An expanded synthetic biology toolkit for gene expression control in *Acetobacteraceae*

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ABSTRACT

The availability of different host chassis will greatly expand the range of applications in synthetic biology. Members of the *Acetobacteraceae* family of Gram-negative bacteria form an attractive class of non-model microorganisms that can be exploited to produce industrial chemicals, food and beverage, and biomaterials. One such biomaterial is bacterial cellulose, which is a strong and ultrapure natural polymer used in tissue engineering scaffolds, wound dressings, electronics, food additives, and other products. However, despite the potential of *Acetobacteraceae* in biotechnology, there has been considerably little effort to fundamentally reprogram the bacteria for enhanced performance. One limiting factor is the lack of a well-characterized, comprehensive toolkit to control expression of genes in biosynthetic pathways and regulatory networks to optimize production and cell viability. Here, we address this shortcoming by building an expanded genetic toolkit for synthetic biology applications in *Acetobacteraceae*. We characterized the performance of multiple natural and synthetic promoters, ribosome binding sites, terminators, and degradation tags in three different strains, namely *Gluconacetobacter xylinus* ATCC 700178, *Gluconacetobacter hansenii* ATCC 53582, and *Komagataeibacter rhaeticus* iGEM. Our quantitative data revealed strain-specific and common design rules for the precise control of gene expression in these industrially relevant bacterial species. We further applied our tools to synthesize a biodegradable cellulose-chitin copolymer, adjust the structure of the cellulose film produced, and implement CRISPR interference for ready down-regulation of gene expression. Collectively, our genetic parts will enable the efficient engineering of *Acetobacteraceae* bacteria for the biomanufacturing of cellulose-based materials and other commercially valuable products.

Keywords: Bacterial cellulose, biomaterials, *Acetobacteraceae*, genetic engineering, synthetic biology, CRISPRi

INTRODUCTION

The rapid development of synthetic biology has enabled the systematic engineering of different living organisms for the production of various commercially valuable industrial chemicals and biofuels[1-2], medicinal compounds[3-5], food and beverage ingredients[6-8], and biomaterials[9-10]. In the early years, the field has focused mainly on a few host chassis, including *Escherichia coli* and *Saccharomyces cerevisiae*, for research and development due to their well-studied physiology and genetics as well as the ready availability of DNA constructs and methods for precise control of gene expression in these microorganisms[11]. However, there are many applications whereby it is advantageous to exploit the natural abilities of non-model organisms to accomplish tasks that a traditional host chassis may find difficulty in carrying out. Hence, more recent work has expanded the scope of synthetic biology research and applications into myriad non-model organisms[12-25]. One such organism is the *Acetobacteraceae* family of Gram-negative bacteria. These microbes play important roles in the production of acetic acid or vinegar, cocoa, keto-L-gulonic acid (a vitamin C precursor), the natural sweetener D-tagatose, shikimate (a key intermediate in the synthesis of various antibiotics), kombucha tea, and different polysaccharides, in particular cellulose.[26-27]

Many members of *Acetobacteraceae*, especially those in the *Gluconacetobacter* or *Komagataeibacter* genus, are capable of producing large quantities of bacterial or microbial cellulose.[28] The core proteins that are used to produce cellulose are encoded in the *acs* (*Acetobacter* cellulose synthase) operon.[29,30] Briefly, cellulose nanofibers are synthesized by the combined action of AcsA (the catalytic subunit) and AcsB (the regulatory subunit that binds the activating cofactor c-di-GMP) using UDP-glucose as a substrate. The fibers are then secreted through pores formed by AcsC with assistance from AcsD, forming pellicles around the cells. While the functional role of bacterial cellulose is currently unclear, it has been proposed that the pellicle helps to (1) maintain these obligate aerobes near the surface of the culture medium where oxygen concentration is highest and (2) protect the bacteria against ultraviolet light and competitors during colonization.[28]

Bacterial cellulose offers several advantages as a starting material for different biotechnology applications. First, it is ultrapure and free from chemical contaminants such as lignin and pectin, which are found abundantly in plant cellulose.[31] Second, it

possesses exceptional mechanical strength,[32,33] due to its ability to self-assemble into a continuous highly interconnected lattice.[30] Third, it can be readily modified in its crystalline structure and combined or functionalized with other materials to form advanced fiber composites.[30, 34] Fourth, it is biocompatible and can be further tailored to be biodegradable *in vivo*, thereby lending itself to medical applications.[35-36] Currently, bacterial cellulose has been used in wound dressings and artificial skin,[30] man-made blood vessels,[35] tissue engineering scaffolds,[36,37] food items,[38] acoustic speakers,[30] electrodes,[39] sensors,[40] and other products.[30,34]

Due to its commercial value, numerous studies have been performed to understand and increase bacterial cellulose production in *Acetobacteraceae*. Most studies have focused mainly on optimizing medium composition, culturing conditions, isolation of novel strains, and bioreactor design.[30, 41] In contrast, there are comparatively fewer reports on the use of genetic engineering techniques to enhance cellulose production,[42-48] despite the availability of several ad hoc plasmids that can function as gene expression systems in different acetic acid bacteria.[27] The main reasons for this are the absence of whole genome information and the lack of a comprehensive, well characterized genetic toolkit for systematic strain engineering. Fortunately, new efforts have been made to address the problems. Genome sequences of two *Acetobacteraceae* family members, namely *Gluconacetobacter hansenii* ATCC 53582[49] and *Komagataeibacter rhaeticus* iGEM,[50] have recently been published. Moreover, a genetic toolkit comprising various plasmids, reporter proteins, synthetic promoters, and inducible promoters have been systematically assembled together for the first time and characterized in the *K. rhaeticus* iGEM strain.[50]

Here, we aim to expand upon the first genetic toolkit for *Acetobacteraceae* by adding and characterizing more parts that can better enable us to precisely control gene expression in multiple cellulose producing strains. We characterized a range of natural and synthetic parts that can regulate gene expression at the transcriptional (promoters and terminators), translational (ribosome binding sites or RBS), and post-translational (degradation tags) levels. With this expanded toolkit, researchers can readily mix-and-match different parts together in order to optimize gene expression and protein production. We envision that our toolkit can also be used to rationally reprogram members of the *Acetobacteraceae* family for other biotechnology applications besides the biomanufacturing of bacterial cellulose.

RESULTS AND DISCUSSION

Characterization of constitutive promoters in *Acetobacteraceae*.

We performed our part characterization in three different bacterial strains, namely *Gluconacetobacter xylinus* ATCC 700178, *Gluconacetobacter hansenii* ATCC 53583, and *Komagataeibacter rhaeticus* iGEM. *G. hansenii* ATCC 53583 and *K. rhaeticus* iGEM were selected because both strains are known to be capable of producing high yields of cellulose[49,50]. In addition, cellulose synthesized by different strains or species can have different structures or properties that may affect downstream processing steps or applications. Therefore, since the properties of the cellulose produced by *G. xylinus* ATCC 700178 under various conditions have been extensively investigated,[51-52] we also included this strain in our study to provide bioengineers with another choice of cellulose-producing candidate.

Promoter is one of the main tools in synthetic biology, which can be manipulated to tune gene expression. The strength of a promoter is specified by its precise sequence composition.[53-55] We selected ten constitutive synthetic minimal *E. coli* promoters from an open-access collection known as the Anderson family for testing (Figure 1a). Each promoter is 35 basepairs (bp) long. We included the strongest member in the family J23119, which contains the -10 (TATAAT) and -35 (TTGACA) consensus sequences. An additional new variant was created by accident during the cloning process and differed from J23119 by only a single nucleotide at the 27th position. Hence, we labeled this new promoter as J23119-A27T.

We evaluated the ability of each of the 11 promoters to activate the expression of a mRFP1 reporter gene (Figure 1b). Although five of these promoters have previously been examined in the *K. rhaeticus* iGEM strain,[50] the remaining promoters have never been tested in any *Acetobacteraceae* bacteria, including J23119. We normalized the strength of each promoter, quantified by the fluorescence intensity per unit OD, to that of the J23119 construct. Overall, we observed that the promoters showed a bimodal distribution (Figure 1c-e). In all the three strains tested, J23104, J23119-A27T, J23102, J23119, and J23100 are strong promoters, while the remaining ones are weak promoters. Notably, unlike *E. coli*, we found that J23119 is not the strongest Anderson promoter in *Acetobacteraceae*. Instead, J23104, J23102, and J23119-A27T are consistently stronger than J23119. Nevertheless, the general trend in promoter strength is similar between *Acetobacteraceae* and *E. coli* – strong

promoters in *Acetobacteraceae* also tend to be strong in *E. coli* and vice versa – although there are some differences in detail between the two families of bacteria (Supplementary Figure S1).

Interestingly, our data revealed that the first 3 bases of the promoter sequence (within the -35 box) play an important role in regulating gene expression in *Acetobacteraceae*. The nucleotides 'T' (thymine) and 'G' (guanine) at positions 1 and 3 respectively are particularly crucial, as both of them are present in all the strong promoters, but absent in all the weak promoters. For example, J23108 differs from J23119 by only one nucleotide at the first position, but its promoter strength is significantly less than that of J23119 in all the three strains tested ($P < 0.001$, Student's t-test). Similarly, J23101 differs from J23119-A27T by only a single nucleotide at the third position, but its promoter strength is at least five-fold less than that of J23119-A27T. In contrast, several nucleotides in the -10 box (underlined in TATAAT) can be mutated without affecting the promoter strength appreciably. Collectively, we have characterized a series of synthetic constitutive promoters in three *Acetobacteraceae* strains and identified the key residues that mediate promoter strength in the bacteria.

Characterization of inducible promoters in *Acetobacteraceae*.

We may want to turn on the synthesis of bacterial cellulose only when necessary for two reasons. First, the bacteria can be cultured and manipulated more easily in the absence of a cellulose pellicle. Second and more importantly, constant production of large quantities of cellulose can impose an undesirably high metabolic burden on the bacteria. Previous studies found that growth of *G. xylinus* under agitated and aerated conditions could lead to the formation of spontaneous cellulose-negative mutants that eventually dominated the culture and caused a reduction in cellulose yield.[56-57] Hence, in order to address these issues, inducible promoters may be used to control the expression of key genes in the cellulose synthesis pathway such as the *acs* operon, so that the biopolymer is only produced after the initial engineering and scaling up phases.

We wondered what inducible promoters may be deployed in *Acetobacteraceae* for the large-scale production of bacterial cellulose. A recent report described the performance of two inducible promoters (P_{Lux} and P_{Tet}) in the *K. rhaeticus* iGEM strain.[50] The P_{Lux} promoter was found to be stronger and less leaky than the P_{Tet}

promoter. However, the chemical inducer of the P_{Lux} promoter, *N*-Acyl homoserine lactone (AHL), is rather expensive and is not cost-effective. Hence, we sought to identify new inducible promoters that are functional in *Acetobacteraceae* and also economically cheaper to use, so that they may serve as a commercially viable option for the industry to produce large amounts of bacterial cellulose.

One potential candidate is the P_{Bad} promoter, which is widely used as an expression part in synthetic biology. Together with the AraC protein, it allows for robust external control of gene expression with the arabinose sugar.[58] Importantly, arabinose is much cheaper than AHL. However, P_{Bad} has never been characterized in *Acetobacteraceae*. Hence, we created two inducible promoter constructs (P_{Bad} and P_{Lux}) and evaluated their performance in *G. xylinus* 700178, *G. hansenii* 53582, and *K. rhaeticus* iGEM using mRFP1 as a reporter. P_{Lux} was included for us to compare P_{Bad} against.

To first characterize our P_{Lux} construct, we used 1 μM AHL to induce mRFP1 expression in the three strains. Consistent with the work of Florea et al.,[50] we observed a significant 26- to 36-fold increase in fluorescence intensity upon addition of the small molecule ($P < 0.005$, Student's t-test) (Figure 2a). Additionally, the fluorescence levels in the three strains were not significantly different from one another in the presence of AHL ($P > 0.2$, Student's t-test), indicating that P_{Lux} can serve as a robust inducible promoter in *Acetobacteraceae*.

