RESEARCH ARTICLE

One step forward, two steps back: Transcriptional advancements and fermentation phenomena in *Actinobacillus succinogenes* 130Z

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Abstract

Within the field of bioproduction, non-model organisms offer promise as bio-platform candidates. Non-model organisms can possess natural abilities to consume complex feedstocks, produce industrially useful chemicals, and withstand extreme environments that can be ideal for product extraction. However, non-model organisms also come with unique challenges due to lack of characterization. As a consequence, developing synthetic biology tools, predicting growth behavior, and building computational models can be difficult. There have been many advancements that have improved work with non-model organisms to address broad limitations, however each organism can come with unique surprises. Here we share our work in the non-model bacterium *Actinobacillus succinognes* 130Z, which includes both advancements in synthetic biology toolkit development and pitfalls in unpredictable fermentation behaviors. To develop a synthetic biology "tool kit" for *A. succinogenes*, information gleaned from a growth study and antibiotic screening was used to characterize 22 promoters which demonstrated a 260-fold range of fluorescence protein expression. The strongest of the promoters was incorporated into an inducible system for tunable gene control in *A. succinogenes* using the promoter for the *lac* operon as a template. This system flaunted a 481-fold range of expression and no significant basal expression. These findings were accompanied by unexpected changes in fermentation products characterized by a loss of succinic acid and increase in lactic acid after approximately 10 months in the lab. During evaluation of the fermentation shifts, new tests of the synthetic biology tools in a succinic acid producing strain revealed a significant loss in their functionality. Contamination and mutation were ruled out as causes and further testing is needed to elucidate the driving factors. The significance of this work is to share a successful tool development strategy that could be employed in other non-model species, report on an unfortunate phenomenon that needs addressed for further development of *A. succinogenes*, and provide a

cautionary tale for those undertaking non-model research. In sharing our findings, we seek
to provide tools and necessary information for further development of *A. succinogenes* as a
platform for bioproduction of succinic acid and to illustrate the importance of diligent and
long-term observation when working with non-model bacteria.

Introduction

Recent research endeavors have turned to generating useful chemicals from biological plat-
forms as an environmentally responsible alternative to non-sustainable sources [1, 2]. Biopro-
duction of industrially important chemicals can utilize organic and renewable feedstocks as
nutrient-sources for microbial fermentation using metabolically engineered strains for opti-
mized production. Examples of bioproduction success stories include the production of arte-
misinin [an anti-malaria drug) from engineered yeast [3] and hydrogen from engineered *E.
coli* [4]. Non-model organisms are becoming increasingly interesting bioproduction platforms
as they would expand the range of metabolic capabilities potentially harnessed for bioproduc-
tion purposes. Specific characteristics that would make an organism a good biological platform
include native abilities to degrade sugar polymers, utilize renewable feedstocks to produce bio-
chemicals of interest, and grow in challenging environments [5]. These unique characteristics
that can be found in non-model microbes go hand-in-hand with unique challenges. The lim-
ited characterization of non-model organisms can raise issues when utilizing synthetic biology
tools in predictable ways, elucidating effective metabolic engineering strategies, and under-
standing complex regulatory behaviors. Advances in computational tools to harness omics
data and synthetic biology have made it possible to begin development of non-model organ-
isms as bioproduction platforms. In fact, a recent review highlights many success stories of
how challenges of working with non-model organisms have been overcome to unlock their
unique potential [6]. Examples include the identification and incorporation in centromeric
regions to solve the problem of low plasmid maintenance in the lipogenic and unconventional
yeast *Yarrowia lipolytica* [7] and the modification of CRISPR/Cas9 plasmid system to reduce
problematic recombination events in non-model actinobacteria producers of diverse natural
products [8] among many others. These innovative solutions can serve as inspiration for work
in other non-model organisms, such as *Actinobacillus succinogenes* 130Z.

A. succinogenes 130Z is a Gram-negative, biofilm-forming, capnophilic, anaerobic and non-
model bacterium identified as a potential bioproduction platform for succinic acid (hereafter
SA) [9] and could also be developed for other products such as itaconic acid or fumarate. Here
we target production of SA; an organic acid that can serve as a precursor for many chemicals
used in the production of various commodities, including biodegradable plastics, active phar-
maceutical agents, and textiles [10–12]. It has been predicted that bioproduction of SA from
complex sugar sources could become the primary mode of production, eventually replacing
current unsustainable methods that rely on declining petroleum sources [13–15]. SA biopro-
duction is supported by *A. succinogenes'* ability to utilize both C5 and C6 sugars derived from
cellulosic biomass [9, 16] and its unique metabolic pathway which includes a truncated TCA
cycle resulting in naturally high production of SA [17] without demonstrating product inhibi-
tion [16]. This bacterium is a biosafety level 1 organism meaning it could be readily incorpo-
rated into any industrial facility. To this date, *A. succinogenes*-driven SA production has
reached a yield of 94% (w/w) from glucose [16, 18] yet has a theoretical yield of 121% (w/w)
from glucose [19]. It has also been demonstrated to grow robustly on corn stover hydrolysate

which contains chemicals that can inhibit microbial growth [20]. This indicates that this bacterium could be an efficient SA producer through its ability to utilize the carbon in hydrolysate without requiring extensive preprocessing. It has been noted that growth condition optimization is not sufficient to obtain maximum SA levels [17], therefore, increasing SA production further will require other strategies such as metabolic engineering using synthetic biology tools, few of which exist for this non-model organism. Previous studies have shown strategies employing endogenous promoters [21] and gene-knock out methods [21–23], but as of yet, exogenous promoters have not been tested or characterized in *A. succinogenes* and no specific inducible promoter has been designed for this bacterium. It is well-known that development of promoters, specifically inducible promoters that can be turned on and off, is one of the easiest and most effective ways to control gene expression [24]. Hence, a wider range of available tools would allow for further fine-tuning of *A. succinogenes*' metabolism for maximizing SA production.

To this end, here we share a case study of both advancements and challenges of working with *A. succinogenes* for SA production. Several steps were taken prior to engineering *A. succinogenes* to increase SA production, including performing small-scale growth studies, identifying effective selection antibiotics, and characterizing and developing synthetic biology tools. We show characterization of 22 constitutive promoters using green fluorescent protein and a flavin-binding fluorescent protein demonstrating a 260-fold range of expression from the weakest to strongest promoter. Additionally, we present characterization of the commonly used inducible *lac* system from *E. coli* and our development of a novel inducible system demonstrating a 481-fold dynamic range following a design strategy that could be applied and tailored to other non-model bacteria of interest. While the progress toward a synthetic biology toolkit for *A. succinogenes* is an important development, we also find SA production being lost over time in the working stock of *A. succinogenes*. This unexpected fermentation shift is yet to be overcome; however, we believe that both the progress and the challenges shared here could aid in future development of *A. succinogenes* as a more stable and efficient producer of SA. These results could simultaneously inspire researchers working in other industrially interesting non-model organisms to adopt practices of more long-term observation. This would benefit not only the stakeholders (i.e., bioprocessing industry and related agricultural markets) but also clear smoother paths for bioproduction efforts using other non-model microbes.

