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ARTICLE

Acetaminophen production in the edible, filamentous cyanobacterium *Arthrospira platensis*

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Abstract

Spirulina is the common name for the edible, nonheterocystous, filamentous cyanobacterium *Arthrospira platensis* that is grown industrially as a food supplement, animal feedstock, and pigment source. Although there are many applications for engineering this organism, until recently no genetic tools or reproducible transformation methods have been published. While recent work showed the production of a diversity of proteins in *A. platensis*, including single-domain antibodies for oral delivery, there remains a need for a modular, characterized genetic toolkit. Here, we independently establish a reproducible method for the transformation of *A. platensis* and engineer this bacterium to produce acetaminophen as proof-of-concept for small molecule production in an edible host. This work opens *A. platensis* to the wider scientific community for future engineering as a functional food for nutritional enhancement, modification of organoleptic traits, and production of pharmaceuticals for oral delivery.

KEYWORDS

Arthrospira platensis, cyanobacteria, spirulina

1 | INTRODUCTION

Arthrospira platensis, commonly referred to and sold commercially as spirulina, is an edible, nonheterocystous, filamentous cyanobacterium of the order *Oscillatoriales*, with species of this genus having been used as traditional food sources in Mexico and Chad for centuries (Ciferri, 1983; Vonshak, 2002). *A. platensis* is considered safe for human consumption and is designated as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (GFA GRN No. 417); it has exceptionally high protein content relative to plants and

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eukaryotic microalgae (Torres-Tiji et al., 2020), and is grown commercially as a food, pigment source, and animal feedstock (Torres-Tiji et al., 2020; Vonshak, 2002). Given its enticing food properties and established commercial growth, as well as applications driven by a photosynthetic metabolism, there is keen interest in genetically modifying this bacterium for enhanced nutrition, modified organoleptic traits, improved growth properties, and pharmaceutical production (Furmaniak et al., 2017; Jester et al., 2022; Torres-Tiji et al., 2020).

While there are clear applications for sustainable biomanufacturing on Earth, the edible nature and photosynthetic metabolism of *A. platensis* makes it well-suited as an alternate food source and microbial host for on-demand pharmaceutical production for in situ resource utilization (ISRU)-based missions for human space exploration and habitation (Mapstone et al., 2022; McNulty et al., 2021; Nangle et al., 2020). As humans delve deeper into space via the Moon and eventually to Mars and beyond, the need to reduce mission launch cost, reduce risk, and anticipate pharmaceutical degradation induced by radiation becomes paramount, further increasing the need for ISRU-driven food and pharmaceutical production (Berliner et al., 2020; Blue, Bayuse, et al., 2019; Blue, Chancellor, et al., 2019; Menezes, Montague, et al., 2015; Menezes, Cumbers, et al., 2015; Nangle et al., 2020). NASA and ESA have both established the utility of this organism for space exploration via their biological life support projects, CELSS and MELISSA, respectively, which used *A. platensis* to photoautotrophically recycle NO_3^- and CO_2 for production of O_2 and biomass for astronaut consumption (Gòdia et al., 2002; Tadros, 1988). Expanding upon its established life support properties to further produce functional foods for nutrition and pharmaceutical delivery will further increase the utility of this organism for deep space missions.

A. platensis has historically remained recalcitrant to the development of genetic tools and a reproducible transformation method (Dehghani et al., 2018; Jeamton et al., 2017; Kawata et al., 2004; Toyomizu et al., 2001). Potential reasons range from its multicellularity, polyploidy, and motility, which combine to inhibit colony formation on plates, and a genome that encodes a high number of restriction-modification systems of types I-IV in addition to several CRISPR/Cas systems (Fujisawa et al., 2010; Silas et al., 2017). However, in a landmark study, Jester et al. (2022) recently engineered *A. platensis* for antibody production targeting campylobacter infections, and showed that their orally-delivered strain prevents disease in mice and is safe for human consumption (Jester et al., 2022). However *A. platensis* has yet to be engineered for exogenous small molecule production.

Here, we independently develop two reproducible transformation methods based on electroporation and natural competence, and use these methods to show that the oscillin gene contributes to motility, express eYFP from the genome, and engineer *A. platensis* to produce acetaminophen as proof-of-concept for small molecule production derived from CO_2, H_2O, and light in an edible host for bioavailable, oral drug delivery.

2 | RESULTS

2.1 | Genetic engineering of *Arthrospira platensis*

We initially developed an electroporation method inspired by the patent literature (Takeuchi & Roberts, 2017) (see Section 4 for details). Due to its filamentous and motile nature, *A. platensis* does not form colonies on plates, and we were unable to obtain filament or cell counts. Thus, we were unable to quantify plating or transformation efficiency, and were therefore dependent on the binary output of growth or no growth, as has been observed in *Phormidium lacuna* (Nies et al., 2020). Based on the predictions of Nies et al. (2020), we confirmed natural competence in *A. platensis*, and established this method as our standard transformation procedure.

