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Research Article

Synechocystis Specifically Inhibits Growth of *Ralstonia eutropha* in an Artificial Microbial Consortium

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ABSTRACT

Petroleum derived plastics are a major contributor to global pollution. There is an urgent need for biodegradable, sustainable plastic alternatives. Cyanobacteria have been studied extensively for photosynthetic production of biofuel precursors including alkanes and free fatty acids. However, large scale production has been slow to emerge from these technologies. Here, we wished to evaluate alternative uses for engineered strains of the cyanobacteria *Synechocystis* PCC 6803 (6803). We investigated the feasibility of using wild type and fatty acid secreting strains of 6803 to support the growth of *Ralstonia eutropha*. This organism is capable of producing polyhydroxyalkanoates (PHAs), which can be used in bioplastic production. Traditional feedstocks for *R. eutropha* include palm oil and other biological precursors that compete with cultivatable land, pitting potential bioplastic production against agricultural demands. Since PHAs are of great interest as plastic alternatives, we co-cultured *R. eutropha* and 6803 strains in the minimal medium BG-11 in an attempt to create carbon neutral PHA from *R. eutropha*. Surprisingly, we observed inhibition of *R. eutropha* growth in co-culture with *Synechocystis* but not another cyanobacterium suggesting further modification of *Synechocystis* is necessary for use as a feedstock.

Keywords: Microbiology; Microbial co-culture; R. eutropha; Synechocystis; Bioplastics, Cyanobacteria

INTRODUCTION

The ecological impacts of plastic waste urgently necessitate plastic alternatives including renewable bioplastics [1,2]. To this end, much work has been done to optimize polyhydroxyalkanoate (PHA) and other bioplastic precursor production by microorganisms. PHAs, including polyhydroxybuterate (PHB), are a class of microbial biomolecules that are suitable replacements for petroleum-based plastics due to their thermoplasticity and biodegradability [3]. However, the high production cost and lack of a sustainable feedstock represent significant challenges to large-scale production of these molecules [4].

The chemolithoautotroph *R. eutropha*, is a subject of biotechnological interest for production of bioplastics due to its ability to produce PHAs in response to nitrogen and/or phosphorous limitation in carbon replete conditions [5,6]. Under environmentally stressful conditions, PHA serve to store carbon in intracellular inclusion bodies [6]. However, *R. eutropha* has been manipulated to overproduce PHAs for biotechnological application. Modification of growth conditions, genetic modification, various feed stocks and co-culture have been explored to optimize PHA production in *R. eutropha* [5,7-10].

Genetic modifications resulted in strains with improved production of PHAs with more uniform molecular weights or higher yields [10]. Optimization of growth conditions has also lead to improved PHA production in *R. eutropha* through the introduction of waste oils or purified, short chain fatty acids to the growth medium [11,12]. Co-culture experiments mixing *R. eutropha* with *B. subtilis* allowed for PHA production at 45% dry cell weight using sugar cane as a carbon source [13]. Similarly, co-cultures with *Lactobacillus delbrueckii* induced *R. eutropha* to produce PHB using glucose as a carbon source [14]. Despite these advances, current, renewable feedstocks are derived from sources such as sugar cane or palm oil that compete for cultivatable land and potable water. This puts bioplastic production in direct competition with agricultural production. Large-scale production of PHAs will require feed stocks that are carbon neutral and sustainable.

Cyanobacteria represent a potential feedstock for *R. eutropha*. Several cyanobacterial species serve as model organisms for the production of useful biological products including alkanes, alcohols and sugars [15]. Of these, *Synechocystis* PCC 6803 (6803) and *Synechoccoccus elongatus* 7942 are among the best studied [16]. Modified 6803 strains have been created that demonstrated enhanced amounts of carbon sources for *R. eutropha*. For instance, the *Synechocystis*

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strain SD249 was engineered to overproduce free fatty acids at a concentration of ~146 mg/L in stationary phase [17]. A modified version of this strain, SD277, secreted ~197 mg/L of FFA. In both cases, the majority of FFAs were comprised of myristate (C12:0), laurate (C14:0), palmitate (C16:0) and oleate (C18:0) [17]. These biomolecules can serve as an organic carbon source for *R. eutropha* growth during PHA production [18,19]. Thus, we wished to see if we could utilize these strains as a sustainable carbon source for PHA production in *R. eutropha*.