Results and discussion

Growth curve

Identifying *A. succinogenes*' growth phases (lag, exponential, and stationary) is important as some molecular biology methods (e.g. electroporation [25]) require their application within certain growth phases. In literature, growth conditions have been described in 500 mL flasks [26], 500 mL Duran bottles [27], bioreactors [18, 21, 26, 28], and test tubes [29]; however, there has yet to be a reported growth curve showing the growth phases that are important for small scale engineering studies. A growth curve for *A. succinogenes* was generated by fitting a logistic model to OD_{600} measurements taken at 30-minute intervals over the course of a 10-hour growth period. Results demonstrated a 2-hour lag phase, followed by a 6-hour exponential growth phase after which the cells entered stationary phase (S1 Fig in S1 File). Early exponential phase, a key point for transformation of the bacterium via electroporation [25], was determined to be between hour 2 and 4 and at 0.4–0.6 OD_{600}.
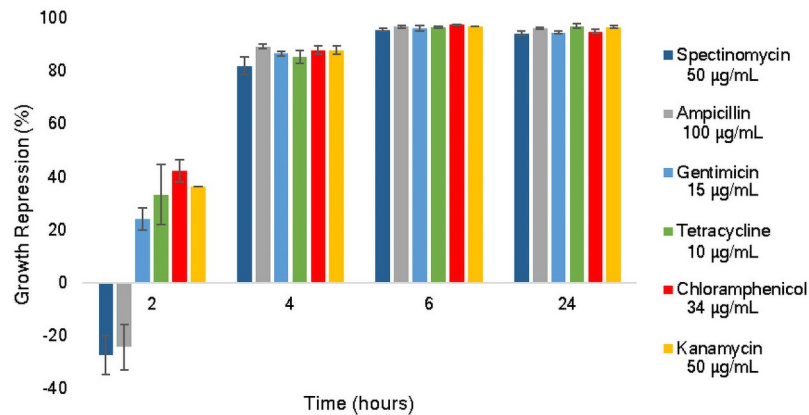
Fig 1. Antibiotic screening. Antibiotic in liquid medium with wild type *A. succinogenes* shown as percent repression of normal growth. Error bars represent one standard deviation.

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Antibiotic screening

Another crucial aspect for developing synthetic biology tools is effective selection antibiotics. Screening of standard antibiotics is needed to provide options that enforce plasmid maintenance for tool testing. Cultures of *A. succinogenes* were grown in the presence of kanamycin (50 μg/mL), tetracycline (10 μg/mL), ampicillin (100 μg/mL), gentamicin (15 μg/mL), spectinomycin (50 μg/mL), or chloramphenicol (34 μg/mL). Antibiotic concentrations were selected within the ATCC recommended concentration range for plasmid maintenance in bacteria containing mid-range plasmid copy number. Previous work with *A. succinogenes* shuttle vectors demonstrated low to medium copy number [30], therefore concentrations within the guidelines for mid-range copy numbers should be sufficient. OD_{600} was measured at 2, 4, 6, and 24 hours. Fig 1 shows the efficacy of each antibiotic displayed as percent growth repression (Materials and Methods). Although spectinomycin and ampicillin both eventually inhibit growth of this fast-growing bacterium equivalently to the other tested antibiotics, neither took effect until after two hours. The delayed response to spectinomycin may be due to slow uptake, which is possible when using aminoglycoside class antibiotics in anaerobic conditions [31]. Similarly, the slow response to ampicillin may be due to difficulty passing through the cell wall, which can be seen in Gram-negative bacteria [32]. Although wild-type *A. succinogenes* cells were eventually killed, using ampicillin for selection posed a problem when selecting successfully transformed colonies of this biofilm-forming bacterium. Resistance to ampicillin is achieved by excretion of β-lactamase which breaks open the antibiotic's β-lactam ring [33]. It is possible that cells containing the plasmid could create an environment for the non-transformed cells to keep growing by excreting the enzyme into the growth medium, therefore making selection difficult. Based on these findings, kanamycin, tetracycline, chloramphenicol, and gentamicin are recommended as selection markers in *A. succinogenes*.

Constitutive promoter library

To enable control of transcription in *A. succinogenes*, 22 constitutive (always expressing) promoters were characterized. The set of promoters included P$_{lac}$, a promoter native to *E. coli* [34]; P$_{pcka}$, a promoter native to *A. succinogenes* [21]; and the family of Anderson promoters, synthetic promoters that were developed in *E. coli* [35]. The Anderson promoter library and the Lac operon's promoter were chosen since they have been shown to function well in multiple bacteria [36–40]. A major goal of this project was to investigate the use of synthetic biology

tools that would be less likely to demonstrate cross talk with native genetic components in *A. succinogenes* [38]. Characterization of these orthogonal tools within *A. succinogenes* was necessary as promoters often do not behave the same across different species, which can be seen in the cyanobacterium *Synechocystis* sp. 6803 [38]. For example, in *Synechocystis*, promoter BBa_J23112 was stronger than BBa_J23100 whereas in *E. coli*, BBa_J23100 produced the strongest expression. Furthermore, P$_{lac}$ was not inducible with IPTG in the cyanobacterium. As shown in Fig 2A, each promoter was inserted into the plasmid SSBIO-AS001 upstream of the modified jellyfish *A. victoria* green fluorescence protein gene (*gFPuv*) [41]. Transformation of the plasmids into *A. succinogenes* created strains sAS100 –sAS122 (S5 Table in S1 File) and normalized expression (Materials and Methods) is shown in Fig 2B. To compare the relative expression of the Anderson promoter library in *A. succinogenes* with activity in *E. coli*, average expression values in *A. succinogenes* were divided by the highest expressing promoter (BBa-J23100) thus setting the maximum expression to 1. These values were compared to reported relative expression values in *E. coli* [35], which were calculated in the same way, as can be seen in Fig 2C. Findings demonstrated that maximal and minimal expressing promoters were the same in both bacteria however, relative expression was not equivalent across the entire promoter set. For instance, BBa_J23119 and BBa_J23100 were expressed similarly in *E. coli* while there was a drastic difference in expression of the two promoters in *A. succinogenes*, revealing unique sensitivities between the two bacteria to sequence variations at different locations within the promoters. A look at how the expression varies within the promoter set reveals a pattern that is evident in both *E. coli* and *A. succinogenes*. A guanine instead of a thymine at the position -12 appeared to hinder expression. This location falls within the -10 hexamer region and the decreased expression may be due to a lower affinity of polymerase binding. Aside from that one consistency, there is not a clear pattern of how the promoter sequence is tied to expression changes, thus reiterating the importance host-specific characterization. A second reporter gene, flavin-binding fluorescent protein [42], hereafter *fbfp*, was inserted in place of *gFPuv* and was tested under the control of a mid-range Anderson promoter (BBa_J23111) and the native promoter (P$_{pcka}$). FbFP was used because the protein can fold in
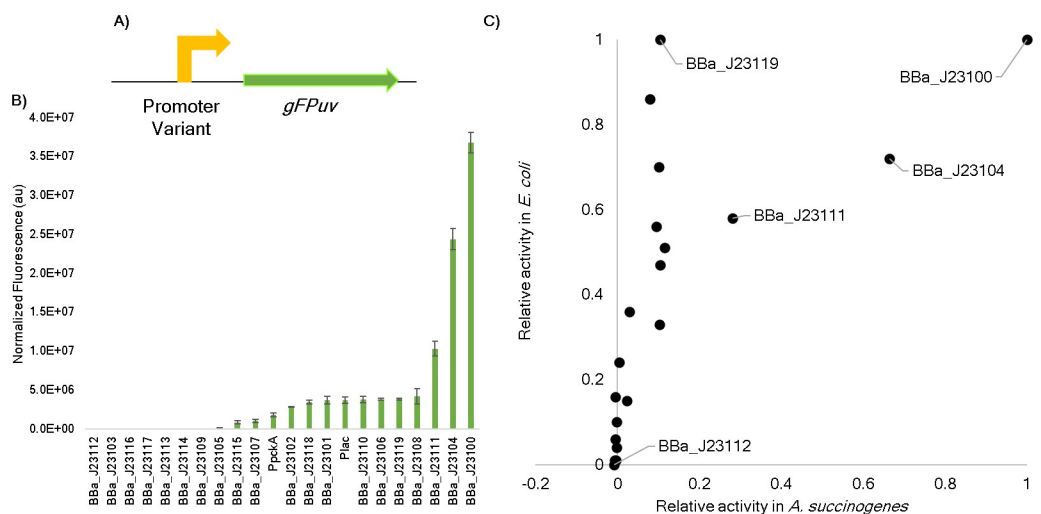
Fig 2. Characterization of 22 constitutive promoters in *Actinobacillus succinogenes* 130Z. (A) Schematic of SSBIO-SA001 expression system. (B) GFPuv expression normalized by absorbance and background control (Materials and Methods). Error bars represent one standard deviation. (C) A comparison of relative expression of the Anderson promoters in *A. succinogenes* and *E. coli*. For both bacteria, relative expression was calculated by dividing all values by normalized expression from the strongest promoter (BBa_J23100).