Cyanobacteria have a wide range of genome copy numbers per cell (Griese et al., 2011), with reported copy numbers of the *Oscillatoriales* ranging from 20 to 90 copies per cell in *Phormidium lacuna* (Nies et al., 2020) to nearly 700 in *Trichodesmium erythreaum* IMS 101 (Sargent et al., 2016). For all transformations resulting in biomass under selection, we observed via PCR that transformant genomes encoded the native locus in addition to the exogenous DNA, suggesting that a single recombination event occurred between the genome and suicide vector. While no counterselection method was present, transformant strains were subcultured until no native locus could be detected via PCR and/or genome sequencing. This took approximately 3–4 months on agar plates and 2–3 months in liquid culture. Jester et al. (2022) reported a segregation time of 8–10 weeks for *A. platensis*, further supporting our observations that exogenous DNA must be selected over long timeframes through a polyploid genome and multi-cell filament to obtain double recombinant mutants that lack the WT locus.

We established a series of parts in *A. platensis* NIES-39: one antibiotic resistance marker, three genomic loci for insertion of exogenous DNA, four endogenous and five exogenous promoters, and one fluorescent reporter (Supplemental Table 1). All parts were integrated into a series of suicide vectors for homologous recombination with the genome (Supplemental Table 2). To screen multiple loci as genomic integration sites, we chose neutral site I (NSI; NIES39_Q01230), a commonly used site in other cyanobacteria for insertion of exogenous DNA (Taton et al., 2014), along with two loci we expected to be involved in motility to potentially create nonmotile strains to aid in plating and genetics. The NSI site in *A. platensis* was found through a homology search to known NSI sites in other cyanobacteria.

Twitching motility in cyanobacteria is dependent on Type IV pili and is light regulated resulting in phototaxis towards or away from light sources (Brahamsha & Bhaya, 2013; Schuergers et al., 2017). Filamentous cyanobacteria from the *Oscillatoriales* and certain species of the *Nostocales* employ surface-dependent gliding motility. The oscillin protein forms fibrils on the surface of *Phormidium uncinatum*, and has therefore been proposed as an essential element in gliding motility (Brahamsha & Bhaya, 2013; Hoiczyk & Baumeister, 1997).

We targeted *pilA* (NIES39_C03030), a central component of the Type-IV pilin system, and an oscillin homolog (NIES39_A01430) as integration sites for exogenous DNA and motility ablation. We knocked out these three loci with the codon-optimized version of *aadA*, *aadA. co*, which encodes for streptomycin/spectinomycin (Sm/Sp) resistance (Figure 1; Supplemental Table 2). The ΔNSI::P*pilA-aadA.co*, Δoscillin::*aadA.co*, and Δ*pilA*::*aadA.co* strains were confirmed by whole genome sequencing (Figure 1a). Of note, Δ*pilA*::*aadA.co* mutants display a "star" morphology when plated on agar, while Δoscillin::*aadA.co* mutants display a "string" morphology (Figure 1). This implies that both genes are involved in motility and/or colony formation on plates.

Deletion of the NSI locus does not result in morphological or growth differences between the wild-type (WT) strain as expected (Figure 1). Light-based motility assays showed that the WT and ΔNSI::P*pilA-aadA.co* strains moved into the illuminated area of the plate within 7 days, while the Δoscillin::*aadA.co* and Δ*pilA*::*aadA.co* strains remained in the shade where they were initially spotted (Figure 1c). While the Δ*pilA*::*aadA.co* strain did not expand beyond its initial spot, the Δoscillin::*aadA.co* mutant moved in a small halo around the initial spot, indicating that motility is severely limited by the loss of oscillin. This indicates that both *pilA* and oscillin genes contribute to phototaxis in *A. platensis*. To our knowledge, this is the first observation that oscillin is directly involved in motility, and, while it is not completely essential for motility, it plays an important role.

2.2 | Expression of a fluorescent reporter system

To test an expression system in *A. platensis*, we designed a series of vectors (Supplemental Table 2) that resulted in mutant strains at the *pilA* locus that had one of three native, strong promoters (P_{psaA}, P_{cpcB}, or P_{rbcL}) (Wang et al., 2012) or one of five J23-series promoters driving the expression of codon-optimized *eYFP* (*eYFP.co*) downstream of the native *pilA* promoter driving the expression of *aadA. co* followed by the native *aadA* terminator (Figure 2a). The fluorescent output of each strain was measured, showing orders of magnitude higher fluorescence in the eight reporter strains than in the WT (Figure 2b). The J23-series promoters displayed a fourfold dynamic range in *A. platensis* compared to a 120-fold dynamic range in both *Synechococcus elongatus* UTEX 2973 and *Synechocystis* sp. 6803 (Vasudevan et al., 2019). Although read-through from the *pilA* promoter may explain some of these differences (See Discussion), these results highlight the need for further development and characterization of *A. platensis*-specific genetic parts. Fluores-

