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Towards stable cyanobacterial cell factories

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2 Nonhierarchical Flux Regulation Exposes the Fitness Burden Associated with Lactate Production in *Synechocystis* sp. PCC6803

Cyanobacteria are mostly engineered to be sustainable cell-factories by genetic manipulations alone. Here, by modulating the concentration of allosteric effectors, we focus on increasing product formation without further burdening the cells with increased expression of enzymes. Resorting to a novel 96-well microplate cultivation system for cyanobacteria, and using lactate-producing strains of *Synechocystis* sp. PCC6803 expressing different L-lactate dehydrogenases (LDH), we titrated the effect of 2,5-anhydro-mannitol supplementation. The latter acts in cells as a nonmetabolizable analogue of fructose 1,6-bisphosphate, a known allosteric regulator of one of the tested LDHs. In this strain (SAA023), we achieved over 2-fold increase of lactate productivity. Furthermore, we observed that as carbon is increasingly deviated during growth toward product formation, there is an increased fixation rate in the population of spontaneous mutants harboring an impaired production pathway. This is a challenge in the development of green cell factories, which may be countered by the incorporation in biotechnological processes of strategies such as the one pioneered here.

Oxygenic photosynthetic organisms have the potential to be used as much-needed sustainable cell factories [51–53], directly converting CO_2 and water into compounds of interest by harvesting energy from light. In recent years, the production of an impressive array of commodity chemicals has been achieved [54]. This inevitably relies on the heterologous expression of production pathways that reroute part of the fixed CO_2 to product. Most often, efforts to increase productivity are centered on the strategy of increasing the levels of the heterologous enzymes without much consideration about the interactions of the "alien" pathway with the native system in which it has been imbedded. Metabolic control analysis applied to these "green" cell factories has shown that, rather rapidly, limitations in the flux toward the product of interest may be shifted to factors other than enzyme levels [55]. At this stage, attention is generally moved to *(i)* increasing substrate availability, by either eliminating competing pathways [56,57] and/or increasing the expression of enzymes that are anticipated to lead to higher concentrations of the substrate [58,59]; or *(ii)* increasing the thermodynamic force driving the reaction by adjusting cofactor specificity and/or availability [17,60,61]. The modulation of flux by making use of allosteric effectors has remained greatly unexplored.

In this study, against the backdrop of a success story—lactate production by *Synechocystis* sp. PCC6803 (*Synechocystis*, Figure 2.1A)—we explored ways to further increase production without resorting to changes in expression of L-lactate dehydrogenase (L-LDH). Cells can achieve changes in their physiology and metabolism through variations of the flux through a biochemical reaction. If such a variation is caused by changes in enzyme levels, cells are using hierarchical regulation [62]. Here, however, we focused on the nonhierarchical control of the flux through LDH. As proof of concept, we used a nonmetabolizable analogue (2,5-anhydro-mannitol, AHM; Figure 2.1B) of one of LDH's known effectors (fructose 1,6- bisphosphate, FBP) [63] to modulate its enzymatic activity. We found that the specific lactate productivity (q_p) can be effectively tuned by the addition of AHM, leading to increases of lactate production of over 2-fold. We also observed that if the system is pushed too hard, this leads to the fixation of mutants in the population that have lost the ability to produce lactate, and thus, can avoid the burden of excessive lactate production.
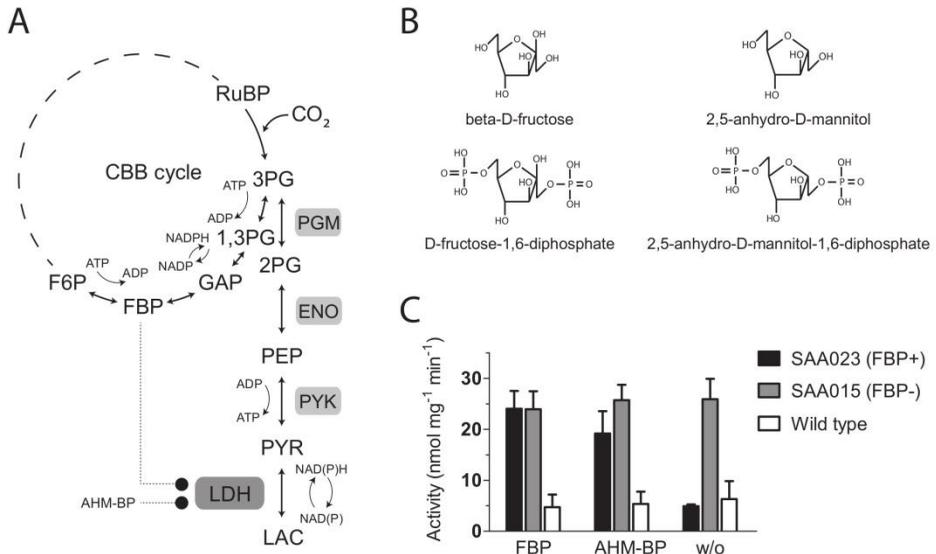
Figure 2.1 Engineered lactate pathway and its dependency on allosteric regulation. (A) Schematic overview of the engineered lactate-forming pathway in *Synechocystis*. The L-LDH of *L. lactis* (FBP+) and *B. subtilis* (FBP−) are complementing the pathway to L-lactate formation utilizing pyruvate as substrate. As schematically depicted the heterologously expressed FBP-dependent (FBP+) LDH is activated through native FBP and AHM-BP (supplied externally in the form of AHM). (B) Chemical structures of both fructose and anhydro-mannitol and the respective dephosphorylated forms showing their structural similarity. (C) FBP and AHM-BP-dependency of LDH activity *in vitro*. Cell-free extracts of *Synechocystis* wild type, SAA023 (FBP+) and SAA015 (FBP−) were subjected to a LDH activity assay. The activity of the LDH originating from *L. lactis* is FBP/AHM-BP dependent, whereas the activity of the LDH from *B. subtilis* is not.