anaerobic conditions [42] unlike GFPuv which requires oxygen [43]. This allowed for fluorescent measurements to be taken immediately after culturing and removed any variation that may have been introduced by the aeration process used when measuring GFPuv expression. Results showed that BBa_J23111 expressed both GFPuv and FbFP approximately 4 times stronger than the native promoter P_{pcka} (4.49 and 4.29 respectively), demonstrating consistently stronger expression with the synthetic promoter. Finally, to determine the range of expression across all tested promoters in *A. succinogenes*, promoters showing no expression were discarded and the remaining 15 were compared to the lowest expressing promoter (BBa_J23105) showing a relative range of 260-fold. The Anderson promoters demonstrate a significantly greater range of expression (up to 2,547-fold) in *E. coli* [35] (verification shown in S2 Fig in S1 File). A possible explanation is that the Anderson promoters were designed from *E. coli*'s consensus sequence (labeled as BBa_J23119 in the Anderson promoter set). This means that minor changes were made to the -10 and -35 hexamer regions of the optimum promoter sequence for transcription within *E. coli*. In contrast, *A. succinogenes*' consensus sequence has not been determined. While *E. coli* and *A. succinogenes* share many characteristics, there may be variation within the replication machinery, such as differences in sigma factors [44], which could account for the lower range of expression observed in *A. succinogenes*.

Characterization of the inducible P_{lac} promoter

While constitutive promoters are useful tools for setting constant gene expression rates, metabolic engineering often requires promoters that can respond to external signals [45]. The *lac* operon from *E. coli* [34] is an inducible system that includes the promoter P_{lac} flanked by operator regions that bind to the repressor protein, LacI. When LacI is bound, transcription is turned off via steric hinderance; however, an inducer molecule, isopropyl β-D-1-thiogalactopyranoside (hereafter IPTG), can bind to LacI and prevent repression of transcription. Therefore, varying levels of IPTG can tune expression of genes under control of P_{lac}. To characterize this inducible system within *A. succinogenes*, the repressor protein's gene, *lacI*, and its native promoter were included with all the same components as SSBIO-AS001, creating SSBIO-AS003 (Fig 3A). Transforming *A. succinogenes* with the plasmid created strain sAS124. Performance of sAS124 induced at 0 and 5 mM IPTG was compared to both wild type *A. succinogenes* and strain sAS120 containing the constitutive system with P_{lac}. Growth was compared across IPTG concentrations and was shown to be consistent (S3 Fig in S1 File). As can be seen in Fig 3B, expression in sSA124 at 0 mM IPTG was not statistically different than wild type background fluorescence and expression in sSA124 at 5 mM IPTG was not statistically different than sAS120. Cultures were grown at various concentrations of IPTG within the range of 0 to 5 mM and expression was measured. Visualization on a logarithmic scale showed a graded response (Fig 3C). While the change in expression from the deactivated to activated state of the inducible system was only 90-fold, the on-state matched the expression level of constitutive P_{lac}, demonstrating complete induction. Additionally, the system did not show leakiness, as no GFPuv expression was observed in the absence of IPTG. While the range of control is limited by the relatively low maximum level of expression of P_{lac} within *A. succinogenes* when compared to other constitutive promoters (see Fig 2B), the binary on and off states make this system promising for development within *A. succinogenes*.

Development of a stronger inducible promoter: P100i

To create an inducible system for *A. succinogenes* with a larger range of expression, the strongest constitutive promoter (BBa_J23100) was added to SSBIO-AS003 in place of the core P_{lac}, sequence creating SSBIO-AS004. In the constitutive system, BBa_J23100 expressed GFPuv ~10
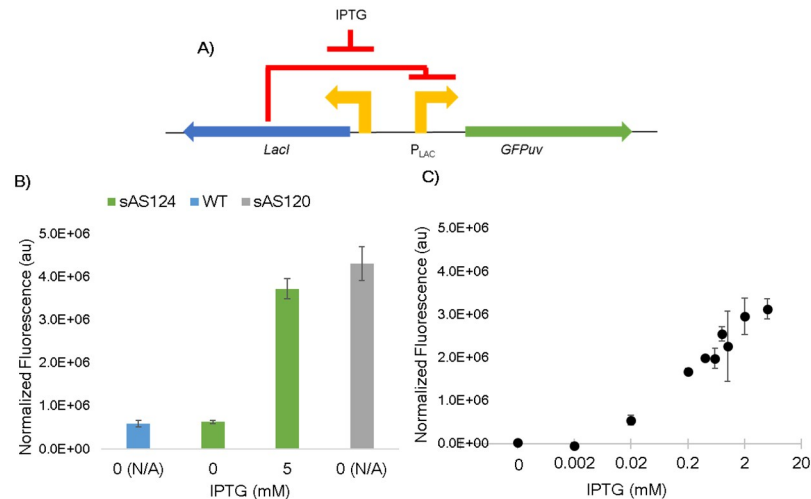
Fig 3. Characterization of strain sAS124 containing SSBIO-AS003 using GFPuv as the reporter protein. (A) Schematic of the expression system used in SSBIO-AS003. (B) Comparison of on and off states of sAS124 at 0 and 5 mM IPTG with wild type *A. succinogenes* and strain sAS120. Values are normalized by absorbance (Materials and Methods). (C) Induction of sAS124 at IPTG levels ranging from 0 to 5 mM. Values are normalized by absorbance and wild-type (Materials and Methods). All error bars represent one standard deviation.

times stronger than P_{lac} and was predicted to set a higher maximum level of expression for the inducible system. The design strategy followed work in cyanobacterium [45, 46] and can be seen in Fig 4A. Due to the differences in length of the core promoter between P_{lac} (36 bp) and BBa_J23100 (35 bp), each possible nucleotide was inserted on the 5' end of the -35 region of BBa_J23100 (hereafter called position -36). Position -36 was chosen to keep the spacing between the operator sites O1 and O3 as well as between the -10 region and the transcription start site equivalent for the new promoter and P_{lac}. As can be seen in Fig 4B, there was significant variation among the four versions (strains sAS125-sAS128). A cytosine allowed for the greatest expression level whereas guanine and adenine showed decreased expression and thymine showed the least expression. Expression tests in *E. coli* revealed the same pattern, suggesting that the single nucleotide was crucial for some aspect of transcription. Sequences upstream of the -35 hexamer can have various regulatory effects due to interactions with transcription factors [47]. For example, the Cyclical-AMP receptor protein (CRP), which enhances polymerase binding, has a binding site in the *lac* system upstream of the -35 hexamer and improves transcription efficiency. It is possible that variations at the -36 site may be changing the binding affinity for CRP. Further exploration of *A. succinogenes* replication machinery will need to be conducted to elucidate the variation caused by the -36 site residues.