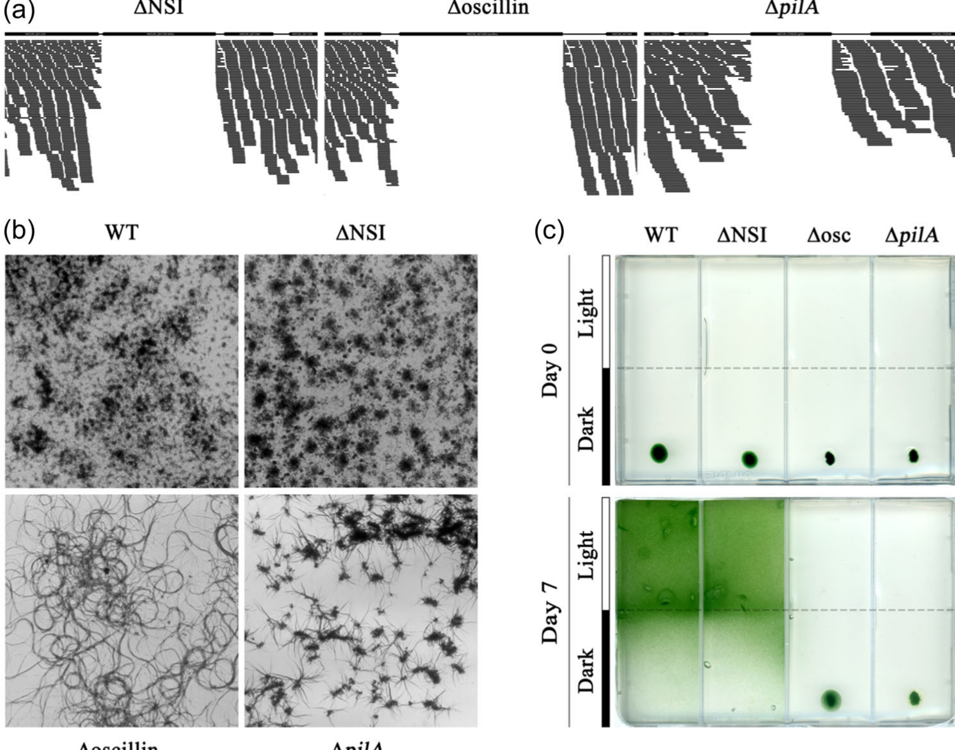

FIGURE 1 Confirmation and phenotyping of deletion mutants at three genomic loci. (a) Downsampled Illumina coverage for each genomic region that was swapped for *aadA.co*. (b) Images showing colony morphology on agar plates for the wild type (WT) and each deletion strain. (c) Images depicting colony position on agar plates at Day 0 (top) and Day 7 (bottom) for the WT and each deletion strain. Strains were plated in the dark, and light was available to the upper half of the plate. NSI, neutral site I.