Here we developed co-cultures of wild type and engineered strains of cyanobacteria with *R. eutropha* and measured cell viability of both species. Our results indicate that both wild type and FFA secreting strains of 6803 inhibit the growth of *R. eutropha* in co-culture. This effect was not observed when co-culture was carried out with *S. elongatus*. Together, these results indicate that strains of 6803 lacking this secreted inhibitor need to be developed for it to be useful as a sustainable feedstock for *R. eutropha*.

MATERIALS AND METHODS

Bacterial Co-Culture Growth Conditions

Cultures used for this study are described in Table 1. *Synechocystis* and *S. elongatus* cultures were grown to late stationary phase in BG-11 [20] with 100 µ Einsteins of light under constant illumination as described [21]. For co-culture experiments, strains of *Synechocystis* were grown to stationary phase in BG-11 with bubbled air. Cultures were back diluted in BG-11 to an OD_{730} of ~0.2.

R. eutropha grown overnight at 30°C with shaking in tryptic soy broth (TSB Fisherscientific.com). One milliliter of *R. eutropha* culture was centrifuged at 17Kxg for 5 minutes, washed three times in BG-11 and added to the *Synechocystis* cultures at a final concentration of ~1x105 CFU/ml. Serial dilutions of the culture were performed and plated onto BG-11 to assess cyanobacterial growth and tryptic soy agar (TSA Fisherscientific.com) to assess *R. eutropha* growth.

Spent Media Growth Experiments

Synechocystis cultures were grown to stationary phase as described above. One milliliter of culture was centrifuged at 17Kxg for 5 minutes. The supernatant was used as a supplement in *R. eutropha* growth assays. Specifically, *R. eutropha* was grown in Tryptic Soy Broth (TSB) overnight at 30°C with shaking. The culture was diluted to and OD_{600} of ~0.1 in 3 ml of TSB. Then, 100 µl of fresh BG-11 or spent medium from *Synechocystis* cultures were added to the culture. Growth was monitored by measuring the OD_{600} over time.

RESULTS

Given the availability of genetically modified strains of 6803, we set out to determine if this species of cyanobacteria would be a sustainable feedstock candidate for PHA production in *R. eutropha*. Therefore, we established a co-culture of wild type 6803 and *R. eutropha* (Figure 1). We observed no notable change in viability between 6803 grown in single culture or co-culture with *R. eutropha*. However, we observed that *R. eutropha* was less viable than when grown in BG-11 alone. The same strain was capable of reaching stationary phase when grown in TSB in single culture.

To determine if the lack of growth in the presence of 6803 could be overcome by the presence of exogenously produced FFA, we set up co-cultures with modified strains of 6803 that overproduce FFA. Specifically, we established co-cultures of *R. eutropha* and SD100, SD249, SD277 (Table 1) or a single culture of *R. eutropha* in BG-11 (Figure 2). We observed moderate growth of *R. eutropha* in BG-11 single culture, but cell viability decreased over the course of 7 days. However, in the presence of all three strains of cyanobacteria we observed a rapid decline in cell viability. These data indicate that the FFA producing strains were not able compensate for the inhibitory effect on *R. eutropha* growth. Intriguingly, the time to loss of viability was slightly longer in FFA producing strains.