Performance of sAS125, containing the cytosine -36 residue, and which is hereafter labeled p100i, induced at 0 and 5 mM IPTG was compared to both wild type *A. succinogenes* and strain sAS100 containing the constitutive system with BBa_J23100. As can be seen in Fig 4C, expression in sSA125 at 0 mM IPTG was not different than wild type and expression in sSA125 at 5 mM IPTG was not different than sAS100. These findings confirmed that p100i was demonstrating a non-leaky off-state and complete induction. Cultures were grown at various concentrations of IPTG within the range of 0 to 5 mM. Visualization on a logarithmic scale showed a graded response (Fig 4D) and 481-fold dynamic range. The non-leaky nature of this inducible system is unique as even *E. coli* shows leakiness of basal expression [48], making this a very
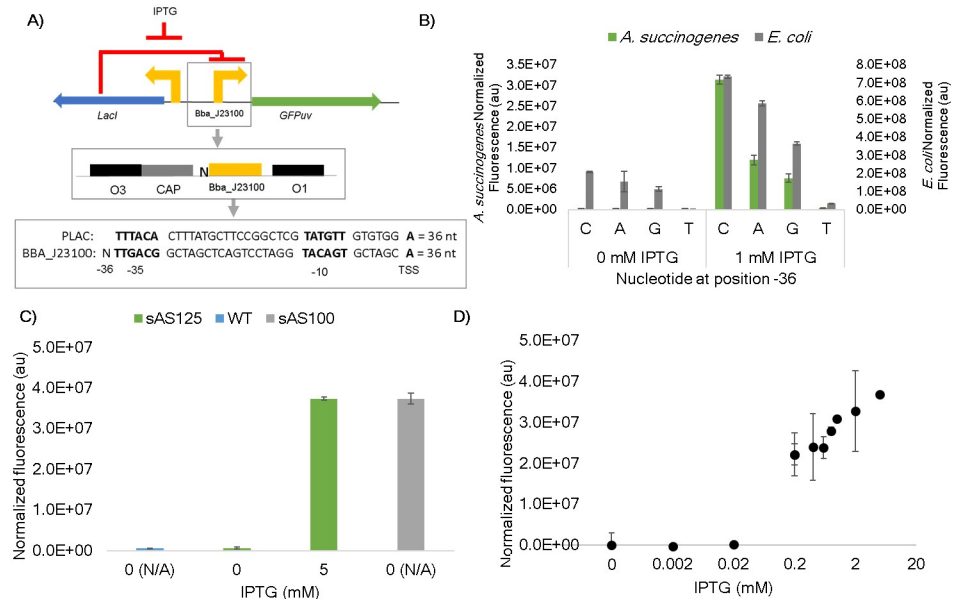
Fig 4. Characterization of strain sAS125 containing SSBIO-AS004 using GFPuv as the reporter protein. (A) Schematic of the expression system used in SSBIO-AS004 showing strategy of where to include the extra nucleotide to make size and spacing equivalent. (B) Comparison of expression variation due to the -36 nucleotide in both *A. succinogenes* and *E. coli* normalized by absorbance and respective wild type (Materials and Methods) at 0 and 1 mM IPTG. (C) Comparison of on and off states of sAS125 at 0 and 5 mM IPTG with wild type *A. succinogenes* and strain sAS100. Fluorescence values are normalized by absorbance (Materials and Methods). (D) Induction of sAS125 at IPTG levels ranging from 0.002 to 5 mM. Fluorescence values are normalized by absorbance and wild-type. All error bars represent one standard deviation.

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exciting development towards *A. succinogenes* metabolic engineering. Such a tool could be very useful for tuning metabolic gene expression within this organism.

Loss of succinic acid production

At this stage, we sought to test the effect of the presence of the plasmids on the production of SA and discovered a decrease in production not only present in the transformed strains, but also in the wild type used for comparison. Fig 5A shows the SA production loss in the working strains at this time point. This loss is presented in relation to fermentation measurements taken in wild type *A. succinogenes* at the beginning of the project (approximately 10 months before synthetic biology toolkit development) and also in relation to a brand-new strain purchased after the loss was realized. What can be seen is that the initial fermentation results and the results from the freshly purchased strain showed comparable levels of SA production after 24 hours. Here we cannot compare the additional organic acids (lactic acid, formic, acid, and acetic acid) because those were not included in the first set of measurements taken in the lab. The wild type strain that had been frozen and used as working stock in the lab for 10 months showed much lower SA production and also an increase in lactic acid. This unsettling finding prompted further investigation. The initial hypotheses were either contamination or mutation causing the decrease in SA production, both of which were tested by sequence comparisons between producing (hereafter SA(+)) and non-producing (SA(-)) strains. Using primers for 16S rRNA, sequencing of the SA(-) strain revealed a 99% match with *A. succinogenes*' reference genome (GCA_000017245.1), making contamination an unlikely cause. Whole genome comparisons between producing and non-producing strains revealed no major mutations, but one
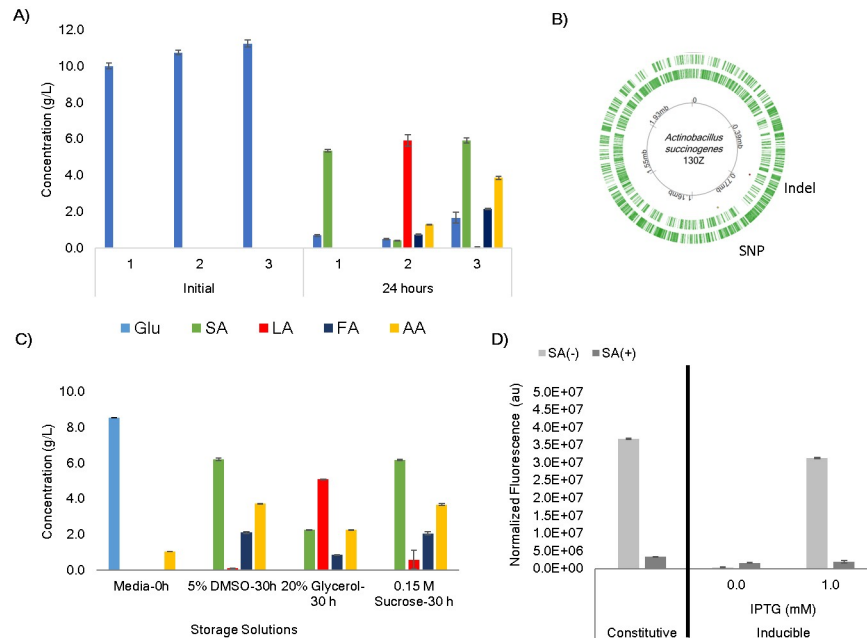
Fig 5. Findings related to succinic acid production loss in *A. succinogenes*. (A) Comparison of organic acid fermentation profiles between *A. succiongenes* working stock at the beginning of use in the lab (labeled 1 on the x-axis), ten months later (labeled 2), and a newly purchased strain (labeled 3) after 24 hours growth. Data was not collected for lactic acid, formic acid, or acetic acid in the first fermentation in the lab (labeled 1). (B) *A. succinogenes* genome map indicating positions of the indel mutation (loss of 5 nts) and SNP mutation (A->G) shown to be different in the SA(-) strain. (C) Production profiles of wildtype, SA(+) *A. succinogenes* after 30-hour fermentation following storage in DMSO, sucrose, and glycerol and being subjected to 10 freeze/thaw cycles. (D) GFP expression data in transformed SA (+) and SA(-) strains under the control of BBa_J23100 and p100i. Glucose (Glu), succinic acid (SA), lactic acid (LA), formic acid (FA), acetic acid (AA). All error bars represent standard deviation.

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small, 5 nucleotide deletion in SA(-) at position 731146 in an intergenic region and a single nucleotide polymorphism (A in SA(+) and reference, G in SA(-)) at position 1004969 at the 5' end of ASUC_RS04870 (Fig 5B). Investigation into these mutations revealed the SNP had been previously shown to have no effect on SA production [49]. While the deletion would have to be investigated to completely rule out mutation as the cause for the loss of SA production, such a minor difference between the two genomes in an intergenic region suggests there may be a better explanation.