Next, we sought to characterize our P_{Bad} construct. We noted that glucose is known to repress the P_{Bad} promoter[58] but it is also the carbon source of our culture media. Although we may reduce the concentration of glucose in the media to allow for higher induction rates, this might reduce cell growth or compromise the cellulose yield. Hence, we aimed to determine the optimal concentration of arabinose that we should use to induce mRFP1 expression at a fixed 2% (w/v) concentration of glucose. We tested three different arabinose concentrations and found that 4% arabinose yielded the highest fluorescence intensity per unit OD in all the bacterial strains used (Figure 2b-d). At this inducer concentration, we obtained an 11-fold increase in mRFP1 expression for the *K. rhaeticus* iGEM strain but only a 5-6-fold increase for the other two *Gluconacetobacter* strains. Although P_{Bad} appears to be not as strong as P_{Lux} upon induction, we noted that the P_{Bad} promoter is significantly less leaky than the P_{Lux} promoter in *Acetobacteraceae* ($P < 0.001$, Student's t-test) (Supplementary Figure S2).

We asked whether the glucose in our culture media may be substituted by other carbon sources. We grew the bacteria strains in HS (Hestrin–Schramm) media supplemented with 2% glucose, 2% sucrose, or 2% fructose and characterized our P_{Bad} construct using the same mRFP1 reporter assay. We found that replacement of glucose with sucrose did not improve the performance of P_{Bad} in any of the strains tested. Instead, replacement of glucose with fructose significantly improved the performance of P_{Bad} in the two *Gluconacetobacter* strains ($P < 0.05$, Student's t-test) (Figure 3a-b). Specifically, when fructose is used as the carbon source, addition of arabinose resulted in a 12-fold and 9-fold induction of mRFP1 expression in *G. xylinus* 700178 and *G. hansenii* 53582 respectively. However, P_{Bad} performed significantly worse in the *K. rhaeticus* iGEM strain when fructose was used instead of glucose ($P < 0.01$, Student's t-test) (Figure 3c).

Presence of arabinose in the culture media might affect the production of cellulose. Arabinose itself is a type of pentose sugar and may compete with the original intended carbon source for enzymes in the cellulose biosynthesis pathway. Hence, we investigated the effect of 4% arabinose on cellulose production, when glucose (Figure 4a), sucrose (Figure 4b), or fructose (Figure 4c) was used as the intended carbon source. Cellulose from uninduced and arabinose-induced cultures were washed, dried, and weighed. Overall, we found that addition of 4% arabinose to cultures containing glucose or fructose as the carbon source did not affect cellulose yield in *Acetobacteraceae* appreciably. However, when sucrose was used as the carbon source instead, addition of arabinose led to a significant loss of cellulose yield by more than 30% in two of the three strains tested ($P < 0.05$, Student's t-test). Arabinose has been reported to inhibit intestinal sucrase activity *in vitro* and reduce blood glucose in human when L-arabinose was added in sugar beverages[59]. Hence, arabinose might be affecting hydrolysis of sucrose in *Acetobacteraceae*, resulting in the lower cellulose yields observed.

Taken together, our results indicate that P_{Bad} is a viable inducible promoter for the control of gene expression in different *Acetobacteraceae* family members. When the P_{Bad} promoter is used, fructose may be a better choice of carbon source for *G. xylinus* 700178 and *G. hansenii* 53582 because it allows for appreciably higher induction rates than glucose or sucrose. For the *K. rhaeticus* iGEM strain, glucose at 2% concentration can still serve as the carbon source because it is not sufficient to prevent 4% arabinose from effectively switching on the P_{Bad} promoter.

Characterization of natural and synthetic terminators in *Acetobacteraceae*.

An efficient or strong terminator is crucial for stopping transcription of DNA by RNA Polymerase (RNAP) in order to avoid interference with the next transcription unit. It is particularly important in synthetic biology to have a wide selection of terminators, as a few terminators may be required in a genetic circuit and can lead to homologous recombination if one terminator is used repetitively.[60-61] To locate useful parts for *Acetobacteraceae*, we selected a set of ten terminators (five natural intrinsic terminators and five synthetic terminators) that have been found to be strong in *E. coli* and do not share a contiguous sequence longer than 25 bp[61] for screening in this study (Figure 5a). To perform our experiments, we inserted each candidate between two fluorescent reporter genes (mCherry and GFP) on a plasmid (Figure 5b).[61-62] A single strong constitutive promoter, J23104, was used to drive expression of the cassette.

Terminator strength was quantified by the relative fluorescence intensities of mCherry and GFP. A strong terminator between the two reporter genes would give rise to a high red fluorescent intensity but a low green fluorescent intensity. We tested all the terminators in *G. xylinus* 700178, *G. hansenii* 53582, and *K. rhaeticus* iGEM. Since the BBa_B0010 natural terminator is commonly used in synthetic constructs due to its strength,[63] we included it as a benchmark to compare the other terminators against. We also have a negative control plasmid that does not contain a terminator between mCherry and GFP. Overall, we observed that rankings of the terminators, based on how well they prevented downstream GFP expression, differed between the three strains (Figure 5c-e). However, we were able to identify six strong terminators from our assays, where mCherry expression was at least seven-fold higher than GFP expression in all the three strains tested. One of them, ECK120010799, consistently exhibited a higher terminator strength than BBa_B0010. Notably, our data revealed three weak terminators (namely ECK120051401, L3S2P24, and L3S2P44) that are not suitable for use in *Acetobacteraceae*, despite being identified as strong terminators in *E. coli*.[61] We further confirmed our fluorescence measurements from the spectrophotometer with microscopy experiments (Supplementary Figure S3). Taken together, we have identified six strong terminators (namely ECK120033736, ECK120033736, ECK120010799, BBa_B0010, L3S2P21, L3S3P21, and L3S1P47) that may be used simultaneously in *Acetobacteraceae* because they have sufficient sequence divergence to minimize homologous recombination.

Characterization of ribosome binding sites in *Acetobacteraceae*.

The ribosome binding site (RBS) is a sequence of nucleotides upstream of the start codon of an mRNA that plays a fundamental role in the initiation of translation in prokaryotes. The 16S rRNA binds to the Shine-Dalgarno sequence, which is central to the RBS and has a consensus sequence AGGAGG, by complementary base pairing and then recruits other subunits of the ribosome to the transcript to begin translation. Since translation initiation is believed to be the most highly regulated step of protein synthesis in prokaryotes,[64] we sought to determine the properties of an optimal RBS in *Acetobacteraceae*.

We screened a library of RBS mutants in *G. xylinus* 700178, *G. hansenii* 53582, and *K. rhaeticus* iGEM using a GFP reporter assay (Figure 6a). As the BioBrick part BBa_B0034 has been widely deployed in synthetic genetic circuits,[65] we used it as a reference and mutated each nucleotide of this RBS to every other possible base. Since this RBS is 12 bp long, we generated a total of 36 novel RBS mutants (Figure 6a and Supplementary Table S1) for testing. The GFP fluorescence intensity that arose from each mutant construct was normalized by that from the original reference plasmid. Readings that are above one indicate that the mutant RBS is stronger than BBa_B0034 and vice versa.

Our data revealed some common features of an optimal RBS among the three strains (Figure 6b-d and Supplementary Figure S4). First, the two guanines ('G') at positions 6 and 7, which are part of the Shine-Dalgarno sequence, are critical for function. Changing them to any other nucleotide significantly reduces GFP expression by at least four-fold ($P < 0.001$, Student's t-test). Second, the nucleotides at positions 4 ('G'), 5 ('A'), and 8 ('A') are also important. Mutating them to any other base generally reduces RBS strength, although to a smaller extent than changing the nucleotides at positions 6 and 7. Third, for position 9, changing the original 'G' to any pyrimidine, namely cytosine ('C') or thymine ('T'), significantly reduces GFP expression by two-fold or more ($P < 0.001$, Student's t-test). However, mutating the 'G' to 'A', both of which are purines, does not have a negative effect on the ribosome strength. This indicates that both A or G are equally favored at position 9 and that there can be non-canonical base pairing between the RBS and the anti-Shine-Dalgarno sequence in the 16S rRNA.[66] Fourth, we found that the nucleotides at positions 11 and 12, both of which are 'A', are sub-optimal. Mutating them to any

other base clearly improves RBS strength, particularly at position 11 where the overall increase in GFP expression is at least two-fold. Fifth, we observed three other specific mutations that can also improve RBS strength in *Acetobacteraceae*, namely a nucleotide change from 'A' to 'C' at position 2, a nucleotide change from 'A' to 'G' at position 3, and a nucleotide change from 'A' to 'G' at position 10. The mutation at position 10 is particularly noteworthy because it creates the perfect Shine-Dalgarno sequence within the RBS.

Taken together, our results suggest that the optimal RBS in *Acetobacteraceae* is C-G-G-**A-G-G-A-G**/A-**G**-B-B. We verified our spectrophotometer measurements by checking the cells under the fluorescence microscope (Supplementary Figure S5). Like *E. coli*, this family of bacteria does exhibit a preference for the consensus Shine-Dalgarno sequence (highlighted in bold). While we are able to decipher design rules for 11 out of the 12 nucleotides, there is no clear base preference for the first position. Changing the original 'A' to 'G' at this position increases GFP expression by approximately 3.5-fold in *G. xylinus* 700178, but decreases GFP expression by approximately 5-fold in *G. hansenii* 53582 instead. Nevertheless, our library of RBS mutants has enabled us to identify variants that are markedly stronger than the commonly used BBa_B0034 genetic part. In contrast, when we tried to apply the same library to a different unrelated host chassis, *Shewanella oneidensis* MR-1, the increase in GFP expression did not exceed 2-fold for any of the mutants tested (Supplementary Figure S6).

Since some single mutations could increase GFP expression in our reporter assay, we asked whether combining two of them together could further enhance the strength of the RBS. We selected four single RBS mutations (1AG, 2AC, 10AG, and 11AC) that exhibited more than a two-fold increase in GFP expression in at least one strain for further testing. At position 11, we focused on the nucleotide change from 'A' to 'C' because it gave the largest average increase in GFP expression, although changing 'A' to either 'G' or 'T' could also more than double the RBS strength. We then paired 11AC with each of the other mutations and evaluated the double mutants in all three *Acetobacteraceae* strains (Figure 6e-g). Combining 10AG with 11AC did not alter the RBS strength appreciably. In contrast, combining either 1AG or 2AC with 11AC led to a clear increase in GFP expression. The result for the 1AG 11AC double mutant was unexpected because 1AG alone did not have a positive effect on

RBS strength in the *G. hansenii* 53582 and *K. rhaeticus* iGEM strains (Figure 6c-d) but yet could boost protein production when combined with the 11AC mutation.

Subsequently, we generated a new RBS variant with three mutations, namely 1AG, 2AC, and 11AC. The strength of this triple mutant was significantly higher than any of the corresponding single mutants ($P < 0.05$, Student's t-test), but was not significantly different from the 2AC 11AC double mutant (Figure 6e-g). Notably, both the RBS triple mutant and the 2AC 11AC double mutant are four- to six-fold stronger than the reference BBa_B0034 genetic part. Taken together, we have generated a series of RBS variants with different strengths that allow us to control target protein levels in *Acetobacteraceae* over a ~50-fold dynamic range.

Characterization of protein degradation tags in *Acetobacteraceae*.

Synthetic genetic circuits, such as the repressilator,[65] commonly rely on the precise modulation of protein levels in the cell. When decreased protein levels are needed, one can switch off transcription of the encoding gene. However, some proteins are long-lived and can persist over multiple generations. To circumvent this problem, one can fuse a degradation tag to the coding sequence in order to earmark the targeted protein for proteolysis.

The most widely used protein degradation tag in synthetic biology is arguably the ssrA tag. In *E. coli*, when a ribosome is stalled on a transcript, the tmRNA system binds to the ribosome and then elongates the C-terminus of the nascent polypeptide with a specific, short peptide tag by switching the ribosome from the transcript to the RNA encoding the tag, thereby rescuing translation.[67-68] This short peptide is known as the ssrA tag and has the sequence AANDENYALAA. Subsequently, the ssrA-tagged protein will be directed to undergo degradation by the ClpAP or ClpXP protease. Hence, the tmRNA system and the ssrA tag perform quality control functions to prevent the accumulation of truncated, non-functional proteins.

In our study, we sought to determine whether the natural *E. coli* ssrA tag and several reported variants[69-70] (Figure 7a) may be utilized in *Acetobacteraceae* to modulate protein levels. Our evaluation platform consisted of a mCherry reporter gene under the control of a strong constitutive promoter, J23104 (Figure 7b). Each candidate degradation tag was fused to the C-terminus of the reporter. The constructs were introduced into *G. xylinus* 700178, *G. hansenii* 53582, and *K. rhaeticus* iGEM

and the measured fluorescence intensity was normalized to that of the untagged control strain.