We then wished to determine if whole cells or a secreted factor(s) were involved diminishing *R. eutropha* viability. Thus, we harvested spent BG-11 from stationary phase cultures of two different strains of 6803: SD100 and SD500 (Table 1). One milliliter of spent media was centrifuged, and 100 µl of the cell-free supernatant was added to a 3ml TSA R. eutropha culture (Figure 3). As a negative control, 100 µl of fresh BG-11 was added to a third culture. In two independent experiments, spent media from 6803 cultures inhibited *R. eutropha* growth. The culture exposed to BG-11 alone grew to an OD_{600} of ~2.5. However, the addition of spent media from either SD100 or SD500 diminished growth by at least three-fold (Figure 3). These data indicate that a soluble factor secreted by 6803 inhibits growth of *R. eutropha*.

Given the potential utility of cyanobacteria as feedstock, we wished to determine if the inhibitory effect we observed was specific to 6803 or general feature of cyanobacteria. To establish this, we set up co-cultures with another species of cyanobacteria, *S. elongatus* SAA017 [22]. We inoculated either BG-11 or BG-11 + *S. elongatus* (OD_{730} 0.2) with *R. eutropha* and measured cell viability (Figure 4). Strikingly, we did not observe the rapid loss of viable *R. eutropha* cells observed in co-cultures with SD100, SD500, SD249 or SD277. In independent experiments, we measured the number of

Table 1: Strains.

Strain	Genotype	Relevant Phenotype	Reference
SD100	PCC 6803	N/A	[28]
SD500	wild type	N/A	[29]
SD249	Δslr1609::PpsbA2'tesAΔ(slr1993-slr1994)::PcpcaccBCPrbcaccDAΔsll1951::PpsbA2U cfatB1PrbcChfatB2Δ(slr2001-slr2002)::PpsbA2ChfatB2Δslr1710::PpsbA2CcfatB1	Fatty Acid Overproduction	[18]
SD277	Δaas-23::$P_{psbA236}$ tesA136 Δ(slr1993-slr1994)-14::P_{cpc39} accB RBS accC70 P_{rbc40} accD RBS accA Δsll1951-15::$P_{psbA210}$ fatB161(Uc) P_{rbc41} fatB262(Ch) Δ(slr2001-slr2002)-17::$P_{psbA211}$ fatB262(Ch) Δslr1710-19::$P_{psbA210}$ fatB163(Cc)Δslr2132-22:: P_{trc} tesA137	Fatty Acid Overproduction	[18]
R. eutropha	Wild typed	N/A	ATCC 17699
SAA017	*Synechocystis* P_{trc}::*ldh*::Ptrc::*sth*::Kmr, L-LDH of B. subtilis, STH of *P. aeruginosa*	Sucrose over production	[22]

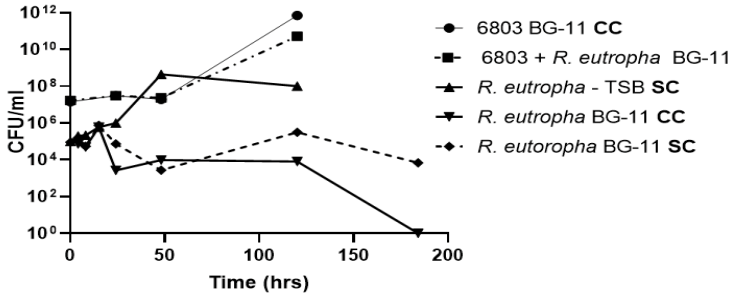

Figure 1: Co-culture with wild type 6803 decreases viability of *R. eutropha*.

Two 15ml cultures were inoculated from 6803 stationary phase cultures to a final concentration of ~1x107 CFU/ml. One of these cultures was also inoculated with *R. eutropha* to a final concentration of 1x105 CFU/ml. Another was left as a monoculture. A 15ml culture of TSB was inoculated with R. eutropha to a final concentration of 1x105 CFU/ml. Cultures were grown at 30^{0}C and bacteria were enumerated by serial dilution and plating on either BG-11 or TSA over the course of 180 hours. Data are representative of at least two experiments. SC = single culture, CC=co-culture.