Since the loss of SA production occurred after several months, one factor may be storage stress. Freeze-dried *A. succinogenes* cells were purchased from ATCC (ATCC 55618), resuspended in liquid culture, and frozen at -80˚C. Cryopreservation at -80˚C has been shown to preserve organisms like *E. coli* for years [50] and can be conducted using a variety of cryo-protectants to avoid water crystallization and subsequent cell damage [51]. For this study, *A. succinogenes* cells had been stored in a final concentration of 20% glycerol as the cryo-protectant and no other cryo-protectants had been tested. To see if a different cryoprotectant would prevent fermentation shifts, aliquots of a newly purchased SA(+) strain was stored in various cryoprotectants including the intracellular protectants glycerol (20%) and DMSO (5%), and the extracellular protectant sucrose (0.15M). Aliquots were then subjected to 10 freeze-thaw cycles to mimic use in the lab and SA fermentation at 30 hours was measured. The findings are shown in Fig 5C and indicate there is some difference in optimality of cryoprotectant used, with both DMSO and sucrose showing better protection than glycerol evidenced by maintenance of SA production. Cells stored in glycerol show the pattern of decreased SA and

increased lactic acid that we saw in the old strains used in the lab. However, there was not a complete loss of SA production and so this was not completely sufficient to explain the shift in fermentation profile seen after 10 months. In addition to investigating cryo-protectant effects, there are other storage strategies that could be tested. For bacterial cultures, ATCC utilizes both freeze drying and cryopreservation in the vapor phase of liquid nitrogen at -130˚C [50]. These strategies are alternatives that could potentially better preserve *A. succinogenes* over time.

At this point, a new SA(+) strain from ATCC was transformed with BBa_J23100 and p100i for further comparisons between SA(+) and SA(-) strains. These promoters were chosen due to the high expression levels of both and the tight inducibility of p100i. These notable characteristics were used to spot check if the developed tools would function the same when *A. succinogenes* produced SA. For the induced expression test, we chose to induce p100i at 0 and 1mM IPTG to see if the tool showed the expected "off" and "on" states respectively. Expression tests revealed that the differences between SA(+) and SA(-) strains extended to how the synthetic biology tools worked. As seen in Fig 5D, the tools were much less effective in the SA(+) strain with both BBa_J23100 and p100i showing much lower expression and p100i losing inducibility. Both promoters produced equivalent expression, with or without IPTG, suggesting that the inducible promoter was not turned off in this system and was operating at maximum, albeit low, expression. We did not try higher levels of IPTG since this showed the expression was already at the maximum possible. This finding adds to the picture of what is happening. Not only are there changes in overflow metabolism, but also changes in the expression of genes on a plasmid. This suggests that gene regulators play a role in the phenotypes of the different strains. Future work could be done to characterize the full set of promoters in an SA(+) strain, however, the underlying causes of the phenotypic changes should first be addressed.

Future work would include long-term studies investigating effects of storage conditions to reveal environmental components contributing the loss of SA production in *A. succinogenes*. Additional studies should include transcriptomics to elucidate the underlying shifts in gene expression that cause *A. succinogenes* to change from SA(+) to SA(-). We hypothesize a transcriptomic comparison between SA(+) and SA(-) will reveal differential gene expression related to sigma factors. Previous studies have shown very drastic fermentation profile changes linked to sigma factor switching in bacteria [52, 53]. Such patterns revealed in *A. succinogenes* could provide information on how SA production changes in this non-model bacterium long-term. It could also be helpful in pinpointing which regulators are key in how the developed synthetic biology tools are expressed. Identifying the factors contributing to both the loss of SA production and the change in the developed tool efficacy could reveal potential interventions that could prevent SA production loss, possibly recover lost SA production, or aid in making the tools more useful in the presence of SA production. This could create a more stable strain of *A. succinogenes* and boost its usefulness as a biological platform for bioproduction.

Conclusion

The findings reported in this work include vital information for researchers seeking to develop *A. succinogenes* as a biological platform. *A. succinogenes*, while a highly interesting bacterium for bioproduction purposes, is not well characterized. A shift in production capabilities following a standard practice for storage is a crucial piece of information for anyone working with this non-model species. In sharing this phenomenon, which has not previously been reported, we have brought to light an area of opportunity for further development of *A. succinogenes* as a stable and useful strain for SA bioproduction.

This work also disseminates information more broadly applicable to non-model bacteria research in general. The development of the inducible promoter system p100i for *A. succinogenes* demonstrates a strategy that could be highly useful in synthetic biology tool development for other non-model bacteria. The described approach for increasing the dynamic range of the inducible *lac* system could be specifically tailored for other organisms of interest.

The loss of SA production can also apply to non-model research in general by serving as a cautionary tale. In research involving non-model organisms, the lack of characterization and long-term studies can lead to unexpected challenges. Such organisms with unique traits can be full of surprises and we propose that careful observation over time should be included in studies seeking to add to their characterization. Specifically, groups working in non-model organisms should focus on their preservation processes and how they could affect phenotype. Taking this into account at the initiation of a project could advance the pace of work within potentially powerful organisms by avoiding "rabbit holes," like the one we have shared here.

The hurdles to developing non-model bacteria are worth overcoming. As has been seen in other non-model systems, time, effort, and innovative solutions have been able to advance work within organisms of interest [5, 6]. By sharing both exciting developments as well as pitfalls, the challenges of non-model systems can be overcome to unlock unique and promising capabilities for use as bio-platforms.

Materials and methods

Strain and media recipes

Actinobacillus succinogenes 130Z (ATCC 55618) was obtained from the American Type Culture Collection (Manassas, VA) and maintained in tryptic soy broth medium (G-Biosciences, St. Louis, MO) with 10 g/L glucose (Fisher Chemical, Hampton, NH) (TSBG hereafter). Seed cultures of *A. succinogenes* were started from 40% glycerol stock in 10 mL medium in 15 mL plastic conical tube and incubated at 37˚C, 250 rpm for 14 hours. Fermentation studies were conducted in fermentation medium containing 16.0 g/L yeast extract (Fisher BioReagents, Pittsburgh, PA), 1.0 g/L sodium chloride (Fisher Chemical), 1.36 g/L sodium acetate (Sigma-Aldric, St. Louis, MO), 0.20 g/L magnesium chloride, hexahydrate (Sigma Life Science, St. Louis, MO), and 0.20 g calcium chloride, dihydrate (Fisher Chemical) with D-glucose (Fisher Chemical) and buffered with magnesium carbonate (Acros Organic, Morris Plains, NJ) at 80% the sugar concentration.

Growth study and antibiotic screening procedures

Cultures for growth curve and antibiotic screening procedures were started from seed culture diluted to 0.02 OD_{600} or 2.5% (v/v), respectively, and were grown in 10 g/L TSBG. New tubes were opened at each time point to maintain anaerobic conditions throughout and all measurements were made in biological triplicate. Antibiotics used included kanamycin (Teknova, Hollister, CA), tetracycline (Thermo Scientific, Waltham, MA), ampicillin (Research Products International, Mount Prospect, IL), gentamicin (Fisher Scientific, Waltham, MA), spectinomycin (VWR, Radnor, PA), and chloramphenicol (VWR, Radnor, PA) at concentrations listed in Results and Discussion. Growth repression for each sample was calculated using Eq 1. Each sample OD_{600} (G_S) was divided by average growth in wild type (G_{WTavg}), converted to a percentage and subtracted from 100 to obtain the percent repression. Growth repression was

averaged across three sample replicates for each treatment.

$$100 - \left(\left(\frac{(G_S)}{(G_{WTavg})} \right) * 100 \right) \tag{1}$$

Plasmid construction

For the purpose of plasmid construction, primers were purchased from Eurofins Genomics (Louisville, KY) with no modifications. PCR templates were amplified with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) and amplified products were purified with PureLink Quick PCR Purification Kit (Invitrogen, Carlsbad, CA) or PureLink Gel Extraction Kit (Invitrogen). Sequences of genetic parts, the promoter library, primers used to amplify target sequences, plasmids constructed, and the resulting strains are listed in S1-S5 Tables in S1 File respectively.