LDHs from different organisms, despite their sequence homology and shared catalytic properties, are allosterically regulated in diverse ways [64]. For instance, while the native LDH of the soil bacterium *Bacillus subtilis* (*B. subtilis*) appears to be FBP-insensitive [65], the one of the lactic acid bacterium *Lactococcus lactis sp. cremoris* (*L. lactis*) requires this metabolite for enzyme activity [66]. *Synechocystis* strains expressing separately the genes encoding either of these enzymes have been constructed in previous studies [60]. We tested the FBP dependency of both these LDHs by determining their activity in cell extracts of either cyanobacterial strains expressing them in the presence, or absence, of FBP. Just like in their native hosts, the LDH originating from *L. lactis* (expressed in strain SAA023) requires FBP to be active, while the one originating from *B. subtilis* (expressed in SAA015) does not (Figure 2.1C). We then examined *in vitro* whether the biphosphorylated form of AHM (AHM-BP) could also selectively act as an activator of the LDH from *L.lactis*, while not affecting the activity of the LDH from *B. subtilis*. The result showed a similar

pattern as the one observed for FBP (Figure 2.1C). This equipped us with an ideal control, which assures that the phenotypes to be observed *in vivo* (see below) are due to the direct effect of the activator on lactate production and not due to other motives.

The effects of varying concentrations of AHM on *Synechocystis* wild type and derivative mutants, harboring different LDHs with different FBP-sensitivities, were quantified for both lactate production and growth rate in a 96-well plate cultivation system. This system has been tested and validated to eliminate positional bias (Supporting Information). After assigning the combinations of different strains with different concentrations of AHM in the plate while following a nonsequential pattern (Figure S2.1), both the growth curves and lactic acid concentrations were monitored. Over the whole range of AHM concentrations tested (0 to 2.5 mM), for both the wild type strain and the FBP-insensitive (FBP−) strain, the growth rates were not impaired at low concentrations of AHM, and only mildly at higher concentrations (Figure 2.2A). The latter inhibition may be due to the regulatory function of FBP at other locations in metabolism [67]. In contrast, for the FBP-sensitive (FBP+) strain, growth rate dropped sharply when the concentration of AHM reached values above 0.3 mM, eventually resulting in no observable growth above 0.6 mM. Concerning the lactate productivity (or lactate yield, Figure S2.2), as expected, AHM has no significant effect on the FBP-insensitive strain (Figure 2.2B). While, for the FBP-sensitive strain, the lactate productivity increased as the AHM concentration was raised up to 0.3 mM, where maximum lactate productivity was observed. With further increasing concentration of AHM, lactate productivity dropped (Figure 2.2B) along with the decreasing growth rates. These results show that AHM can indeed be utilized *in vivo* to regulate the flux toward product formation for the FBP-sensitive strain. We then decided to test whether the boosting effect on lactate production would also be observed in a larger lab-scale cultivation setup. The concentrations of 0.1 and 0.3 mM of AHM were chosen to be further tested, since in those cultures (on a 96-well microplate scale) we could see enhanced lactate productivity with negligible negative effects on growth kinetics.