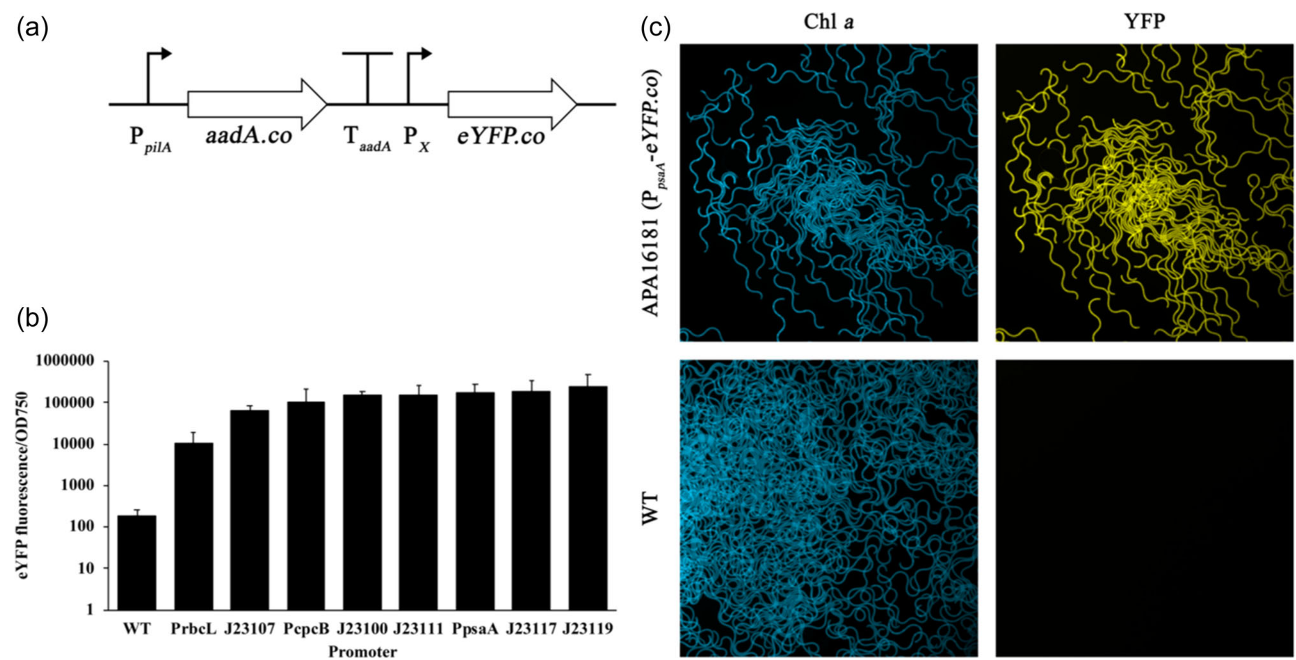

FIGURE 2 Fluorescent reporter and promoter characterization. (a) Schematic of the mutant genotypes for *eYFP.co* expression at the *pilA* locus: P_{pilA} drives expression of *aadA.co*, which is terminated by T_{aadA}. Downstream, one of three native promoters drives expression of the *eYFP.co* gene. (b) Relative promoter strength for three native *A. platensis* promoters and the five J23-series promoters. Error bars represent standard deviations of technical replicates. (c) Fluorescence microscopy of the Δ*pilA*::*aadA.co*-P*psaA*-*eYFP.co* and wild type (WT) strains showing Chl *a* autofluorescence of filaments under excitation at 587 nm, and YFP fluorescence under excitation at 485 nm.

cence was manually checked under a fluorescence microscope to confirm expression of eYFP.co in the reporter strains; no fluorescence was detected in the WT strain (Figure 2c).

2.3 | Acetaminophen production in *A. platensis*

For the biosynthesis of acetaminophen (APAP; Figure 3a), chorismate is converted into para-aminobenzoic acid (PABA) by the multienzyme complex PabABC, composed of aminodeoxychorismate synthase (*pabAB*) and 4-amino-4-deoxychorismate lyase (*pabC*). PABA is converted into 4-aminophenol by the 4-aminobenzoate hydrolase (4ABH) from *Agaricus bisporus*, which is subsequently converted to APAP by *N*-hydroxyarylamine *O*-acetyltransferase (NhoA) from *Escherichia coli* (Anderson, 2017). A series of 38 suicide vectors encoding variations on the synthetic APAP biosynthesis pathway with codon-optimized genes (Figure 3a) were designed and screened in *A. platensis* (Supplemental Table 3). Of these, 23 resulted in biomass that displayed the WT morphology under selection at 1 μg mL^{-1} Sm/Sp; no transformants for any vector could be recovered at higher selection strengths.

For every transformation, a negative control was run to ensure that the selection killed WT cells, and pJMH024 was used as a positive control to ensure that cells were transformable. PCR screens of genomic DNA isolated from biomass that grew under selection indicated the presence of *pilA*, *aadA.co*, *4ABH*, and *nhoA*, suggesting an initial integration of the suicide vector into the genome. However, subculturing these strains into the same conditions resulted in no growth. The only way we could recover a fully segregated strain, APA16206 (Figure 3b), was under 16 h light:8 h dark cycles illuminated with ~20–30 μmol m^{-2} s^{-1} (μEi) of photosynthetically active radiation (see Section 4) for 3 months until star morphology appeared. Tufts of star morphology were subcultured into fresh selective medium under the same conditions. Upon reaching mid to late log phase, biomass was subcultured and grown under 24 h light illuminated with ~70–100 μEi. Biomass was regularly subcultured under these conditions upon reaching mid to late log phase until fully segregated (Supplemental Figure 1).

As PABA supplementation was previously shown to increase APAP titer (Anderson, 2017), we screened APAP production in the WT and APA16206 strains with and without the addition of the precursor compound, PABA (Figure 3c–e; Supplemental Figure 2). The WT strain did not produce APAP under any condition, while the mutant produced APAP only when PABA was added to the medium (Figure 3c). The APAP titer and production rate in APA16206 were dependent on the amount of PABA supplied, with titer and rate increasing with increasing PABA concentrations. The maximal titer and rate were 2.9 mg L^{-1} and 0.018 mg L^{-1} h^{-1}, respectively, when supplied with 2.74 g L^{-1} PABA. Growth rate was slowed by increasing PABA concentration, but did not impact the final biomass yield, except for two of the replicates supplemented with 2.74 g L^{-1} PABA, which prematurely reached stationary phase and died (Figure 3e). The APAP production rate appeared dependent on the growth rate.