Sequences and original sources of key genetic components are listed in S1 Table in S1 File. Hot fusion [54] was used to construct plasmid SSBIO-AS001. Parts included the *E. coli* and *A. succinogenes* origins of replication (originally from the PLGZ920 plasmid [55] generously donated by Dr. Gregg Beckham at the National Renewable Energy Lab in Golden, CO), *gFPuv* (originally from plasmid pBbB7a-GFP [56] purchased from Addgene 35358, Watertown, MA) under the control of P*lac* (originally from *E. coli* strain MG1655 purchased from Yale's Coli Genetic Stock Center https://cgsc.biology.yale.edu/), and a kanamycin resistance (from the plasmid pBBR1MCS-2 [57] purchased from Addgene 85168). All parts except kanamycin were amplified from a plasmid constructed from a previous project AS-Plac(c)-GFPuv-amp (unpublished, Saha Lab at UNL). The Anderson promoters from the constitutive promoter library (S2 Table in S1 File), were inserted into SSBIO-AS001 by first linearizing the vector, excluding the P*lac* promoter region, using primer pairs that included tails with the promoter library sequences. PCR products were treated with DpnI (Thermo Fisher Scientific, Waltham, MA), gel extracted, phosphorylated with T4 PNK (Thermo Fisher Scientific), and formed into plasmids with T4 DNA ligase (Thermo Fisher Scientific) following the user guide for self-circularization of linear DNA. As the native promoter, P*pcka* (the sequence of which was previously described [21], amplified from A. succiniogenes genomic DNA (extracted with Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit) was longer, hot fusion was used to combine it with linearized SSBIO-AS001 excluding the P*lac* promoter region. To construct SSBIO-AS002, the promoter variants P*pcka* and BBa-J114 of SSBIO-AS001 were linearized by PCR amplification excluding the *gFPuv* gene region. The flavin binding fluorescent protein gene was synthesized from previously known sequence [58] (Thermo Fisher Scientific) and the plasmid was formed using the hot fusion method. To construct plasmid SSBIO-AS003, the *E. coli* origin of replication, *A. succinogenes* origin of replication, and *gFPuv* under the control of P*lac* as well as the *lacI* gene (originally from *E. coli* strain MG1655) were amplified from the plasmid AS-Plac(i)-GFPuv-amp (unpublished, Saha Lab at UNL) in two pieces. These parts were combined with the previously used kanamycin resistance gene via the hot fusion method. To construct plasmid SSBIO-AS004, SSBIO-AS003, excluding the promoter region, was linearized using primers which contained tails with the sequence for promoter BBa-J23100. Blunt end ligation was used to form the plasmid.

Plasmids expressed in *A. succinogenes* were obtained from cultures of *E. coli* DH10B strain (NEB #C3019 New England Biolabs, Ipswich, MA) using PureLink Quick Plasmid Miniprep Kit (Invitrogen) and were sequence verified. Plasmids were inserted into *A. succinogenes* using

a room-temperature electroporation method [25] and strains sAS100 –sAS128 (S5 Table in S1 File) were frozen at -80˚C in 40% glycerol for further analysis.

Expression testing conditions

Expression tests were performed in 10 mL cultures started from 2.5% v/v dilutions of seed cultures and were conducted in biological triplicate. Strains containing the variants of SSBIO-AS001 and SSBIO-AS002 were grown in 10 mL TSBG with 50 µg/mL kanamycin at 37˚C, 250 rpm for 8 hours. Strains containing plasmids SSBIO-AS003 and SSBIO-AS004 were grown in 10 ML TSBG with 50 µg/mL kanamycin at 37˚C, 250 rpm for 1 hour, IPTG was added in concentrations ranging from 0–5 mM, and cultures were then grown for an additional 8 hours. 500 µL of each culture was pelleted and washed in 500 µL 1x phosphate buffer saline (hereafter PBS) two times. The washed pellets were resuspended in 200 µL 1x PBS and analyzed.

Fluorescence measurements

Measurements were taken using a Molecular Devices SpectraMax i3x Multi-Mode Microplate Detection Platform. Absorbance was measured at 600 nm. GFPuv expression was measured at excitation 395 nm and emission 509 nm after 12 hours of aeration. FbFP expression was measured at excitation 450 nm and emission 495 nm immediately follwing washing. Relative expression for each sample was calculated as shown in Eq 2. Fluorescence and absorbance of each sample (F_S and A_S respectively) had average PBS values for fluorescence and absorbance removed (F_{Pavg} and A_{Pavg} respectively) and then fluorescence was divided by absorbance. When comparisons were not made to wild type, expression of a wild type control (calculated using fluorescence, F_{WT}, and absorbance, A_{WT}) averaged across 3 replicates was then subtracted to remove background expression. All reported expression values were averaged across 3 replicates.

$$\left(\frac{(F_{Sn} - F_{Pavg})}{(A_{Sn} - A_{Pavg})} \right) - \left(\left(\frac{(F_{WT} - F_{Pavg})}{(A_{WT} - A_{Pavg})} \right)_{avg} \right) \tag{2}$$

Succinic acid batch fermentation

Seed cultures of *A. succinogenes* were diluted to 2.5% in 100mL of fermentation medium in rubber stoppered 125 mL Erlenmeyer flasks in triplicate with between 10 and 50 g/L. The varying sugar concentrations reported are because the data came from comparing a glucose control to another carbon source that varied. Due to making the comparisons across time, we were not able to go back and repeat the studies with equivalent amounts of glucose. The flasks were equipped with check valves to relieve pressure buildup. Cultures were grown at 37 ˚C and 150 rpm until sampling. Samples were either drawn up through a valve or when cultures were sacrificed. Stoppers were not removed at any point during the fermentation. Growth phase was determined by measuring OD600 of the samples in a spectrophotometer (Thermo Scientific Genesys 10S UV-Vis). Then, cells were pelleted (15 min at 3,000 g) and filtered through 0.45-micron filter into a clean vial.

Concentration of glucose, SA, lactic-, formic- and acetic- acid in the samples were determined by high- performance liquid chromatography (HPLC). A 10 µL cell-free sample was injected into a BioRad chromatographic column (Aminex HPX-87H, 7.8 mm 300 mm, Biorad, USA). The HPLC was equipped with a P680 pump, Dionex, USA and a refractive index

detector, RI101 (Shodex, USA). The column temperature was set at 65˚C and the eluant used was 0.01 N H2SO4 at a flow rate of 0.6 ml/min.

Genomic sequencing and alignment

Cultures of SA(+) and SA(-) strains were grown to stationary phase and the genomic DNA was extracted (Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit) for concentrations of 279.1 and 248.9 ng/μl respectively. The samples were sent to the University of Nebraska Medical Center Sequencing Core, Omaha, NE and whole genome sequence data was obtained by Illumina paired end sequencing with read sizes of 150 bases and 100x coverage. The sequence libraries have been registered in the Sequence Read Archive (accession number PRJNA713368) for the SA(+) and SA(-) strains of *A. succinogenes* (BioSample accession numbers SAMN18247005 and SAMN18247006, respectively). For each strain, reads were trimmed, an index was constructed, and reads were aligned using Bowtie [59] to the *A. succinogenes* reference genome (GCA_000017245.1). The SA(+) strain had 2792897 raw read pairs, 2694757 trimmed reads, and an overall alignment rate of 99.92%. The SA(-) strain had 2457603 raw read pairs, 2385139 trimmed reads, and an overall alignment rate of 91.15%. Alignments that had a mapping score <30 were removed. To perform variant calling analysis, Samtools [60] was used to convert from SAM to BAM files and sort them by coordinates. Bcftools was then used to generate the pileups, call SNP and indels, filter high quality loci, and output the variant calling file. SNPs and INDELs for which the q-value was <20 OR the depth <100 were removed. The significant variants that were different in the mutant at positions 731146 and 1004969 had read depths of 178 and 248 respectively.