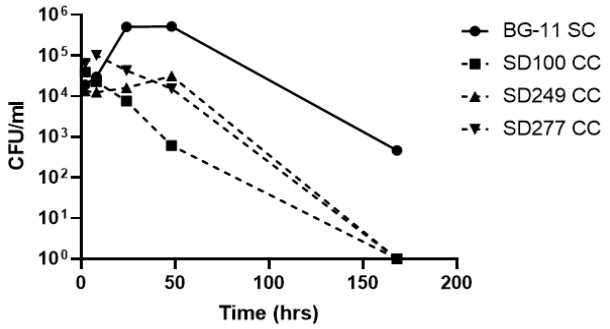

Figure 2: Co-culture with FFA secreting strains decreases viability of *R. eutropha*

R. eutropha was grown to stationary phase and used to inoculate cultures to a final density of 1x104-1x105 CFU/ml into BG-11 or BG-11 containing ~1x107 CFU/ml of SD249 or SD277. Bacteria were enumerated by serial dilution and plating on TSA plates over the course of 7 days. SC=Single Culture, CC=Co-culture.

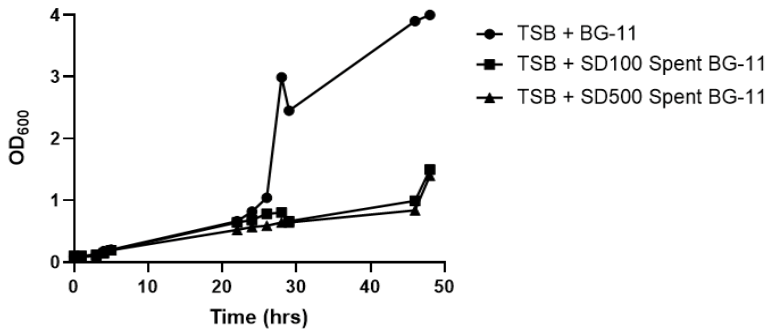

Figure 3: *Synechocystis* secretes a soluble factor that inhibits growth of *R. eutropha*

Three *R. eutropha* cultures were inoculated to a final OD_{600}. Each culture received 100µl of centrifuged, spent media from stationary phase cultures of SD100 (squares), SD500 (triangles) or fresh BG-11 (circles). Cultures were grown at 30°C with shaking. Bacteria were enumerated by serial dilution and plating on TSA over the course of 7 days. Data shown are representative of two independent experiments.

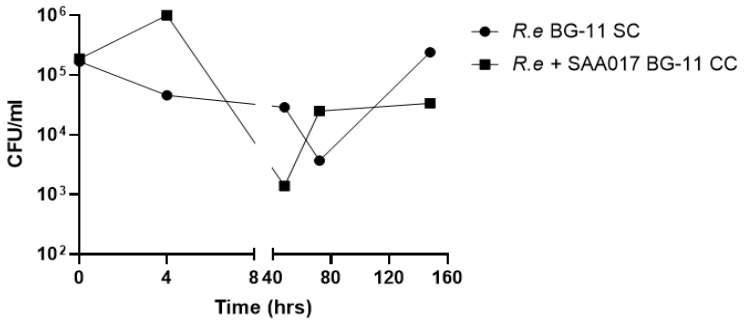

Figure 4: *S. elongatus* does not decrease viability of *R. eutropha* in BG-11

A stationary phase cultures of *S. elongatus* SAA017 was used to inoculate a 15ml culture in BG-11 to a final concentration of ~1x107 CFU/ml. ~1x105 CFU/ml *R. eutropha* was added to the cyanobacterial culture or to plain BG-11. Bacteria were grown with aeration under 100µ Einsteins of light with constant illumination. *R. eutropha* were enumerated by serial dilution and plating on TSA over the course of 7 days. Data are representative of two independent experiments. SC = single culture, CC=co-culture.

viable *R. eutropha* cells. Intriguingly, there was little change in viable cell count in the presence of *S. elongatus*. These data indicate that inhibition of *R. eutropha* growth is specific to 6803. Furthermore, they provide evidence that *S. elongatus* may be a more suitable feedstock for large scale production of PHA in *R. eutropha*.