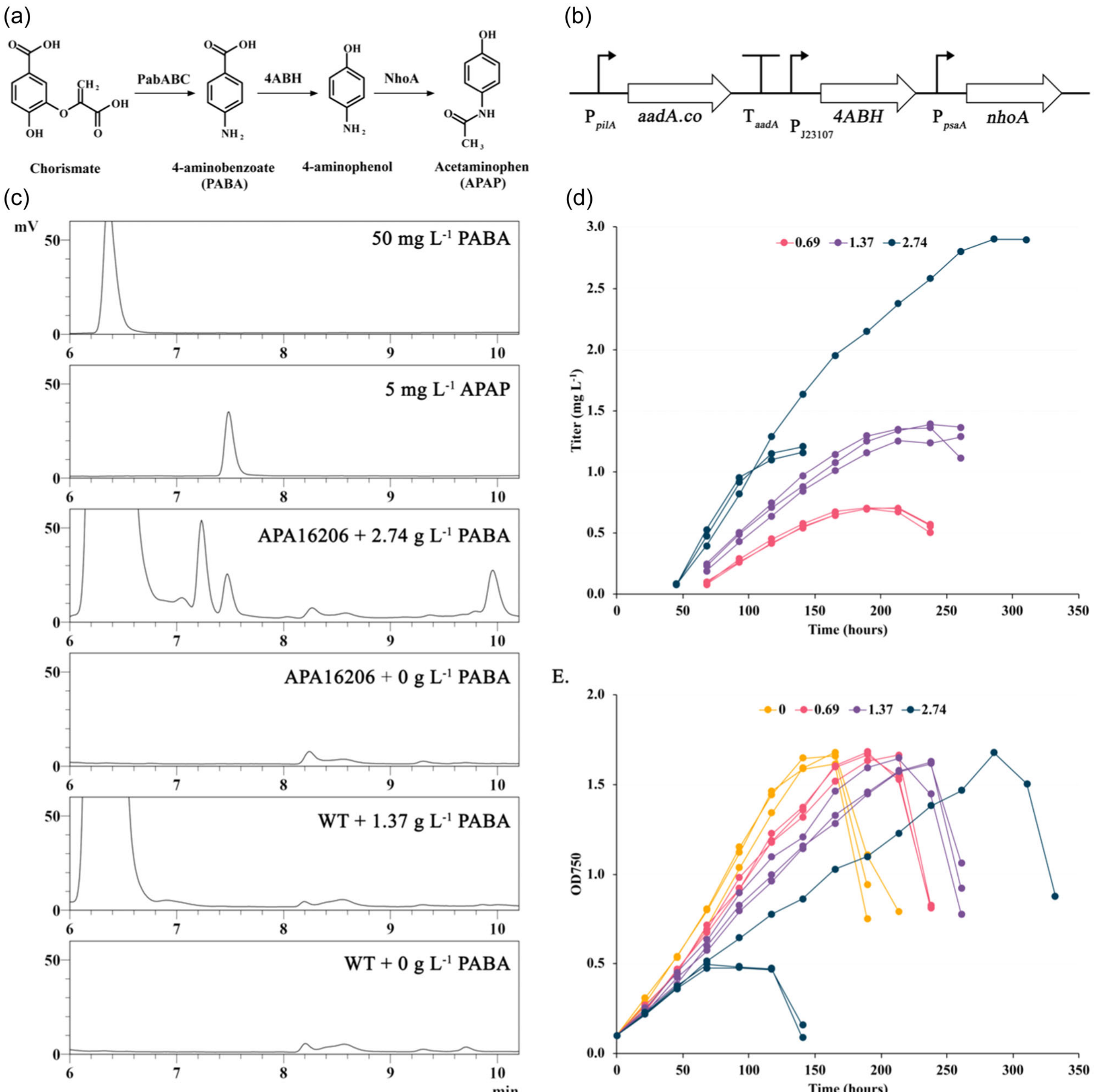

FIGURE 3 Acetaminophen (APAP) production in *A. platensis* APA16206. (a) The pathway for APAP production from para-aminobenzoic acid (PABA) uses the enzyme 4ABH to convert PABA to 4-aminophenol, which is then converted to APAP by NhoA. (b) Schematic for the APAP production pathway at the *pilA* locus in APA16206. (c) High-performance liquid chromatography (HPLC) chromatograms showing peaks eluting between 6 and 10.2 min for PABA and APAP standards along with APA16206 and wild-type (WT) strains with and without PABA supplementation. (d) APAP titer over time in APA16206 when supplemented with 0.69, 1.37, and 2.74 g L^{-1} PABA. (e) Growth of APA16206 with and without PABA supplementation at 0, 0.69, 1.37, and 2.74 g L^{-1}.

3 | DISCUSSION

Here we independently establish the reproducible transformation of *A. platensis* via electroporation and natural competence, show that oscillin is important to motility, detectably express eYFP from the genome, and produce APAP as a proof-of-concept pharmaceutical for oral delivery. Our methods help unlock this bacterium for future engineering efforts for sustainable biomanufacturing on Earth and ISRU-based food and pharmaceutical production in space by providing a reproducible blueprint. Building on recent work (Jester et al., 2022; Nies et al., 2020), we help establish genetic tractability in the *Oscillatoriales*, an order of cyanobacteria that have been historically understudied through genetic methods. Members of this order, such as the diazotrophic genus *Trichodesmium*, are major contributors to the biological nitrogen cycle.