Overall, we found that the different ssrA tags promoted degradation of the mCherry reporter to variable extents (Figure 7c-e). The most effective tags were the original peptide and a variant with the last three residues changed from LAA to LVA. The fluorescence intensities of mCherry-LAA and mCherry-LVA were less than 40% that of the untagged mCherry protein in *G. xylinus* 700178 and *G. hansenii* 53582. Interestingly, we observed that the DAS and the DAS+2 tags, which were designed to increase the dependence of degradation on a SspB adaptor protein,[70] exhibited only moderate strength in the two *Gluconacetobacter* strains (Figure 7c-d). The fluorescence intensities of mCherry-DAS and mCherry-DAS+2 were around 40-80% that of the untagged mCherry reporter. This result suggests that the level of SspB may not be very high in the tested bacterial strains or that the adaptor protein is not well conserved between *E. coli* and *Gluconacetobacter*. Additionally, we found that all the ssrA tags exhibited at most a weak effect in the *K. rhaeticus* iGEM strain (Figure 7e). Although the degradation profiles were similar among the different *Acetobacteraceae* strains, none of the examined tags were able to reduce mCherry fluorescence by more than 25% in *K. rhaeticus* iGEM. This suggests that the endogenous proteases or adaptor proteins in *K. rhaeticus* iGEM have diverged sufficiently from those in *E. coli* such that they are less able to recognize the *E. coli* ssrA tag. Taken together, our results are consistent with previous data obtained in *E. coli*[69-70] and indicate that the various ssrA tags can be used to effectively modulate protein levels at least in *G. xylinus* 700178 and *G. hansenii* 53582.

Synthesis of a cellulose-chitin copolymer

Bacterial cellulose can be combined with other materials, such as collagen or gelatin, to form nanocomposites with improved mechanical, electrical, or biological properties.[71-73] In particular, previous studies have demonstrated that in the *G. xylinus* ATCC 10245 strain, *N*-acetylglucosamine (GlcNAc) may be incorporated into cellulose fibres during biosynthesis to form a cellulose-chitin copolymer that is susceptible to degradation in the mammalian body, since the bacteria's cellulose synthase can use both UDP-glucose and UDP-*N*-acetylglucosamine (UDP-GlcNAc) as substrates.[74-76] However, the incorporation rate is extremely low because *G. xylinus* cannot convert GlcNAc to activated UDP-GlcNAc efficiently. Hence, to enhance this

conversion, three genes from *Candida albicans* for UDP-GlcNAc synthesis (NAG5, AGM1, and UAP1) have to be heterologously expressed in the bacteria (Figure 8a).[76]

To demonstrate the utility of our genetic toolkit, we explored the formation of the cellulose-chitin composite in *G. xylinus*. We sought to address several questions. First, could the same strategy be implemented in a different strain from ATCC 10245? Second, in earlier work, besides ectopic expression of the three *Candida* genes, the authors also inactivated the *nagA* gene because it enabled the bacteria to divert GlcNAc away from the chitin biosynthetic pathway into the glucose assimilation pathway instead.[76] However, absence of NagA had been found to have a negative impact on cell growth.[77-78] Hence, could the cellulose-chitin composite be robustly produced simply by expressing the *Candida* genes without knocking out the *nagA* gene? Third, could we generate modified cellulose with variable chitin content, which would be expected to exhibit variable degrees of biodegradability?

We cloned the three *Candida* genes (NAG5, AGM1, and UAP1) under the control of a single promoter so that they formed an operon (Figure 8b). The gene fragments were amplified from *Candida* genomic DNA and assembled into the pSEVA331Bb plasmid backbone[50] by Gibson assembly.[79] Based on our characterization data (Figure 6b), we selected a strong RBS for each of the genes to ensure robust translation. Moreover, to enable us to tune the rate of GlcNAc incorporation, we generated a low expression plasmid using the J23101 promoter and a high expression plasmid using the J23104 promoter (Figure 1c).

Subsequently, we transformed our NAG5-AGM1-UAP1 expression plasmids into wildtype *G. xylinus* ATCC 700178 (with the *nagA* gene intact) and cultured the cells with either glucose or GlcNAc as the carbon source. Consistent with our earlier results (Figure 4a), the bacteria produced more than 1mg/ml cellulose in the presence of 2% glucose, regardless of which additional plasmid they carried. However, when the glucose was substituted completely with GlcNAc, the cellulose yield dropped by more than 10-fold for all tested strains. Nevertheless, the yield recovered to intermediate levels when the cells were grown in a mix of 1% glucose and 1% GlcNAc. Interestingly, we further observed that the strain carrying the high expression plasmid always formed larger cellulose pellicles than the strain carrying the low expression plasmid, irrespective of the carbon source (Figure 8c).

Since the lectin wheat germ agglutinin (WGA) binds specifically to GlcNAc residues, we checked for the presence of GlcNAc in our polymeric films by staining

them with fluorescent dye-conjugated WGA (Figure 8d). As expected, the lectin failed to bind to all films produced with a 2% glucose feed. Additionally, no GlcNAc was detected in the cellulose pellicle produced by wildtype *G. xylinus* cells that were cultured in 1% glucose and 1% GlcNAc. Moreover, these wildtype cells did not even produce a visible pellicle with only 2% GlcNAc as the carbon source. In contrast, our engineered strains produced polymeric films that were bound by fluorescent WGA in the presence of 2% GlcNAc. The lectin staining was visibly brighter for the film synthesized by cells carrying the high expression (J23104) plasmid than that synthesized by cells carrying the low expression (J23101) plasmid. Even with a mixed 1% glucose and 1% GlcNAc feed, the polymeric film synthesized by cells expressing high levels of the *Candida* genes was also clearly stained by lectin, although less brightly than when the same cells were grown in 2% GlcNAc. Taken together, our results show that it is possible to control the rate of GlcNAc incorporation into the cellulose fibres by tuning the expression of NAG5-AGM1-UAP1 or by adjusting the concentration of GlcNAc monomers in the culture media. Furthermore, our data indicate that the strategy of heterologously expressing *Candida* genes for UDP-GlcNAc synthesis is viable for different *G. xylinus* strains and that the *nagA* gene does not need to be inactivated for the strategy to work.

Modulation of the bacterial cellulose biosynthetic pathway

In a recent structural study, the AcsD subunit from *G. hansenii* ATCC 23769 was found to assemble into an octamer with central pores that formed four inner passageways to facilitate the extrusion of four distinct glucan chains in cellulose production.[80] Importantly, the N-terminus of AcsD was shown to be particularly important for binding to the glucan chains. Deletion of the first six amino acids dramatically reduced cellulose yield, as Lys6 (together with Asp9 and Thr11) were predicted to form hydrogen bonds with the polysaccharide.

As deletion of the first six amino acids did not interfere with the oligomerization of AcsD,[80] we hypothesized that the truncated mutant would be able to exert a dominant negative effect on the octamer complex and we may use it to modulate the process of cellulose biosynthesis. Hence, to evaluate our hypothesis, we first cloned this dominant negative AcsD (dnAcsD) under the control of a strong promoter J23104 (Figure 1). To ensure complete loss of binding to the glucan chains, we introduced an additional Thr11Ala mutation along with the deletion of the first six

amino acids. Furthermore, we also cloned the full-length wildtype AcsD under J23104, which we could utilize to compare the dnAcsD construct against. We used the sequence of AcsD from the *G. hansenii* ATCC 53582 strain, since the AcsD protein from a *G. hansenii* strain was investigated in previous structural work.[80]

Next, we transformed our set of plasmids into the *G. hansenii* 53582 strain. The original J23104-mRFP1 construct (Figure 1) was used as a control. When we examined the colonies that formed on our solid agar plates, we observed a clear phenotype (Figure 9a). For the cells expressing mRFP1 or wildtype AcsD, the colonies were hard as expected due to the secretion of a dense cellulose matrix. Hence, each bacterial colony could be readily picked off the plate in its entirety. However, for the dnAcsD-expressing cells, the colonies were soft and it was not possible to pick a whole colony off the plate. Similar observations were made when we used another strong promoter J23119 to express mRFP1, wildtype AcsD, and dnAcsD (Supplementary Figure S7).

Subsequently, we measured the amount of cellulose produced by the different bacterial strains (Figure 9b). Compared to control, the cellulose yield of the cells overexpressing wildtype AcsD was around 13% higher. In contrast, the cellulose yield of the cells overexpressing dnAcsD was around 15% lower instead. Our results are consistent with previous studies that inactivated the AcsD subunit and found a drop in cellulose productivity as well.[29, 80-81]

We performed additional characterization of the cellulose pellicles using the scanning electron microscope (SEM) (Figure 9c). Based on the images, we observed that the cellulose fibres from cells overexpressing wildtype AcsD appeared to be denser than those from control cells. Strikingly, the SEM images also revealed that the cellulose film produced by the dnAcsD-overexpressing cells consisted of thinner fibres than those produced by the other bacterial strains. Analysis of fibre thickness using the image processing software ImageJ further confirmed these observations (Figure 9d). Hence, the presence of dnAcsD might disrupt the crystallization of thicker subelementary fibrils after glucan chain extrusion. Taken together, the SEM data are in agreement with our prior measurements of cellulose yields (Figure 9b). Future work can involve tailoring the structure of the cellulose film by tuning the ratio of wildtype AcsD to dnAcsD in the bacteria and can also involve investigating the mechanical properties of the altered bacterial cellulose.

Implementation of CRISPR interference

The ability to readily knock down the expression of any gene is useful not just for basic biomedical research but also for synthetic biology applications. One key technology for doing so in bacteria is CRISPR interference (CRISPRi), which relies on a catalytically dead Cas9 protein to block transcription.[82] To our knowledge, this technology has so far not been demonstrated in the *Acetobacteraceae* family of Gram-negative bacteria. Hence, we wondered if CRISPRi could be implemented in *Acetobacteraceae* for robust sequence-specific gene repression.

We sought to generate an all-in-one vector that could be easily reprogrammed to target any genomic locus-of-interest (Figure 10a). We employed the Cas9 enzyme from *Streptococcus pyogenes* because it is well characterized, exhibits robust activity, and possesses a broad targeting range.[83-85] First, we cloned a Cas9-3xFLAG fusion gene under the control of a strong promoter J23104 (Figure 1). Next, we introduced a single guide RNA (sgRNA) expression cassette into the same pSEVA331Bb plasmid backbone.[50] The cassette included a BsmBI-dependent cloning site for the spacer sequence. Additionally, we utilized the native tracrRNA promoter from *S. pyogenes* to drive the expression of the sgRNA. However, there may be potential disparities in the promoter strength between *S. pyogenes* and *Acetobacteraceae*. Hence, following a previous report,[86] we compensated for such possible differences by cloning the sgRNA expression cassette just downstream of the Cas9 gene so that the two were polycistronic. Lastly, we employed a strong terminator BBa_B0010 (Figure 5) to stop transcription of the CRISPR-Cas9 system.

As a proof-of-concept, we targeted the endogenous *acs* operon in *G. hansenii* ATCC 53582, since the genome sequence of this strain was available.[49] We designed two different sgRNAs, one targeting the start of the *acsAB* gene and the other targeting the start of the *acsD* gene (Figure 10b). After transforming our plasmids into the bacterial strain, we assayed the expression of *acsAB* by quantitative real-time PCR (qRT-PCR). For the former sgRNA, we observed a significant decrease in *acsAB* expression by more than two-fold ($P < 0.001$, Student's t-test), while for the latter sgRNA, there was no significant change in expression as expected (Figure 10c). We further analysed the cellulose yield of our transformed bacterial strains. For the *acsAB*-targeting sgRNA, the yield decreased significantly by around 15% ($P < 0.005$, Student's t-test), while for the *acsD*-targeting sgRNA, the yield only dropped by around 5% (Figure 10d). The modest changes in cellulose productivity could be due

to incomplete repression of gene expression or due to compensatory effects from two other putative *acsAB* genes identified in the genome of *G. hansenii* ATCC 53582.[49] Future work can involve optimizing the location of the sgRNA and multiplexed targeting of all annotated *acsAB* genes in the genome. Taken together, our results indicate that CRISPRi can be successfully deployed in *Acetobacteraceae* for programmable regulation of gene expression.