Supporting information

S1 File.
(PDF)

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References

1. Herman BG, Blok K, Patel MK. Producing bio-based bulk chemicals using industrial biotechnology saves energy and combats climate change. Environmental Science and Technology. 2007; 41 (22):7915–21. https://doi.org/10.1021/es062559q PMID: 18075108

2. Becker J, Lange A, Fabriuse J, Wittmann C. Top value platform chemicals: bio-based production of organic acids. Current Opinions in Biotechnology. 2015; 36:168–75. https://doi.org/10.1016/j.copbio.2015.08.022 PMID: 26360870

3. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature. 2006; 440(7086):940–3. https://doi.org/10.1038/nature04640 PMID: 16612385

4. Maeda T, Sanchez-Torres V, Wood TK. Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*. Applied Microbiology and Biotechnology. 2007; 77(4):879–90. https://doi.org/10.1007/s00253-007-1217-0 PMID: 17938909

5. Yan Q, Fong SS. Challenges and Advances for Genetic Engineering of Non-model Bacteria and Uses in Consolidated Bioprocessing. 2017(1664-302X (Print)).

6. Fatma Z, Schultz JC, Zhao H. Recent advances in domesticating non-model microorganisms. Biotechnology progress. 2020;n/a(n/a):e3008. https://doi.org/10.1002/btpr.3008 PMID: 32329213

7. Liu L, Otoupal P, Pan A, Alper HS. Increasing expression level and copy number of a Yarrowia lipolytica plasmid through regulated centromere function. FEMS Yeast Research. 2014; 14(7):1124–7. https://doi.org/10.1111/1567-1364.12201 PMID: 25154452

8. Mo J, Wang S, Zhang W, Li C, Deng Z, Zhang L, et al. Efficient editing DNA regions with high sequence identity in actinomycetal genomes by a CRISPR-Cas9 system. Synth Syst Biotechnol. 2019; 4(2):86–91. https://doi.org/10.1016/j.synbio.2019.02.004 PMID: 30891508

9. Beauprez JJ, De Mey M, Soetaert WK. Microbial succinic acid production: natural versus metabolic engineered producers. Process Biochemistry. 2010; 45:1103–14.

10. Zeikus JG, Jain MK, Elankovan P. Biotechnology of succinic acid production and markets for derived industrial products. Applied Microbiology and Biotechnology. 1999; 51(5):545–52.

11. Bechthold I, Bretz K, Kabasci S, Kopitzky R, Springer A. Succinic acid: a new platform chemical for bio-based polymers from renewable resources. Chemical Engineering and Technology. 2008; 31:1–14.

12. Cukalovic A, Stevens CV. Feasibility of production methods for succinic acid derivatives: A marriage of renewable resources and chemical technology. Biofuels, Bioproducts, and Biorefining. 2008; 2(6):505–29.

13. Song H, Lee SY. Production of succinic acid by bacterial fermentation. Enzyme and Microbial Technology. 2006; 39(3):352–61.

14. McKinlay JB, Vieille C, Zeikus JG. Prospects for a bio-based succinate industry. Applied Microbiology and Biotechnology. 2007; 76:727–40. https://doi.org/10.1007/s00253-007-1057-y PMID: 17609945

15. Bozell JJ, Peterson GR. Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chemistry. 2010; 12.

16. Guettler MV, Rumler D, Jain MK. *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. International journal of systematic bacteriology. 1999; 49:207–16. https://doi.org/10.1099/00207713-49-1-207 PMID: 10028265

17. McKinlay JB, Laivenieks M, Schindler BD, McKinlay AA, Siddaramappa S, Challacombe JF, et al. A genomic perspective on the potential of A*ctinobacillus succinogenes* for industrial succinate production. BMC Genomics. 2010; 11. https://doi.org/10.1186/1471-2164-11-680 PMID: 21118570

18. Brink HG, Nicol W. Succinic acid production with A*ctinobacillus succinogenes*: rate and yield analysis of chemostat and biofilm cultures. Microbial Cell Factories. 2014; 13:111. https://doi.org/10.1186/s12934-014-0111-6 PMID: 25259880

19. van Heerden CD, Nicol W. Continuous and batch cultures of *Escherichia coli* KJ134 for succinic acid fermentation: metabolic flux distributions and production characteristics. 2013; 12(80):1–10.

20. Bradfield MF, Mohagheghi A, Salvachua D, Smith H, Black BA, Dowe N, et al. Continuous succinic acid production by *Actinobacillus succinogenes* on xylose-enriched hydrolysate. Biotechnology for biofuels. 2015; 8:181. https://doi.org/10.1186/s13068-015-0363-3 PMID: 26581168

21. Guarnieri MT, Chou Y, Salvachúa D, Mohagheghi A, John PC, Peterson DJ, et al. Metabolic engineering of *Actinobacillus succinogenes* provides insights into succinic acid biosynthesis. Applied and Environmental Microbiology. 2017; 83(17). https://doi.org/10.1128/AEM.00996-17 PMID: 28625987

22. Joshi RV, Schindler BD, McPherson NR, Tiwari K, Vieille C. Development of a markerless knockout method for A*ctinobacillus succinogenes.* Applied and Environmental Microbiology. 2014; 80:3053–61. https://doi.org/10.1128/AEM.00492-14 PMID: 24610845

23. Zhang W, Yang Q, Wu M, Liu H, Zhou J, Dong W, et al. Metabolic Regulation of Organic Acid Biosynthesis in Actinobacillus succinogenes. Frontiers in Bioengineering and Biotechnology. 2019; 7(216). https://doi.org/10.3389/fbioe.2019.00216 PMID: 31620431

24. Keasling JD. Synthetic biology and the development of tools for metabolic engineering. Metabolic Engineering. 2012; 14:189–95. https://doi.org/10.1016/j.ymben.2012.01.004 PMID: 22314049

25. Tu Q, Yin J, Fu J, Herrmann J, Li Y, Yin Y, et al. Room temperature electrocompetent bacterial cells improve DNA transformation and recombineering efficiency. Nature Scientific Reports. 2016; 6. https://doi.org/10.1038/srep24648 PMID: 27095488

26. Li Q, Wang D, Song Z, Zhou W, Wu Y, Xing J, et al. Dual-phase fermentation enables *Actinobaccilus succinogenes* 130Z to be a potential role for high-level lactate production from the bioresource. Bioresource technology. 2010; 101(19):7665–7. https://doi.org/10.1016/j.biortech.2010.04.058 PMID: 20483592

27. Almqvist H, Pateraki C, Alexandri M, Koutinas A, Liden G. Succinic acid production by *Actinobacillus succinogenes* from batch fermentation of mixed sugars. Journal of Industrial Microbiology and Biotechnology. 2016; 43(8):1117–30. https://doi.org/10.1007/s10295-016-1787-x PMID: 27255975

28. Pateraki C, Patsalou M, Vlysidis A, Kopsahelis N, Webb C, Koutinas AA, et al. Actinobacillus succinogenes: Advances on succinic acid production and prospects for development of integrated biorefineries. Biochemical Engineering Journal. 2016; 112:285–303.