We originally began this work using electroporation with the goal of replacing *pilA* to remove motility so that this organism was easier to plate for genetics. However, given that we discovered that this organism was naturally competent, replacing *pilA* may impede subsequent natural transformations as type IV-pili (T4P) typically play a large role in natural transformation. If a nonmotile strain that is capable of natural competence is desired, the oscillin deletion strain or targeting one or more of the hormogonium polysaccharide (HPS) genes may be needed. HPS is a is a motility-associated polysaccharide and inactivation of some *hps* genes can result in no effect on the T4P systems, with the expectation that natural competence would be undisrupted; the *A. platensis* genome encodes for orthologs of most of the *hps* genes found in *N. punctiforme* (Zuniga et al., 2020).

While our proof-of-concept biosynthesis of APAP is an important step for metabolically engineering *A. platensis*, continued improvement in the genetic tools available is paramount to decrease the design-build-test-learn cycle time in this bacterium. Crucial to this endeavor is the development of new promoters to overcome the limited dynamic range we observed, which may be achieved through the characterization of alternate native promoters, synthetic promoters engineered from native promoters, or the establishment of inducible promoters (Behle et al., 2020). We acknowledge that we did not control for possible readthrough of the *pilA* promoter in our mutant strains at this locus. In terms of the promoters we used in this study (Figure 2), we expect that the native promoters used (i.e., *cpcB*, *psaA*, and *rbcL*) do indeed express eYFP as these are three of the canonically strong promoters used throughout cyanobacteria and do show reproducible differences in the expression of eYFP between them. We cannot say if the J23-series promoters have low dynamic range due to read-through from the *pilA* promoter or due to other reasons. Future work should take potential read-through issues into account when characterizing new promoters. Outside of promoters, operationalizing a CRISPR/Cas system (Ungerer & Pakrasi, 2016) would provide a locus-independent counterselection that may decrease the segregation time, as the Cas enzyme would be expected to cut all, or most, genome copies present in the cell, and would allow for installation of more complex pathways. Developing self-replicating vectors for the expression of pathways or a CRISPR/Cas system would bypass the need for genome integration, and allow for rapid screening of pathway topologies and simpler CRISPR/Cas-based genome engineering.

APAP is the first reported exogenous small molecule produced in *A. platensis* and provides a milestone in the long-term efforts to genetically engineer this organism. Given that a dose of APAP is 325 mg, our maximum titer of 2.9 mg L^{-1} would require ~112 L for a single dose, assuming complete bioavailability. Thus, higher titer will need to be achieved to make manufacturing a dose of APAP feasible if oral delivery via spirulina is the goal. To this end, there are several routes that should be explored.

Of the 38 APAP pathway topologies we transformed into *A. platensis*, we could only recover one fully segregated mutant that, in turn, could only be recovered under day/night cycles. This was in stark contrast to the creation of other mutants reported here, which proceeded without difficulty, and suggests that enzyme toxicity from 4ABH and/or NhoA, due to their products or the enzymes themselves, inhibited integration and/or segregation of the pathway into and through the genome. Several additional, unknown chromatography peaks were observed in APA16206 when PABA was supplied (Figure 3c), and may contribute to the observed pathway toxicity. These unknown peaks eluted at similar times as PABA and APAP, implying a similar chemical structure. These peaks may be due to enzyme promiscuity of 4ABH and/or NhoA, or from APAP degradation products; these peaks are not from the intermediate 4-aminophenol as the standard for this molecule produced two peaks at elution times of 2.9 and 3.3 min. If these compounds are produced from enzyme promiscuity, engineering a strain to produce only APAP would likely increase the titer of APAP by avoiding flux of PABA into these accessory compounds. This could be done through enzyme engineering, removing biosynthesis pathways from the genome for substrates that these enzymes may promiscuously convert, or screening 4ABH and/or NhoA homologs for improved selectivity towards APAP production. Decreasing the potential pathway toxicity through use of weaker promoters, enzyme scaffolding, or enzyme engineering for higher product selectivity may make this pathway more engineerable for future iterations towards a higher titer.

PABA was not detected in either strain without PABA supplementation. This may be due to natively low flux through PABA or a high rate of PABA turnover. However, while we were able to identify a homolog for PabAB (NIES39_E04160), we were unable to identify a homolog for characterized PabC proteins (de Crécy-Lagard et al., 2007; Green et al., 1992; Satoh et al., 2014) in the *A. platensis* genome. While there may be an uncharacterized PabC, there is a chance that PABA is not natively produced by *A. platensis*, which would be unexpected as PABA is a precursor for folate. Establishing and/or increasing endogenous PABA biosynthesis could increase APAP titer, or, crucially, allow for production without PABA supplementation. As the APAP yield from supplied PABA was ~0.1%, a sufficient intracellular production rate of PABA may be easily achievable to reach APAP production rates observed with supplementation. This may also alleviate the observed toxicity from PABA when supplied at high extracellular concentrations (Figure 3d). Engineering *A. platensis* for sufficient PABA production would also remove the economic burden of PABA supplementation for scale-up.