In conclusion, we have expanded the genetic toolkit for *Acetobacteraceae* by characterizing a variety of constitutive and inducible promoters, terminators, ribosome binding sites, and protein degradation tags. We generated quantitative data on how these components behave in three different strains. Importantly, the data will allow fine-tuning of gene expression at the transcriptional, translational, and post-translational levels. Our collection of genetic parts is a valuable resource for synthetic biologists who are interested in engineering *Acetobacteraceae* family members for the biomanufacturing of cellulose and potentially other commercially valuable products.

MATERIALS AND METHODS

Bacterial strains and media.

G. xylinus ATCC 700178 and *G. hansenii* ATCC 53582 were purchased from ATCC (cat. number ATCC® 700178™ and ATCC® 53582™ respectively), while *Komagataeibacter rhaeticus* iGEM was a generous gift from Tom Ellis (Imperial College London). Strains and plasmids used in this study are listed in Supplementary Tables S2 and S3 respectively. *E. coli* Top10 was used for general molecular cloning. Top10 was routinely grown in LB (Luria Broth) medium. *Gluconacetobacter* strains were streaked on HS (Hestrin–Schramm) agar containing 2 % (w/v) glucose and incubated at 30 °C for at least 72 h. Single colonies were selected and grown in HS broth supplemented with 2 % (w/v) glucose, 30 °C for at least 48 h, unless otherwise stated, with appropriate antibiotics. Where appropriate, antibiotics were added to the following concentrations: chloramphenicol (Cm, 30 μg/ml for *E. coli*, 140 μg/ml for *G. xylinus* 700178 and *G. hansenii* 53582, 245 μg/ml for *K. rhaeticus* iGEM), or kanamycin (Km, 50 μg/ml)

Plasmid Construction.

For construction of various ribosome binding site (RBS) mutants, individual site-directed mutagenesis was performed on P_{lac}-RBS-GFP-terminator-pHG101 to mutate every single nucleotide of the 12bp RBS (AAA GAG GAG AAA, BBa_B0034) into another three nucleotides, generating a total of 36 single RBS mutants (Supplementary Table S1). Site-directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) according to manufacturer's instructions together with the primers given in Supplementary Table S4. Subsequently, the various P_{lac}-RBS-GFP-terminator mutated constructs were digested with *EcoR*I-*Spe*I restriction enzymes and sub-cloned into likewise digested pSEVA331Bb plasmid (generous gift from Tom Ellis, Imperial College), yielding the different P_{lac}-RBS-GFP-terminator-pSEVA331Bb constructs.

For construction of various constitutive synthetic promoter constructs, the J23104-mRFP1-pSEVA331Bb plasmid (generous gift from Tom Ellis, Imperial College) was first digested with *EcoR*I-*Spe*I. Subsequently, the original J23104 promoter was replaced with other constitutive synthetic promoters (Figure 1a) via oligos annealing (Supplementary Table S5).

For construction of terminator constructs, the J23104-RBS-mCherry fragment was cloned into the promoter-less RBS-GFP-terminator-pSEVA331Bb construct, yielding J23104-mCherry-GFP-pSEVA331Bb [pMYMH461]. A *Sbf*I restriction site was introduced downstream of P_{lac} in the *EcoR*I-P_{lac}-RBS-GFP-terminator-pSEVA331Bb construct [pMYMH75] via site-directed mutagenesis, and the P_{lac} promoter was removed by digesting the construct with *EcoR*I-*SbfI* restriction enzymes. On the other hand, promoter J23104 was cloned into pSB1C3-RBS-mCherry-terminator (BBa_J06702) via *EcoR*I-*Xba*I sites, generating pSB1C3-*EcoR*I-J23104-*Xba*I-RBS-mCherry-terminator [pMYMH455]. An *Sbf*I restriction site was then introduced into pMYMH455 (downstream of mCherry) and the resulting construct was digested with *EcoR*I-*Sbf*I. The *EcoR*I-J23104-RBS-mCherry-*Sbf*I fragment was then ligated with the likewise digested RBS-GFP-terminator-pSEVA331Bb (from above), generating J23104-RBS-mCherry-*Sbf*I-RBS-GFP-terminator-pSEVA331Bb. A *BamH*I restriction site was subsequently introduced upstream of *Sbf*I, resulting in J23104-RBS-mCherry-*BamH*I-*Sbf*I-RBS-GFP-terminator-pSEVA331Bb construct. Various natural and synthetic terminators (Figure 5a) to be examined were cloned into the construct via oligos annealing through the *BamH*I-*Sbf*I restriction sites (Supplementary Table S5).

For construction of various degradation tag constructs, a *BamH*I restriction site was introduced into pSB1C3-J23104-RBS-mCherry-*Sbf*I-terminator (upstream of the TAATAA stop codons of mCherry), yielding pSB1C3-J23104-RBS-mCherry-*BamH*I-TAATAA-*Sbf*I-terminator. This construct was digested with *EcoR*I-*Spe*I restrictions enzymes and the J23104-RBS-mCherry-*BamH*I-TAATAA-*Sbf*I-terminator fragment was sub-cloned into likewise digested pSEVA331Bb, generating J23104-RBS-mCherry-*BamH*I-TAATAA-*Sbf*I-terminator-pSEVA331Bb. The construct was digested with *BamH*I-*Sbf*I restriction enzymes and various degradation tags (Figure 7a) were cloned into the construct via oligos annealing (Supplementary Table S5).

For construction of various inducible promoters (P_{Lux} and P_{Bad}), various repressor genes (LuxR and AraC) were PCR amplified from different sources using the primers given in Supplementary Table S6 and subsequently cloned into *Spe*I-*Sbf*I digested J23119-mRFP1-*Sbf*I-pSEVA331Bb. LuxR (BBa_C0062) was amplified from Part BBa_F2620 and AraC was amplified from Part BBa_K731201. The ECK120010799 terminator-P_{Lux}/P_{Bad} gBlocks were cloned into J23119- LuxR/AraC-

*Xba*I-*Sbf*I-mRFP1-pSEVA331Bb plasmids via *Xba*I-*Sbf*I restriction sites, to create P_{Lux} and P_{Bad} inducible promoters that control mRFP1 expression in respective constructs. The J23119 constitutive promoter of the P_{Lux} construct was later replaced by P_{Tet} (Part BBa_R0040) via oligo duplex insertion.

Preparation of *Gluconacetobacter* electro-competent cells and electroporation.

Single colonies of *G. xylinus* ATCC 700178, *G. hansenii* ATCC 53582 and *K. rhaeticus* iGEM were inoculated in 5 ml HS broth-0.2 % (w/v) glucose and grown at 30 °C for 72 h. The cultures were vortexed for 3 min prior to sub-culturing 1:20 in 4 tubes of 30 ml fresh HS broth-0.2 % (w/v) glucose and grown at 30 °C for 24 h, with aeration. Then, 0.2 % (v/v) cellulase (*T. reesei* ATCC 26921 cellulase, Sigma, #C2730) was added into the cultures and continued to grow at 30 °C for 4 h, with aeration, to breakdown cellulose pellicles. Cultures were vortexed to further dissolve the cellulose. Bacteria were centrifuged at 4100 rpm, 12 min, 4 °C. Supernatant was discarded carefully and pellets were resuspended in 10 ml ice-cold 1 mM HEPES (pH 7.0). All cultures were pooled together in one tube and repeated centrifugation as above. Then, the cultures were washed similarly in 10 mL 1 mM HEPES. Bacteria were pelleted again at 4100 rpm, 12 min, 4 °C. Lastly, supernatant was discarded and pellets were resuspended in 1.5 ml (for ATCC 700178 and 53582) or 4 ml (for *K. rhaeticus* iGEM) of 15 % (v/v) glycerol. Competent cells were used fresh or kept at -80 °C.

4-7 µl of pure plasmids were added into 100 µl electro-competent cells and transferred into a 0.1 cm Gene Pulser Electrocuvette (Bio-Rad). *Gluconacetobacter* was electroporated at 2.5 kV, 5-6 ms. The cells were recovered by adding 800 ul HS broth-0.2 % (w/v) glucose supplemented with 0.2 % (v/v) cellulase and grown at 30 °C for 16 h, with aeration. Following overnight recovery, the cells were spread onto HS agar-0.2 % (w/v) glucose supplemented with 140 µg/ml Cm or 245 µg/ml Cm.

Characterization of RBS variants, constitutive Anderson promoters, degradation tags, inducible promoters, and terminators constructs.

For characterization of RBS variants, constitutive Anderson promoters, degradation tags, and terminators constructs, 3 ml HS-0.2 % (w/v) glucose supplemented with 140 µg/ml or 245 µg/ml Cm was inoculated with single colonies and grown at 30 °C for 72 h. The cultures were vortexed and diluted 1:20 in 15 ml

HS-0.2 % (w/v) glucose supplemented with 140 µg/ml or 245 µg/ml Cm and 0.2 % (v/v) cellulase, and grown at 30 °C for 24 h. Overnight cultures were standardised to OD_{600} = 0.1 and grown for 4 h at 30 °C with aeration, in triplicates. 300 µl samples were taken after 4 h, pelleted (15, 000 rpm, RT, 10 min) and resuspended in 300 µl PBS. 200 µl samples were then loaded into 96-well plates. GFP (excitation 480 nm, emission 532 nm), mRFP1 (excitation 532 nm, emission 590 nm) and mCherry (excitation 587 nm, emission 610 nm) expression were measured with Spectramax M5 or TECAN microplate reader.

For characterisation of inducible promoters, cultures were prepared as mentioned above, except that inducer was added to inducible cultures to 1 µM for AHL (*N*-(β-Ketocaproyl)-L-homoserine lactone) (Sigma, #K3007) and 2 %, 4 % or 6 % (w/v) for Arabinose (Gold Biotechnology, #A-300-100), where applicable. Samples were collected 4 h post-induction and prepared as mentioned above.

Determination of cellulose pellicle weight.

Single colonies were inoculated into culture media supplemented with various carbon sources (2% (w/v) glucose, 2% (w/v) sucrose or 2% (w/v) fructose, where applicable) and incubated at 30 °C for 3 days. The cultures were then subcultured into 20 ml of varying carbon sources placed in 50 ml Falcon tubes. 4% (w/v) arabinose was added where applicable. The cultures were grown statically at 30 °C for six days. The cellulose pellicle was harvested and washed twice with distilled water, followed by immersion in 0.1M NaOH and incubated at 60 °C for 4 h. It was washed twice again with distilled water. The cellulose pellicle was then freeze-dried overnight (Alpha 2-4 LSC, Martin Christ) and the dry weight of cellulose was measured.

Fluorescence microscopy.

To visualize signals from fluorescent proteins, bacteria (3 µl) was spotted and immobilised on a 1% (w/v) agarose gel pad, before being viewed on a Leica SFL4000/7000 instrument.

Scanning electron microscopy.

To obtain structural details of the polymeric films, cellulose pellicles were freeze dried, coated with palladium, and imaged with Field Emission Scanning Electron Microscope (JSM-6700F, JEOL, Japan) at 15,000X magnification.

Lectin staining.

Colonies were grown in HS broth for 4 days, then 0.2% (v/v) cellulase was added into the broth and the cells were cultured for another 24 hours at 30ºC. Cells were then diluted to OD 0.3 in 30 ml and grown in a 250ml bottle. Cellulose films were harvested on day 4 and washed with 70% ethanol for 4 hours followed by 0.1 M NaOH for 4 hours. Subsequently, films were stained with Alexa-Flour 488 conjugated Wheat-Germ Agglutinin at 50 µg/ml in PBS for 30 minutes. Stained films were then washed 3 times with PBS, with each time lasting at least 10 minutes. Images were taken with the EVOS FL Imaging System at 20X magnification.

Quantitative real-time PCR (qRT-PCR)

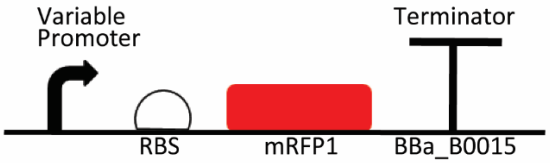
Total RNA was extracted using the Direct-zol RNA Kit (Zymogen) according to manufacturer's instructions. Next, reverse transcription was carried out using qScript cDNA Supermix (Quanta Biosciences) following manufacturer's instructions. Subsequently, quantitative PCR was performed using Perfecta SYBR Green Supermix (Quanta Biosciences) on the CFX96 instrument (Bio-Rad) following manufacturer's reaction protocol (initial denaturation - 95°C for 3min; PCR cycling - 40 cycles of 95°C for 15s, 60°C for 1min, collect data). Ct values of *acsAB* were normalized against *gyrB*, as previously described.[87] The primer sequences used are as follows – *gyrB*_Forward: 5'-TCTCGTCACAGACCAAGGACAAG-3', *gyrB*_Reverse: 5'-CTTCCTTGGGGTGGGTTTCAAAC-3', *acsAB*_Forward: 5'-ACAATGGGCTGGATGGTCGA-3', *acsAB*_Reverse: 5'-ACCCGCAAAAGAAGGTCGCA-3'.