29. McKinlay JB, Zeikus JG, Vieille C. Insights into *Actinobacillus succinogenes* fermentative metabolism in a chemically defined growth medium. Applied and Environmental Microbiology. 2005; 71(11):6651–6. https://doi.org/10.1128/AEM.71.11.6651-6656.2005 PMID: 16269693

30. Jang YS, Jung YR, Lee SY, Kim JM, Lee JW, Oh DB, et al. Construction and characterization of shuttle vectors for succinic acid-producing rumen bacteria. Applied Environmental Microbiology. 2007; 73(17). https://doi.org/10.1128/AEM.01382-07 PMID: 17616626

31. Krause KM, Serio AW, Kane TR, Connolly LE. Aminoglycosides: An Overview. Cold Spring Harbor Perspectives in Medicine. 2016; 6(6). https://doi.org/10.1101/cshperspect.a027029 PMID: 27252397

32. Anderson KN, Kennedy RP, Plorde JJ, Shulman JA, Petersdorf RG. Effectiveness of Ampicillin Against Gram-Negative Bacteria: In Vitro and In Vivo Studies of a New Antibiotic. JAMA. 1964; 187(8):555–61.

33. Richmond MH, Sykes RB. The β-lactamases of Gram-negative bacteria and their possible physiological role. Advances in Microbial Physiology. 1973; 9:31–88. https://doi.org/10.1016/s0065-2911(08)60376-8

34. Cooper TG, Magasanik B. Transcription of the *lac* operon of *Escherichia coli*. The Journal of Biological Chemistry. 1974; 249:6556–61. PMID: 4609086

35. Promoters/Catalog/Anderson [Internet]. 2008. Available from: http://parts.igem.org/Promoters/Catalog/Anderson.

36. Bagdasarian MM, Amann E, Lurz R, Ruckert B, Bagdasarian M. Activity of the hybrid trp-lac (tac) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled expression vectors. Gene. 1983; 26:273–82. https://doi.org/10.1016/0378-1119(83)90197-x

37. Radeck J, Kraft K, Bartels J, Cikovic T, Durr F, Emenegger J, et al. The Bacillus BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*. Journal of Biological Engineering. 2013; 7(1):29. https://doi.org/10.1186/1754-1611-7-29 PMID: 24295448

38. Camsund D, Heidorn T, Lindblad P. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. Journal of Biological Engineering. 2014; 8(4).

39. Cook TB, Rand JM, Nurani W, Courtney DK, Liu SA, Pfleger BF. Genetic tools for reliable gene expression and recombineering in *Pseudomonas putida*. Journal of Industrial Microbiology and Biotechnology. 2018; 45(7):517–27. https://doi.org/10.1007/s10295-017-2001-5 PMID: 29299733

40. Li S, Sun T, Xu C, Chen L, Zhang W. Development and optimization of genetic toolboxes for a fast growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Metabolic Engineering. 2018; 48:163–74. https://doi.org/10.1016/j.ymben.2018.06.002 PMID: 29883802

41. Crameri A, Whitehorn Ea Fau—Tate E, Tate E Fau—Stemmer WP, Stemmer WP. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat Biotech. 1996; 14(3):315–9. https://doi.org/10.1038/nbt0396-315

42. Mukherjee A, Weyant KB, Walker J, Schroeder CM. Directed evolution of bright mutants of an oxygen-independent flavin-binding fluorescent protein from Pseudomonas putida. Journal of Biological Engineering. 2012; 6(1):20. https://doi.org/10.1186/1754-1611-6-20 PMID: 23095243

43. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science. 1994; 263:802–5. https://doi.org/10.1126/science.8303295 PMID: 8303295

44. Browning DF, Busby SJW. The regulation of bacterial transcription initiation. Nature Reviews Microbiology. 2004; 2:57–65. https://doi.org/10.1038/nrmicro787

45. Markley AL, Begemann MB, Clarke RE, Gordon GC, Pfleger BF. Synthetic biology toolbox for controlling gene expression in the cyanobacterium *Synechococcus* sp. strain PCC 7002. ACS Synthetic Biology. 2015; 4(5):595–603. https://doi.org/10.1021/sb500260k PMID: 25216157

46. Albers SC, Gallegos VA, Peebles CAM. Engineering of genetic control tools in Synechocystis sp. PCC 6803 using rational design techniques. Journal of biotechnology. 2015; 216:36–46. https://doi.org/10.1016/j.jbiotec.2015.09.042 PMID: 26450561

47. Davis JH, Rubin AJ, Sauer RT. Design, construction and characterization of a set of insulated bacterial promoters. Nucleic Acids Research. 2011; 39(3):1131–41. https://doi.org/10.1093/nar/gkq810 PMID: 20843779

48. Rosano G, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and challenges. Frontiers in Microbiology. 2014; 5:172. https://doi.org/10.3389/fmicb.2014.00172 PMID: 24860555

49. McPherson N. Novel Insights Into Sugar and Succinate Metabolism of Actinobacillus succinogenes from Strains Evolved for Improved Growth on Lignocellulose Hydrolysate Sugars: Michigan State University; 2017.

50. FAQs-Long term storage of cultures 2014 [updated 01/14/2014. https://www.atcc.org/support/faqs/f5ad2/Long%20term%20storage%20of%20cultures-976.aspx.

51. Brockbank KGM, Covault JC, Taylor MJ. Cryopreservation Guide. 2007.

52. Qiu Y, Nagarajan H, Embree M, Shieu W, Abate E, Juárez K, et al. Characterizing the interplay between multiple levels of organization within bacterial sigma factor regulatory networks. Nature Communications. 2013; 4(1):1755. https://doi.org/10.1038/ncomms2743 PMID: 23612296

53. Taniguchi H, Wendisch VF. Exploring the role of sigma factor gene expression on production by Corynebacterium glutamicum: sigma factor H and FMN as example. Frontiers in microbiology. 2015; 6:740-. https://doi.org/10.3389/fmicb.2015.00740 PMID: 26257719

54. Fu C, Donovan WP, Shikapwashya-Hasser O, Ye X, Cole RH. Hot Fusion: An Efficient Method to Clone Multiple DNA Fragments as Well as Inverted Repeats without Ligase. PLoS ONE. 2014; 9(12). https://doi.org/10.1371/journal.pone.0115318 PMID: 25551825

55. Kim P, Laivenieks M, McKinlay J, Vieille C, Gregory Zeikus J. Construction of a shuttle vector for the overexpression of recombinant proteins in Actinobacillus succinogenes. Plasmid. 2004; 51(2). https://doi.org/10.1016/j.plasmid.2003.11.003 PMID: 15003707

56. Lee TS, Krupa RA, Zhang F, Hajimorad M, Holtz WJ, Prasad N, et al. BglBrick vectors and datasheets: A synthetic biology platform for gene expression. Journal of Biological Engineering. 2011; 5(1):12. https://doi.org/10.1186/1754-1611-5-12 PMID: 21933410

57. Kovach ME, Elzer PH, Steven Hill D, Robertson GT, Farris MA, Roop RM, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 1995; 166(1):175–6. https://doi.org/10.1016/0378-1119(95)00584-1 PMID: 8529885

58. Immethun CM, Ng KM, DeLorenzo DM, Waldron-Feinstein B, Lee YC, Moon TS. Oxygen-responsive genetic circuits constructed in *Synechocystis* sp. PCC 6803. Biotechnology and Bioengineering. 2016; 113(2). https://doi.org/10.1002/bit.25722 PMID: 26264210

59. Langmead B, Trapnell C, Pop M, Salzberg SI. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Gen Biol. 2009; 10. https://doi.org/10.1186/gb-2009-10-3-r25 PMID: 19261174

60. Li H, Handsaker B, Wysoker A, Fennel T, Ruan J, Homer N, et al. The Sequence alignment/map (SAM) format and SAMtools. Bioinformatics. 2009; 25:2078–9. https://doi.org/10.1093/bioinformatics/btp352 PMID: 19505943