The methods we provide here pave the way for the diversification of commodity chemicals and biologics to be produced in *A. platensis* beyond APAP and the expressed proteins of Jester et al. (2022). Decisions on which molecules to produce would benefit from a combination of technoeconomic and life cycle analyses targeting high-value products such as pharmaceuticals and cannabinoids. This work further opens *A. platensis* as an engineerable, functional food. Modification of organoleptic traits and nutritional properties may lead to more palatable and nutritious strains for improved consumption by humans or for animal feedstocks. Improved genetic tools along with host optimization; for example, through the removal of restriction-modification systems, genome copy number control, and

morphological control, may further improve the engineerability of this organism. The present work is thus an important step towards realizing the potential of an industrially grown, edible, photosynthetic organism for the oral delivery of pharmaceuticals and nutritional compounds.

4 | ONLINE METHODS

4.1 | Growth and transformation of *Arthrospira platensis*

A list of strains used in this study can be found in Supplemental Table 2. WT *A. platensis* NIES-39, obtained from the NIES culture collection, was grown in Zarrouk's Medium (ZM) (Madkour et al., 2012) at 30°C under ~70–100 µmol photons $m^{-2} s^{-1}$ (µEi) of photosynthetically active radiation produced by cool, white LEDs (compact LED bars from Photon Systems Instruments controlled by their LC 200 light controller) with shaking at 200 rpm unless stated otherwise. Working streptomycin/spectinomycin (Sm/Sp) concentrations ranged from 1 to 100 µg mL^{-1}.

For electroporations, WT *A. platensis* NIES-39 cells were grown to early log phase (OD_{750} ~ 0.2–0.3), washed three times with room temperature, sterile water and concentrated to 150 µg Chl *a* mL^{-1} in sterile water. Chl *a* concentration was measured based on the methods of Meeks and Castenholz (1971). For each electroporation reaction, 400 µL concentrated cells were mixed with 10 µg plasmid on ice, transferred to a cold 2 mm electroporation cuvette, and electroporated at 600 V with a time constant of ~6–7 ms^{-1} on a BTX Gemini electroporator. Cells were recovered in a final volume of 1 mL ZM, and grown overnight (~15–20 h) at 30°C under ~20–30 µEi with shaking. The following day, cells were washed once with ZM + Sm/Sp (Torres-Tiji et al., 2020), and plated onto selective 1.5% Bacto agar (BD #214010) plates or into selective liquid ZM in multiwell (96-, 24-, 12-, and/or six-well) plates. Biomass was selectively passaged onto plates and into liquid media, and monitored via PCR and/or Illumina sequencing until fully segregated. No counter-selectable marker was used in this process.

For natural transformations, WT *A. platensis* NIES-39 cells were grown to early log phase (OD_{750} ~ 0.2-0.3) and concentrated to 150 µg Chl *a* mL^{-1} in ZM. 100 µL concentrated cells were mixed with 2.5 µg plasmid DNA, and incubated for 45 min at room temperature. Cells were recovered in a final volume of 1 mL ZM, and grown overnight (~15–20 h) at 30°C under ~20–30 µEi with shaking. Recovered cells were added to 99 mL ZM + Sm/Sp (1–10 µg mL^{-1}) in 250-mL flasks, and incubated under ~20–30 µEi until growth appeared. Biomass was subcultured under increasing concentrations of Sm/Sp (up to 100 µg mL^{-1}) until fully segregated as monitored by PCR.

4.2 | Genomic DNA extraction

gDNA was extracted as follows: 1-mL aliquots of culture were harvested by centrifugation. Pelleted cells were resuspended in 300 µL ELB (20 mM Tris–HCl, 2 mM Na-EDTA, 1.2% Triton-X). Cell suspension was incubated at room temperature for 30 min with 2500 units of Ready-Lyse lysozyme (Lucigen; #R1804M) and 200 µg RNase A (Qiagen; #19101). An additional 2500 units of lysozyme were added and incubated for 30 min at room temperature. Fifteen microliters of Proteinase K (20 mg mL^{-1}; Qiagen; #19157) were added to the suspension and incubated at 65°C for approximately 1 h. Four hundred microliters of phenol:chloroform:isoamyl alcohol (25:24:1 v/v; Sigma; #P3803) were mixed with the cell suspension, and centrifuged for 5 min at 21,130*g*. Thirty microliters of 3 M sodium acetate + 0.1 M EDTA were added to the aqueous phase, followed by 660 µL cold 100% ethanol, and centrifuged for 15 min at 21,130*g*. Supernatant was removed, and the pellet was washed once with 1 mL 70% EtOH. Following removal of the wash, the pellet was air dried for 5 min before resuspension in nuclease-free TE buffer (Thermo Fisher Scientific; #J75793).