FIGURES

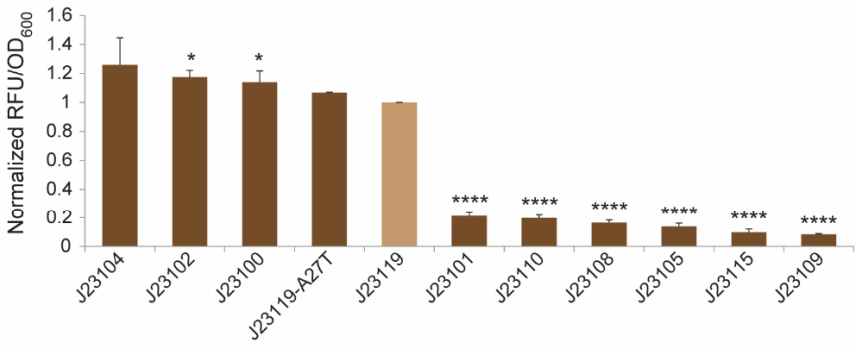
a

Anderson Promoter	Sequence (5' – 3')
J23100	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC
J23101	TTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGC
J23102	TTGACAGCTAGCTCAGTCCTAGGTACTGTGCTAGC
J23104	TTGACAGCTAGCTCAGTCCTAGGTATTGTGCTAGC
J23105	TTTACGGCTAGCTCAGTCCTAGGTACTATGCTAGC
J23108	CTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGC
J23109	TTTACAGCTAGCTCAGTCCTAGGGACTGTGCTAGC
J23110	TTTACGGCTAGCTCAGTCCTAGGTACAATGCTAGC
J23115	TTTATAGCTAGCTCAGCCCTTGGTACAATGCTAGC
J23119	TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGC
J23119-A27T	TTGACAGCTAGCTCAGTCCTAGGTATTATGCTAGC

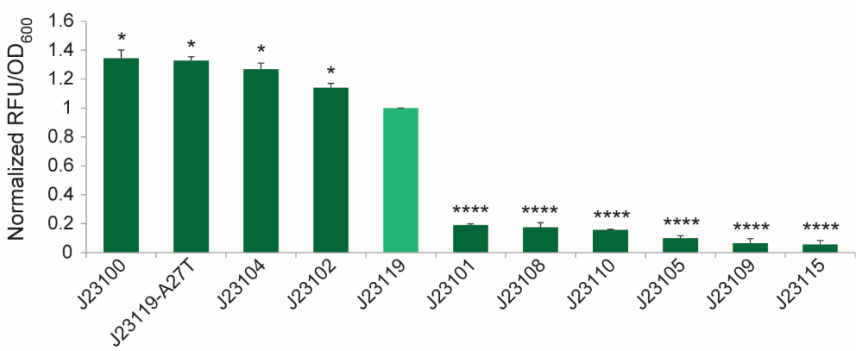
b



c



d



e

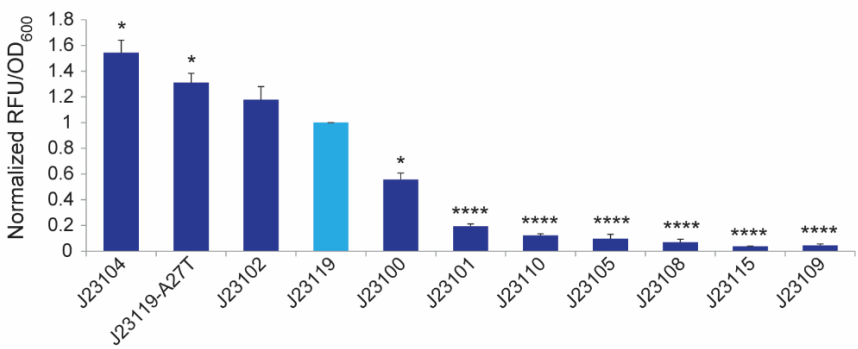
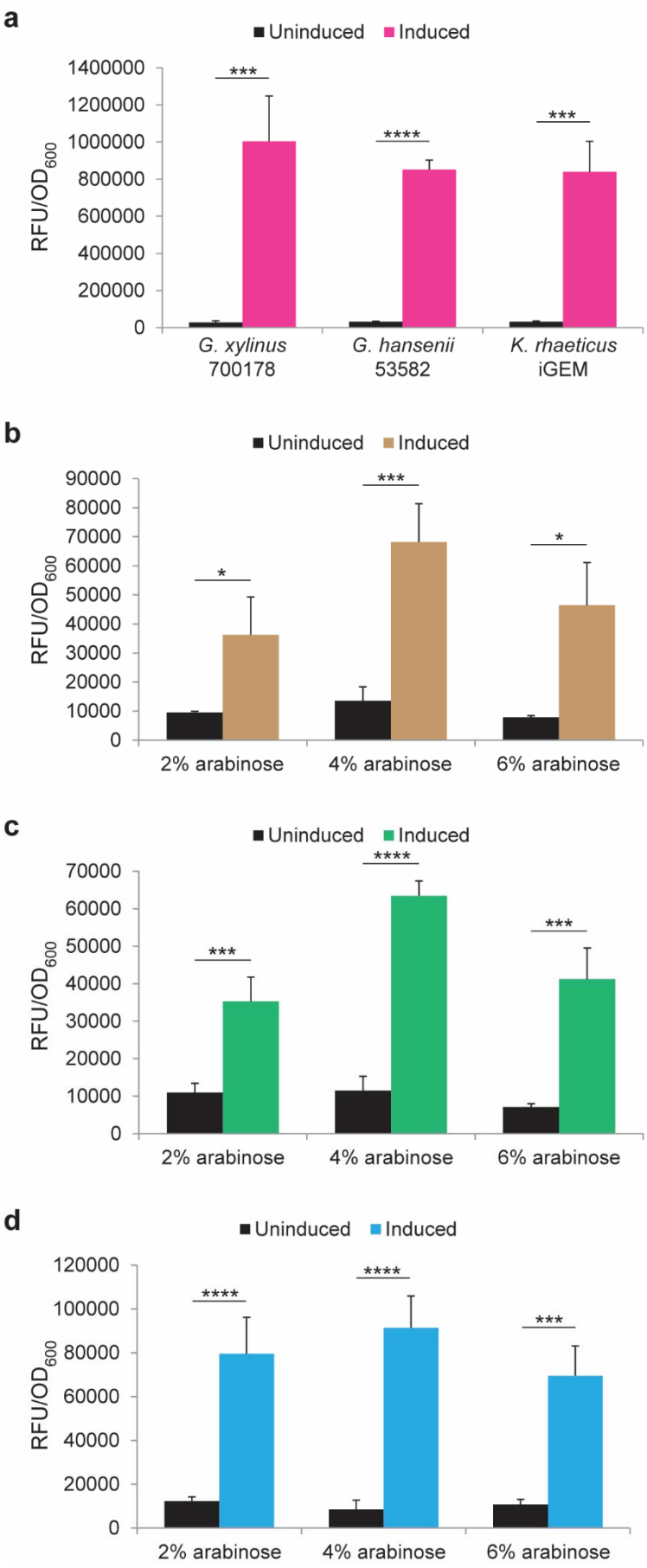


Figure 1. Characterization of constitutive promoters in *Acetobacteraceae*.
(a) List of Anderson promoters that were evaluated in this study.
(b) A schematic depicting the mRFP1 reporter assay used to determine the strength of each candidate promoter. The RBS and terminator in the testing platform were the BioBrick parts BBa_B0034 and BBa_B0015 respectively.
(c-e) Strengths of the various Anderson promoters in (c) *G. xylinus* 700178, (d) *G. hansenii* 53582, and (e) *K. rhaeticus* iGEM, as measured by the total mRFP1 fluorescence per cell. Promoter strengths were quantified in the presence of cellulase to remove any cellulose fibrils that may interfere with the spectrophotometer readings. Our results obtained in the *K. rhaeticus* iGEM strain are consistent with a previous study.[50] Data represent mean ± S.D. (n = 3 biological replicates). (* $P < 0.05$, **** $P<0.001$; Student's t-test)


a
Uninduced
Induced
RFU/OD600
1400000
1200000
1000000
800000
600000
400000
200000
0
***
****
***
G. xylinus 700178
G. hansenii 53582
K. rhaeticus iGEM
b
Uninduced
Induced
RFU/OD600
90000
80000
70000
60000
50000
40000
30000
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2% arabinose
4% arabinose
6% arabinose
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Uninduced
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RFU/OD600
120000
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80000
60000
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20000
0
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2% arabinose
4% arabinose
6% arabinose

Figure 2. Characterization of inducible promoters in *Acetobacteraceae*.
(a) Conditional regulation of mRFP1 reporter gene expression by the P_{Lux} promoter. Gene expression was activated by the addition of 1 μM AHL. Promoter strengths were quantified in the presence of cellulase to remove any cellulose fibrils that may interfere with the spectrophotometer readings. Data represent mean ± S.D. (n = 3 biological replicates). (*** $P < 0.005$, **** $P<0.001$; Student's t-test)
(b-d) Conditional regulation of mRFP1 reporter gene expression by the P_{Bad} promoter in the (b) *G. xylinus* 700178, (c) *G. hansenii* 53582, and (d) *K. rhaeticus* iGEM strains. Gene expression was activated by the addition of 2%, 4%, or 6% arabinose. The cells were treated with cellulase before measurements were taken. Our results indicate that 4% arabinose is the optimal concentration for mRFP1 expression in all three strains. Data represent mean ± S.D. ($n \geq 3$ biological replicates). (* $P < 0.05$, *** $P < 0.005$, **** $P<0.001$; Student's t-test)

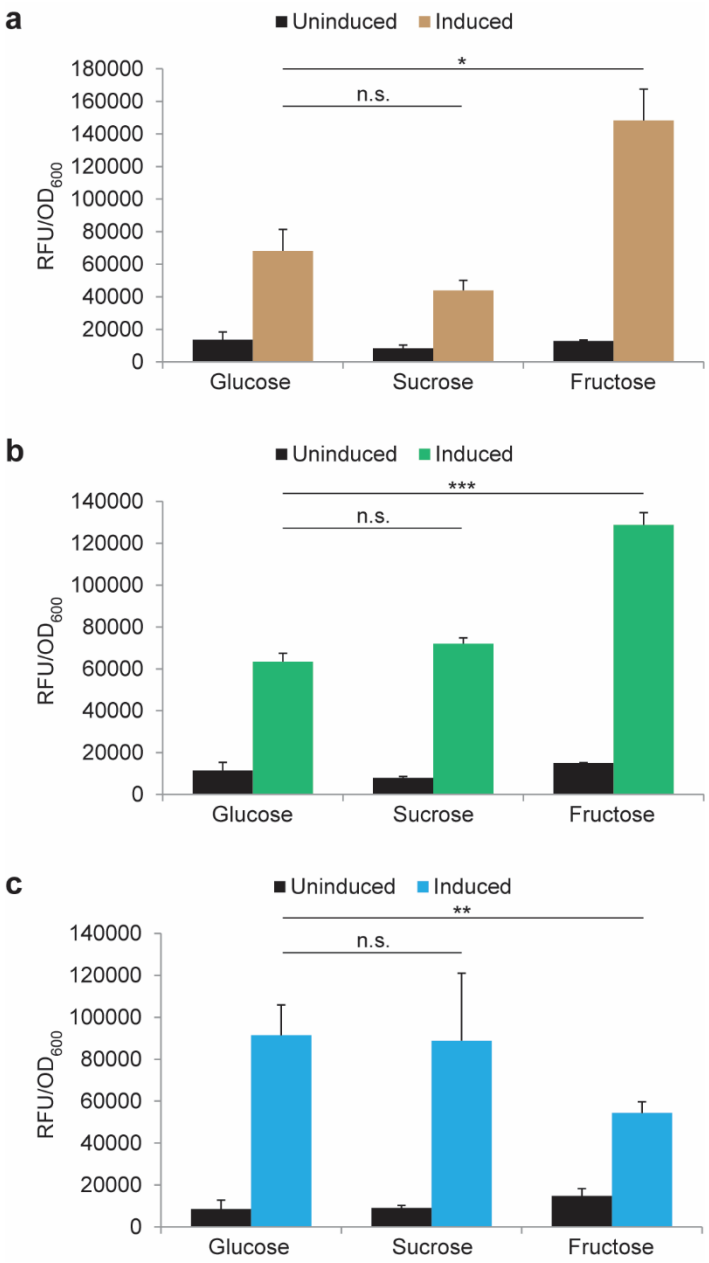


Figure 3. Effect of different carbon sources on the P_{Bad} promoter.