DISCUSSION

Here we observed that 6803 inhibited growth of *R. eutropha* in co-culture when grown under optimal conditions for cyanobacterial proliferation. Both wild type and engineered strains of 6803 demonstrated this phenotype. This effect was not observed when *R. eutropha* was co-cultured with *Synechoccoccus elongatus*. We confirmed that growth inhibition was due to a soluble factor that was either actively secreted or passively shed from *Synechocystis*. Specifically, we showed that *R. eutropha* grown in TSB along with a 1:300 dilution of BG-11 from a 6803 culture showed a 3.5-fold decrease in growth.

When grown in single culture in BG-11, *R. eutropha* demonstrated an initial increase, then decrease in CFU count prior to recovery at 40 hours post inoculation (Figures 1 and 4). One explanation for the initial growth is carryover of nutrients from the inoculating culture. However, for these experiments' cells were washed three times in BG-11 prior to inoculation in fresh BG-11. Thus, it may be that *R. eutropha* was utilizing intracellular stores of energy for growth in BG-11. The exhaustion of these stores would then explain the subsequent decrease in CFU until 40 hours post inoculation. At this point we consistently observe an increase in the number of viable CFU. We postulate that this is the time required for *R. eutropha* to modulate its transcriptional profile to accommodate survival in the minimal medium BG-11. In co-culture experiments, however, we observed that after 40 hours, *R. eutropha* growth was inhibited.

The inhibitory effect on *R. eutropha* was observed with four different 6803 strains. However, the time to death was slower with SD249 and SD277 (Figure 2). The number of viable *R. eutropha* cells began decreasing within 24 hours when mixed with wild type strains of 6803. This was not observed in the FFA secreting strains until 48 hours after co-inoculation. There are two likely reasons for this delay. Both SD277 and SD249 exhibit a long lag phase [18]. To create our co-culture, we concentrated SD249 and SD277 from cultures that were at a lower cell density than SD100 or SD500. Thus, it may be that the inhibitory factor may be secreted optimally only at high cell density. Alternatively, the genetic modifications of the FFA secreting strains may decrease the production and/or secretion of the soluble inhibitor.

Although we had not anticipated the inhibitory effect of 6803 on *R. eutropha*, we wished to know if it was common to multiple species of cyanobacteria. To this end, we co-cultured *R. eutropha* with a strain of *S. elongatus*, SAA017. Although SAA017 can be induced to secrete mM concentrations of sucrose under salt stress and at high cell density [22], we did not expect SAA017 to support robust growth of *R. eutropha* for two reasons. Firstly, SAA017 was not induced for sucrose overproduction. Secondly, *R. eutropha* cannot metabolize sucrose directly [23]. However, co-culture with this strain illustrates that the deleterious effects observed when 6803 was co-cultured with *R. eutropha* is specific to *Synechocystis* and not a general feature of cyanobacteria.

A tremendous body of work has been amassed toward using cyanobacteria as biofuel generators [15,24,25]. Indeed, several engineered 6803 strains produce elevated levels of hydrocarbons including alkanes, short and long chain fatty acids [15,18,26]. Similarly, strains of *S. elongatus* were constructed to overproduce a variety of potentially useful molecules including sucrose, ethanol and gaseous hydrogen [15]. Their ability to photosynthetically produce a variety of biologically valuable molecules make cyanobacteria attractive candidates for feedstocks [25]. However, the data presented here highlight significant challenges to using *Synechocystis* in this capacity.

It is of interest to determine whether the same inhibitory effect observed with *R. eutropha* is true for other heterotrophic organisms. For instance, we wish to investigate whether 6803 co-culture systems can support the growth of heterotrophic organisms such as *K. hansenii*, which has been engineered for hyper-production of cellulose [27]. However, for *Synechocystis* to be useful in a *R. eutropha* co-culture system, strains of 6803 that do not secrete the inhibitor(s) need to be developed.

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