4.3 | Next generation sequencing

Library preparation and 250 bp paired-end sequencing on an Illumina HiSeq. 4000 was completed by the QB3 Genomics, UC Berkeley, RRID:SCR_022170.

4.4 | Data analysis

Raw Illumina NovaSeq sequencing reads were processed as follows. Burrows-Wheeler Alignment tool (BWA) (Li & Durbin, 2009) was used to index the reference genome AP011615, align sequencing reads and generate a sam file. Samtools (Danecek et al., 2021) was used to convert the data to a bam file and then sort and index the alignments. Picard's DownsampleSam (https://broadinstitute.github.io/picard/) was used to downsample the BAM files to ~10% of the total depth for visualization in Geneious. Geneious was used to visualize the alignments and generate figures.

4.5 | Motility assays

ZM solidified with 0.5% agar was poured into each lane of a four-well plate. Cultures of each strain to be assayed were inoculated to a starting OD_{750} of 0.05 and allowed to grow until mid-log phase (OD_{750} = 0.5–0.7). A volume of each culture equal to 750 µL OD_{750} = 1.0 was pelleted by centrifugation and the supernatant was removed. The cell pellets were collected and dispensed at one end of an agar lane in the four-well plate. The plate was sealed and imaged immediately. The plate was moved to a 30°C room and placed directly under cool, white LEDs at a flux of 50 µEi. The half of the plate containing the cell pellets was placed in a protective covering to prevent direct exposure to light and encourage growth to the far end of the plate. The plate was imaged every 24 h for 7 days.

4.6 | Vector design and synthesis

A list of vectors can be found in Supplemental Table 2. All genes were codon optimized, purged of the restriction-modification (RM) sites (see below), synthesized, and cloned by Genscript Corp. The remaining parts were synthesized and cloned by Genscript Corp. The purged RM sites can be found in Supplemental Table 4. This list was compiled from literature and homology searches (Fujisawa et al., 2010; Roberts et al., 2015; Shiraishi & Tabuse, 2013). 1000 bp upstream and downstream of the target genes (*pilA*, NSI, or oscillin) were used as homology arms for recombination.

4.7 | Fluorescence microscopy

Mid-log phase cultures were imaged on a Zeiss Observer D1 fluorescence microscope. Chlorophyll autofluorescence was imaged under excitation at 587 nm and emission at 610 nm and eYFP fluorescence was measured under excitation at 495 nm and emission at 527 nm. Images were falsely colored using Zeiss Zen Pro version 3.5.

4.8 | Quantification of promoter strengths

A. platensis WT and mutant strains were each grown in eight wells of triplicate 96-well plates under ~20–30 µEi without shaking at 30°C. OD_{750} and eYFP fluorescence (excitation at 485 nm and emission at 535 nm) were measured daily on a Tecan Spark M10 plate reader, and these values were normalized to blank measurements.

4.9 | High-performance liquid chromatography (HPLC)

Acetaminophen (APAP) and para-aminobenzoic acid (PABA) were quantified on a Shimadzu Prominence HPLC system equipped with a photodiode array detector for UV detection. Compounds were separated on a Zorbax StableBond Plus C18 5 µm with detection at 240 nm. The elution protocol was based on a gradient method using (A) 0.1% phosphoric acid and (B) methanol as follows: 0 min − 95% A, 5% B; 15 min 100% B; 16–20 min 95% A, 5% B at a flow rate of 1 mL min^{-1}.

For sample preparation, 1 mL culture was transferred to a BeadBug bead beating tube (Millipore Sigma; #Z763748) and vortexed on high for 10 min. Samples were then centrifuged for 10 min at 14 k rpm. Eight hundred microliters of supernatant were filtered through 13 mm 0.2 µm PVDF syringe filters (Pall; #4406). Standards were prepared in volumetric flasks using APAP (Sigma; #A5000) and PABA (Sigma; #A9878), and serially diluted in ZM. The 4-aminophenol (Sigma; #A71328) standard was prepared similarly using methanol as the solvent.

AUTHOR CONTRIBUTIONS

Jacob M. Hilzinger, Jeffrey M. Skerker, and Adam P. Arkin conceived the project. Jacob M. Hilzinger, Skyler Friedline, Jeffrey M. Skerker, and Adam P. Arkin designed experimental work. Jacob M. Hilzinger, Skyler Friedline, Divya Sivanandan, Shunsuke Yamazaki, and Jeffrey M. Skerker performed experimental work. Jacob M. Hilzinger, Skyler Friedline, Douglas S. Clark, Jeffrey M. Skerker, and Adam P. Arkin analyzed data. Ya-Fang Cheng developed the HPLC method. Jacob M. Hilzinger, Skyler Friedline, Jeffrey M. Skerker, Divya Sivanandan C., Douglas. S. Clark, and Adam P. Arkin wrote the manuscript.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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