Three different bacterial strains, namely (a) *G. xylinus* 700178, (b) *G. hansenii* 53582, and (c) *K. rhaeticus* iGEM, were cultured in HS (Hestrin–Schramm) medium supplemented with 2% glucose, 2% sucrose, or 2% fructose as the carbon source. The expression of the mRFP1 reporter gene was activated by the addition of 4% arabinose. Promoter strengths were quantified in the presence of cellulase to remove any cellulose fibrils that may interfere with the spectrophotometer readings. Data represent mean ± S.D. ($n \geq 3$ biological replicates). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, n.s.: not significant; Student's t-test)

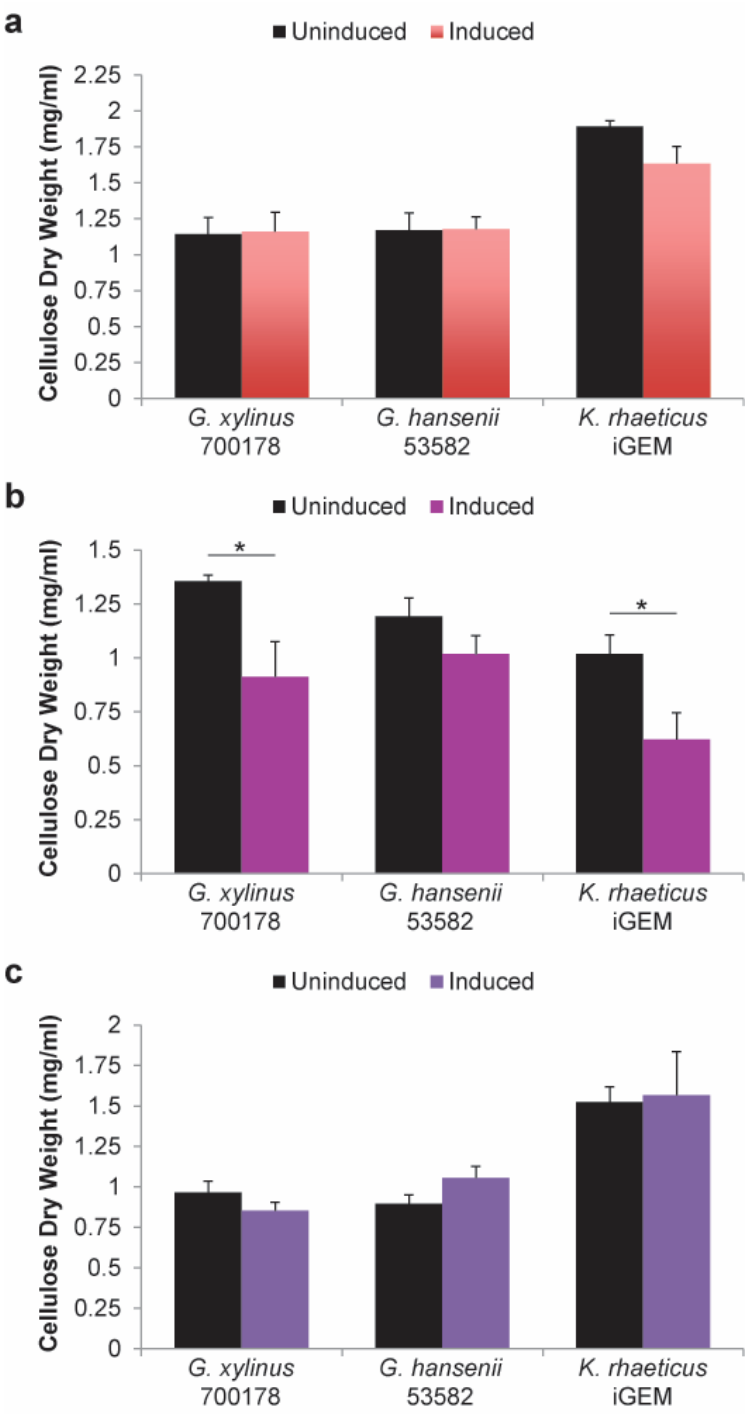


Figure 4. Effect of 4% (w/v) arabinose on bacterial cellulose yield.

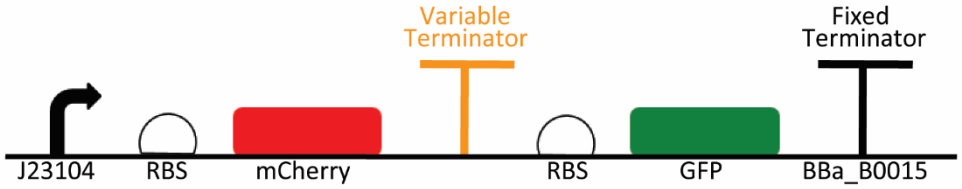
The cellulose productivity of *G. xylinus* 700178, *G. hansenii* 53582, and *K. rhaeticus* iGEM were examined in the absence (uninduced, black bars) or presence (induced, colored bars) of 4% arabinose. Each bacterial strain was cultured in HS medium supplemented with (a) 2% glucose, (b) 2% sucrose, or (c) 2% fructose as the intended carbon source. Data represent mean ± S.D. (n = 3 biological replicates). (* $P < 0.05$; >20% change in cellulose yield; Student's t-test)

a

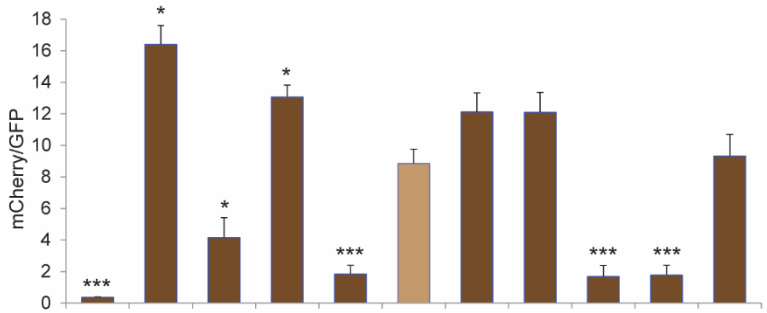
Natural Terminator	Sequence (5' – 3')
ECK120033736	AACGCATGAGAAAGCCCCCGGAAGATCACCTTCCGGGGGCTTTTTTATTGCGC
ECK120010818	GTCAGTTTCACCTGTTTTACGTAAAAACCCGCTTCGGCGGGTTTTTACTTTTGG
ECK120010799	GTTATGAGTCAGGAAAAAAGGCGACAGAGTAATCTGTCGCCTTTTTTCTTTGCTTGCTTT
ECK120051401	CGCAGATAGCAAAAAAGCGCCTTTAGGGCGCTTTTTTACATTGGTGG
BBa_B0010	CCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTG TTTGTCGGTGAACGCTCTC

Synthetic Terminator	Sequence (5' – 3')
L3S2P21	CTCGGTACCAAATTCCAGAAAAGAGGCCTCCCGAAAGGGGGGCCTTTTTTCGTTTTGGTCC
L3S3P21	CCAATTATTGAAGGCCTCCCTAACGGGGGGCCTTTTTTTGTTTCTGGTCTCCC
L3S2P24	CTCGGTACCAAATTCCAGAAAAGACACCCGAAAGGGTGTTTTTTCGTTTTGGTCC
L3S2P44	CTCGGTACCAAACCAATTATTGAAGACGCTGAAAAGCGTCTTTTTTTGTTTCGGTCC
L3S1P47	TTTTCGAAAAAAGGCCTCCCAAATCGGGGGGCCTTTTTTTATAGCAACAAAA

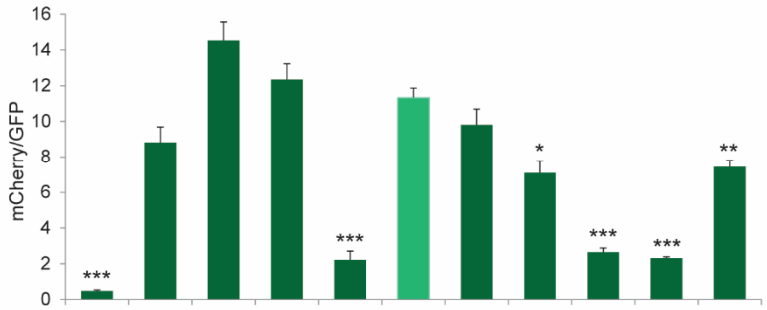
b



c



d



e

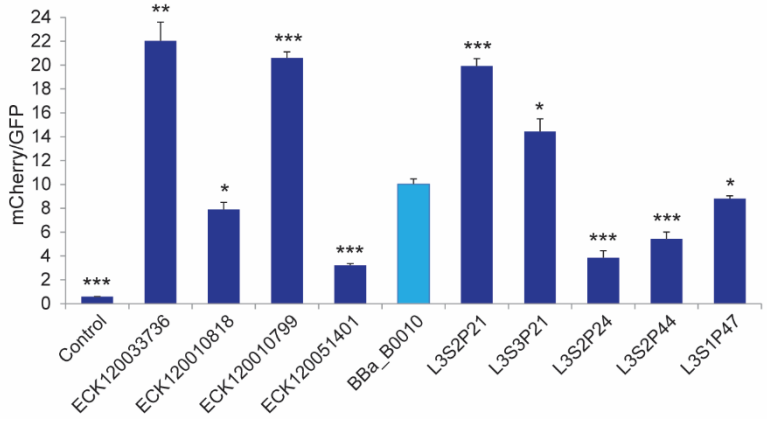


Figure 5. Characterization of terminators in *Acetobacteraceae*.

(a) List of natural and synthetic terminators that were tested in this study.

(b) A schematic depicting the evaluation platform used to determine the strength of each candidate terminator. The BioBrick RBS part, BBa_B0034, was placed in front of each reporter gene (mCherry or GFP). At the start of the cassette was a strong Anderson promoter, J23104, while at the end of the cassette was a fixed double terminator, BBa_B0015.

(c-e) Strengths of the various terminators in (c) *G. xylinus* 700178, (d) *G. hansenii* 53582, and (e) *K. rhaeticus* iGEM, as measured by the ratio of the total mCherry fluorescence per cell to the total GFP fluorescence per cell. Terminator strengths were quantified in the presence of cellulase to remove any cellulose fibrils that may interfere with the spectrophotometer readings. Data represent mean ± S.D. (n = 3 biological replicates). (* $P < 0.05$, ** $P < 0.01$, *** $P<0.005$; Student's t-test)

