 14048 genome sequence (GenBank accessions CP016345 and CP016346, corresponding to chromosomes I and II, respectively) was screened for the presence of homologues to a subset of negative effectors known to limit the productivities of *E. coli*-based CFPS lysates (*endA, lon, mazF, ompT, rna, rnb, glpK, gor, gshA, tnaA*) using a tBLASTn search with *E. coli* gene sequences as the query (Sequence List S1). As an additional confirmation of the identity of potential homologues, the gene annotation engine present in the Archetype Genomics Discovery Suite software package (Synthetic Genomics, Inc.) was used to assign putative functions to the identified *V. natriegens* genes. Using this approach, likely homologues to *endA, lon, rnb, glpK, gor, gshA*, and *tnaA* were identified in *V. natriegens* (Sequence List S2). Natural competence-mediated homologous recombination was used (essentially as described in Dalia *et al.*[70]) to generate the knockout strains. Briefly, *V. natriegens* cells rendered naturally competent were transformed with DNA cassettes composed of a chloramphenicol resistance gene bounded by 3 kb homology arms to the genetic loci of interest, resulting in the replacement of the putative negative effector gene with the chloramphenicol resistance marker. The resulting chloramphenicol-resistant transformants were screened by colony PCR to confirm the desired knockout.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00252.

Supplemental Figures S1−S6, describing some of the optimizations attempted to further improve the *V. natriegens* CFPS platform; Supplemental Tables S1−S3 listing the antimicrobial peptides synthesized in this study, the genomic knockout targets described, and the bacterial strains utilized; Supplemental Sequence Lists S1−S2 presenting sequences associated with knockout bioinformatics searches (PDF)

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B.J.D. and M.C.J. designed the experiments. B.J.D. and S.R.D. conducted the experiments. B.J.D. and M.C.J. wrote the paper. M.T.W. and D.G.G. performed bioinformatic analysis to identify putative negative effectors and generated the library of knockout strains. M.C.J. supervised the research.

Notes

The authors declare the following competing financial interest(s): M.T.W. and D.G.G. are employees of Synthetic Genomics, Inc., a synthetic biology company commercializing products and platform technologies based on novel host organisms, including *V. natriegens*.

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■ ABBREVIATIONS

CFPS, cell-free protein synthesis; sfGFP, superfolder green fluorescent protein; BHI, brain-heart infusion; AMP, antimicrobial peptide; *Vnat*, *Vibrio natriegens*

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