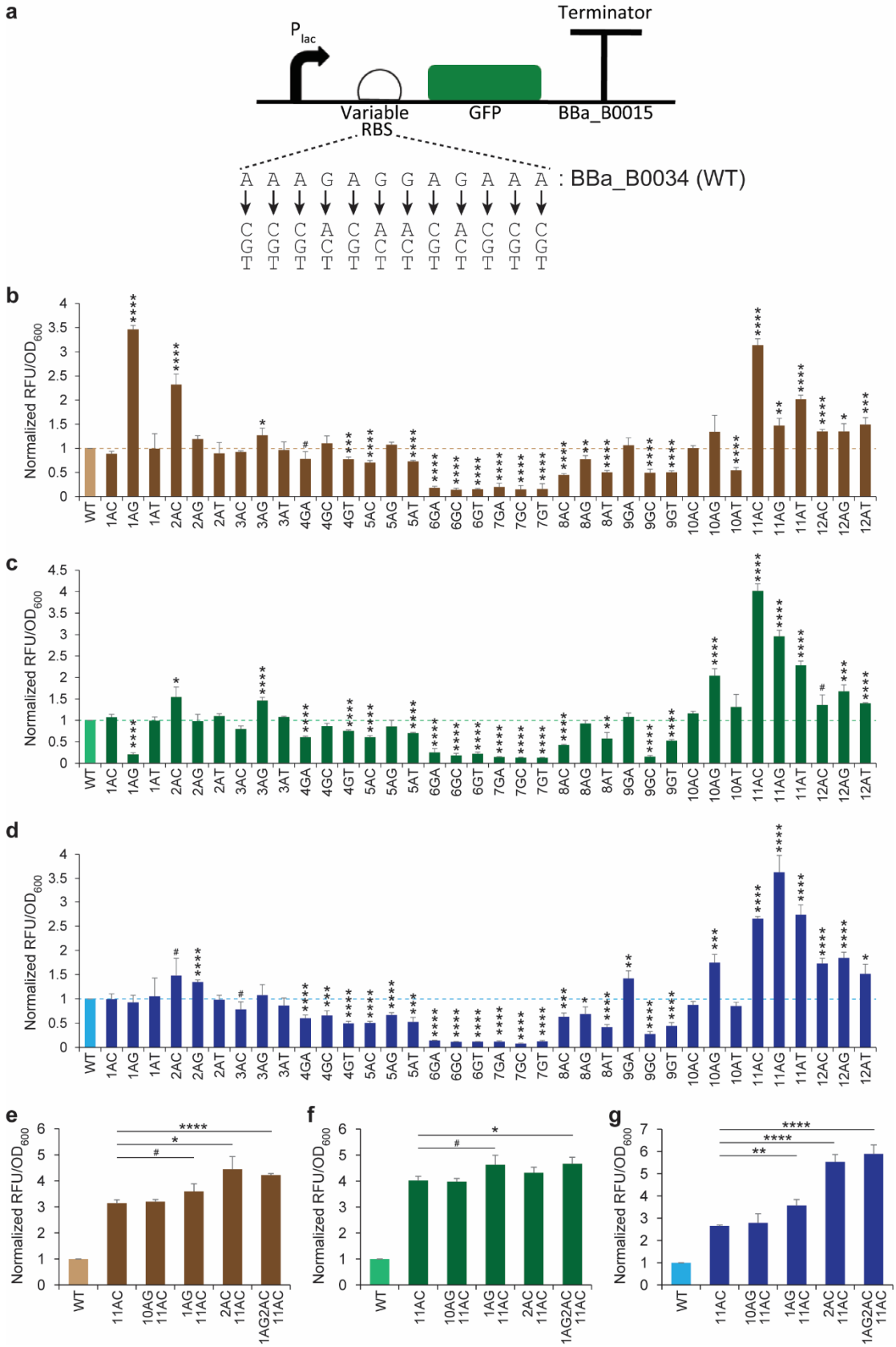
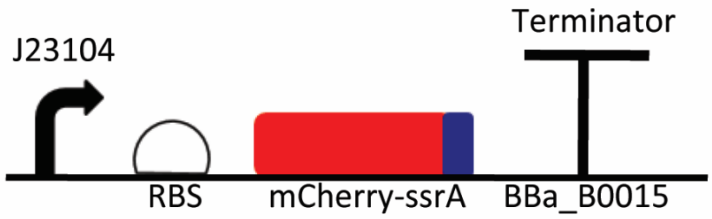

a
Terminator
P lac
Variable RBS
GFP
BBa_B0015
A A A G A G G A G A A A : BBa_B0034 (WT)
C G T C G T A C T C G T A C T C G T A C T C G T C G T
b
Normalized RFU/OD600
WT 1AC 1AG 1AT 2AC 2AG 2AT 3AC 3AG 3AT 4GA 4GC 4GT 5AC 5AG 5AT 6GA 6GC 6GT 7GA 7GC 7GT 8AC 8AG 8AT 9GA 9GC 9GT 10AC 10AG 10AT 11AC 11AG 11AT 12AC 12AG 12AT
c
Normalized RFU/OD600
WT 1AC 1AG 1AT 2AC 2AG 2AT 3AC 3AG 3AT 4GA 4GC 4GT 5AC 5AG 5AT 6GA 6GC 6GT 7GA 7GC 7GT 8AC 8AG 8AT 9GA 9GC 9GT 10AC 10AG 10AT 11AC 11AG 11AT 12AC 12AG 12AT
d
Normalized RFU/OD600
WT 1AC 1AG 1AT 2AC 2AG 2AT 3AC 3AG 3AT 4GA 4GC 4GT 5AC 5AG 5AT 6GA 6GC 6GT 7GA 7GC 7GT 8AC 8AG 8AT 9GA 9GC 9GT 10AC 10AG 10AT 11AC 11AG 11AT 12AC 12AG 12AT
e
Normalized RFU/OD600
WT 11AC 10AG 11AC 1AG 11AC 2AC 11AC 1AG2AC 11AC
f
Normalized RFU/OD600
WT 11AC 10AG 11AC 1AG 11AC 2AC 11AC 1AG2AC 11AC
g
Normalized RFU/OD600
WT 11AC 10AG 11AC 1AG 11AC 2AC 11AC 1AG2AC 11AC

Figure 6. Characterization of ribosome binding sites (RBS) in *Acetobacteraceae*. (a) A schematic depicting the GFP reporter assay used to determine the strength of each candidate RBS. The promoter and terminator used in the assay were P_{lac} and the BioBrick part BBa_B0015 respectively. We noted that the published genomes of *G. hansenii* 53582[49] and *K. rhaeticus* iGEM[50] do not contain any *lac* repressors. Hence, P_{lac} can be used as a constitutive promoter in our experiments, as evidenced by the strong GFP expression in many of our strains carrying the reporter constructs without any IPTG induction. The BioBrick part, BBa_B0034, served as our reference RBS, from which we generated other RBS variants by site-directed mutagenesis. (b-d) Strengths of the various RBS in (b) *G. xylinus* 700178, (c) *G. hansenii* 53582, and (d) *K. rhaeticus* iGEM, as measured by the total GFP fluorescence per cell normalized to that of BBa_B0034. Here, each RBS variant carried only a single mutation and the type of mutation is indicated as the x-axis labels. RBS strengths were quantified in the presence of cellulase to remove any cellulose fibrils that may interfere with the spectrophotometer readings. Data represent mean ± S.D. ($n \geq 3$ biological replicates). ($^{\#}$ $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P<0.005$, **** $P < 0.001$; >20% difference in RBS strength from that of BBa_B0034; Student's t-test) (e-g) Strengths of RBS variants carrying the 11AC mutation either by itself or in combination with other types of mutations. The RBS strengths were measured in the (e) *G. xylinus* 700178, (f) *G. hansenii* 53582, and (g) *K. rhaeticus* iGEM strains. Data represent mean ± S.D. ($n \geq 2$ biological replicates).

a

Degradation tag	Protein sequence
LAA (Wildtype)	AANDENY--ALAA
AAV	AANDENY--AAAV
LVA	AANDENY--ALVA
DAS	AANDENY--ADAS
DAS+2	AANDENYNYADAS

b



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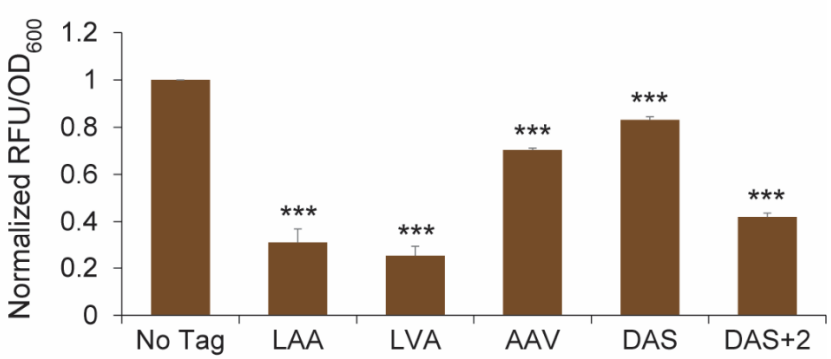
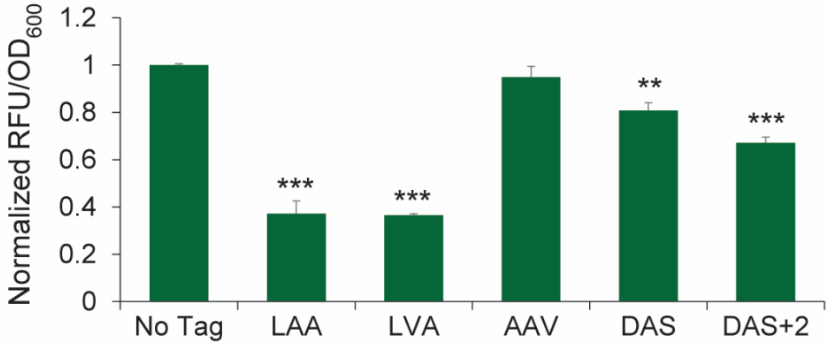


d



e

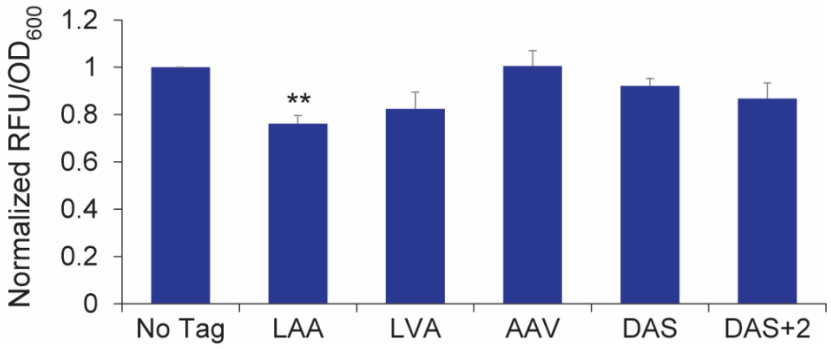


Figure 7. Characterization of ssrA degradation tags in *Acetobacteraceae*.
(a) List of ssrA tags that were evaluated in this study. The last three pink letters of each protein sequence indicate the ClpX-interacting residues.
(b) A schematic depicting the evaluation platform used to assess the effectiveness of each degradation tag (blue) in promoting proteolysis of the mCherry reporter (red). The strong Anderson promoter, J23104, was used to drive the expression of the mCherry gene, while the RBS and terminator in the testing platform were the BioBrick parts BBa_B0034 and BBa_B0015 respectively.
(c-e) The total fluorescence intensity per cell was measured for each mCherry-ssrA construct in (c) *G. xylinus* 700178, (d) *G. hansenii* 53582, and (e) *K. rhaeticus* iGEM and normalized to that for the untagged mCherry control. A value much less than one would indicate that the fused tag at the C-terminus was effective in enhancing the proteolysis of the mCherry reporter. All measurements were taken in the presence of cellulase to remove any cellulose fibrils that may interfere with the spectrophotometer readings. Data represent mean ± S.D. (n = 3 biological replicates). (** $P < 0.01$, *** $P < 0.005$; ≥ 10% reduction in fluorescence intensity per cell; Student's t-test)

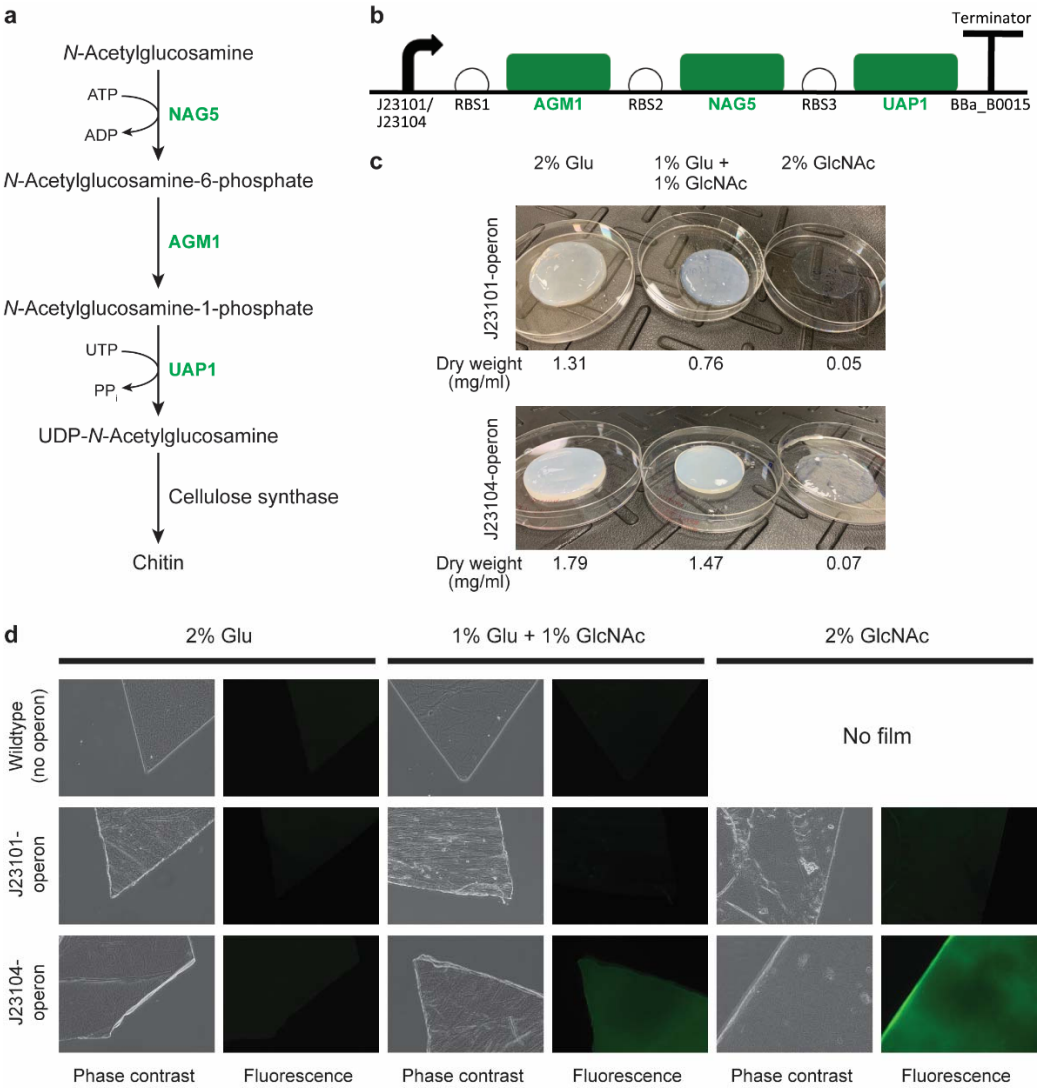


Figure 8. Biosynthesis of a cellulose-chitin copolymer.

(a) The biosynthesis pathway of chitin from *N*-acetyl-glucosamine (GlcNAc).

(b) Schematic of the operon, containing three genes from *Candida albicans* (AGM1, NAG5, and UAP1), that was constructed to enable *Acetobacteraceae* to synthesize a cellulose-chitin composite in the presence of GlcNAc. The three genes were expressed from either a weak promoter (J23101) or a strong promoter (J23104). The sequences of RBS1, RBS2, and RBS3 were AAAGAGGAGAAA (BBa_B0034), GAGGAGGAGGAA, and GCAGAGGAGGCA respectively. Different RBS sequences were used to facilitate cloning by Gibson assembly.

(c) Images showing the polymeric sheets produced using different concentrations of glucose (Glu) or GlcNAc.

(d) Lectin staining to demonstrate differential incorporation of GlcNAc into the cellulose matrix.

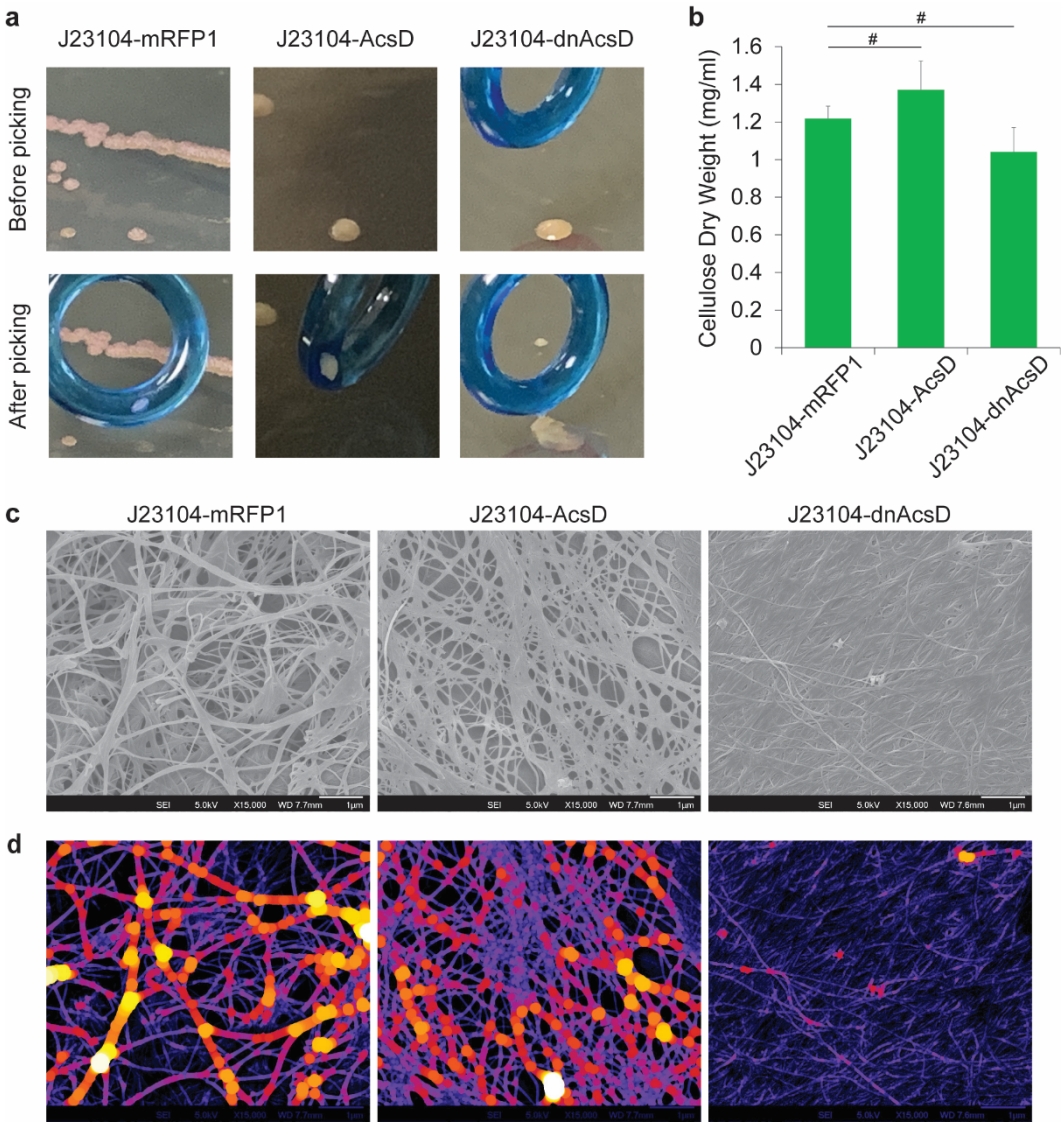


Figure 9. Effect of dominant negative AcsD (dnAcsD) expression.

(a) Due to the secretion of a dense cellulose matrix, a typical *Gluconacetobacter* colony on an agar plate is hard and can be easily picked off the plate. This was the case for the strains bearing either a J23104-mRFP1 plasmid or a J23104-AcsD (wildtype) plasmid. However, we found that for the strain carrying a J23104-dnAcsD plasmid, soft colonies were formed instead.

(b) Measurements of cellulose productivity. Data represent mean ± S.D. (n = 3 biological replicates). ($^{\#}$ $P < 0.1$; Student's t-test)

(c) Scanning electron micrographs of purified bacterial cellulose sheets.

(d) The SEM images were analysed using a publicly available ImageJ plugin called "Local Thickness". A dim purple colour indicates thin fibres, while a bright orange or yellow colour indicates thick fibres.

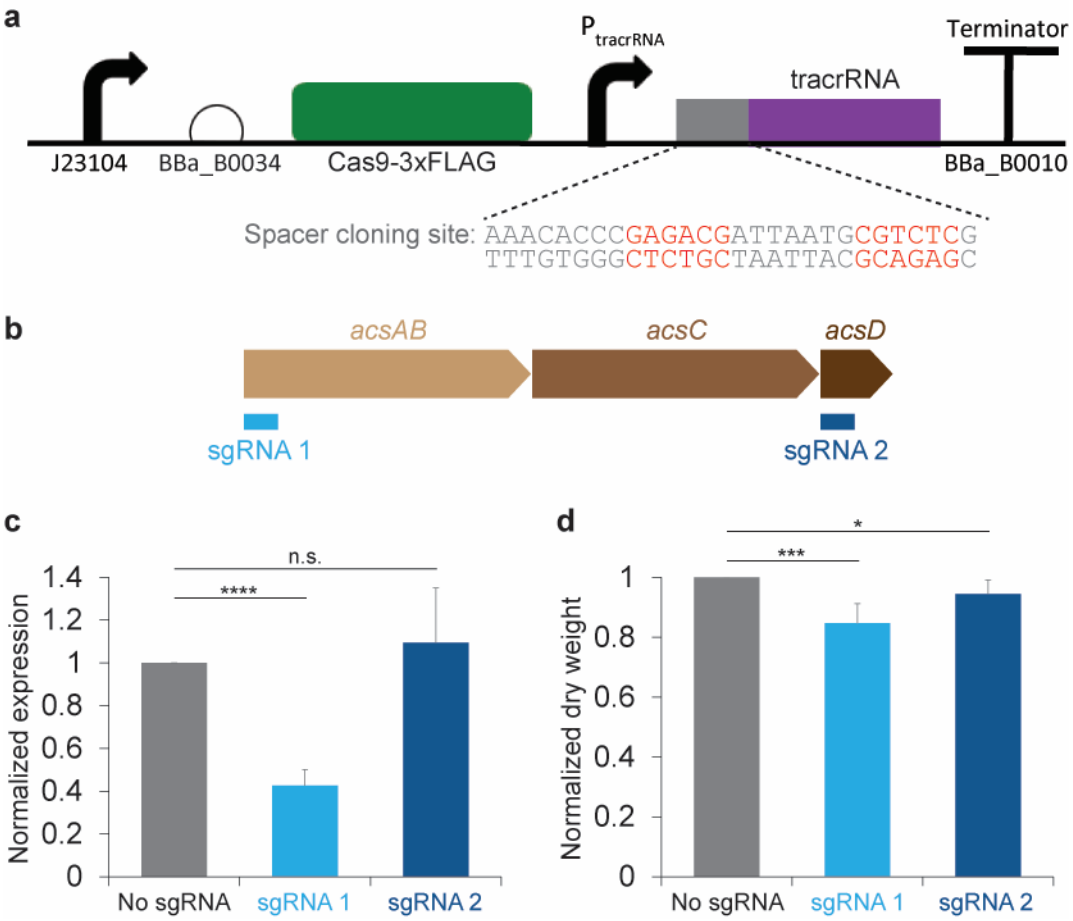


Figure 10. Implementation of CRISPR interference (CRISPRi) in *Acetobacteraceae*.

(a) Schematic of our all-in-one CRISPR-Cas9 construct. The sequences highlighted in red indicate BsmBI restriction sites.

(b) Schematic of the main *acs* operon in *G. hansenii* ATCC 53582. We targeted Cas9 to the start of either *acsAB* (using sgRNA 1) or *acsD* (using sgRNA 2).

(c) Measurements of *acsAB* expression by qRT-PCR. Data represent mean ± S.D. (n = 3 biological replicates). (**** $P < 0.001$, n.s.: not significant; Student's t-test)

(d) Measurements of cellulose productivity. Data represent mean ± S.D. (n = 4 biological replicates). (*** $P < 0.005$, * $P < 0.05$; Student's t-test)

Author Contributions

M.H.T. conceived the project and supervised the research. M.Y.T., K.H.O., S.X.D.T., and M.H.T. designed the experiments. M.Y.T., K.H.O., S.X.D.T., and M.E.B.M. performed the experiments, with help from W.Z.S.L. and M.H.T. M.Y.T. and M.H.T. wrote the manuscript.

Acknowledgement

M.H.T. is supported by a National Research Foundation grant (NRF2013-THE001-046) and a startup grant from Nanyang Technological University (NTU). The authors thank NTU Institute of Structural Biology for use of the TECAN spectrophotometer, Dr. Tom Ellis from Imperial College London for the *K. rhaeticus* iGEM strain and various plasmids, as well as Mr. Sundaravadanam Vishnu Vadanan and Ms. Krishnamoorthi Shalini for technical support.

Supporting Information

The Supplementary Information file contains Supplementary Results, Supplementary Figures S1-S7, and Supplementary Tables S1-S6. The Supporting Information is available free of charge on the ACS Publications website at DOI: X.

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