The Multi-Cultivator MC 1000, which allows for 8-parallel cultivations in a tightly controllable environment, was employed for validation of the effects of AHM on lactate production for the FBP+ strain. Both the growth kinetics and the lactate production were monitored at a concentration of 0, 0.1, and 0.3 mM of AHM, (Figure 2.3A and B). Surprisingly, the addition of 0.3 mM AHM suppressed cell growth for approximately 200 h, after which, growth started recovering. No significant differences in maximal growth rate for the cells with 0 and 0.1 mM AHM was observed, corroborating observations from the 96-well plate growth

assay (Figure 2.2A). However, the culture treated with 0.1 mM AHM showed an earlier slowing down of growth and a lower final optical density than the culture without AHM. As for lactate production, the cultures with 0.1 and 0.3 mM AHM display higher lactate production than the cultures without AHM for the first 200 h of the cultivation. Thereafter, the lactate production rate dropped in particular in the culture treated with 0.3 mM AHM. Thus, while higher AHM concentrations led to higher production in the early stages of growth, they subsequently resulted in a sharp drop of lactate production at later growth stages. In fact, when closely examining the relationship between growth and lactate production, we find that cultures in the presence of 0.3 mM AHM recovered from the growth suppression exactly as they stopped producing high amounts of lactate. We hypothesized that the observed reduction in lactate production might then be a consequence of the accumulation of naturally occurring mutations that render the gene cassette nonfunctional. In line with known trade-offs in cellular resource allocation [68], the higher the amount of AHM added, the bigger the burden for the cell will be, since more carbon is deviated from anabolic processes toward product formation. The chance of fixation of burden-releasing mutations is highest when the difference in relative fitness (here, growth rate) is greatest. This would mean that the higher the amount of AHM added, the higher the probability that during the cultivation mutants hampered in lactate production would start to dominate the population.
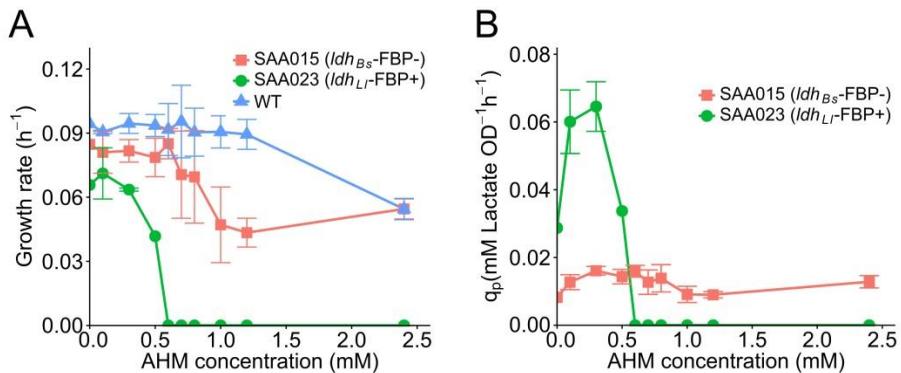
Figure 2.2 *In vivo* allosteric regulation of lactate production in a 96-well format cultivation system under constant light conditions of approximately 50 µE m^{-2} s^{-1}. (A) Effects of AHM on growth rate for wild type, SAA015 (FBP−) and SAA023 (FBP+); (B) effects of AHM on lactate productivity for SAA015 (FBP−) and SAA023 (FBP+). Error bars indicate standard deviation of at least 3 independent replicate cultures, except for SAA023 cultured with 0.5 mM AHM, where growth rate could be reliably calculated only for one of the replicates.

Figure 2.3 Lactate production in the Multi-Cultivator for SAA023 (FBP+) with different amounts of AHM and mutation analysis during the later growth stage under constant light illumination of 120 μE m^{-2} s^{-1} supplied with 99% N$_2$ and 1% CO$_2$. (A) Growth curves and (B) lactate concentration per biomass unit over time in the Multi-Cultivator; (C) the annotated conserved functional domains of the L-lactate dehydrogenase (from NCBI database) and (D) different types of mutations based on translation analysis from DNA sequence of (E) the isolated single colonies. Error bars indicate the standard deviation of 2 replicates for cultures without AHM, and 3 replicates for the cultures with 0.1 and 0.3 mM AHM. The red arrows in (B) indicate the sampling time points. "tt" in (C) stands for transcriptional terminator.

To test this hypothesis, DNA samples at two distinct time points from three independent biological replicates were collected. Sequence analysis was performed either directly at the population level, or individually on single colony isolates. The gene encoding L-lactate dehydrogenase, along with its upstream promoter region, was sequenced. Primers were designed such that they are binding at different locations of the gene cassette covering approximately 600 bp stretches (Table S2.1). The sequencing results from the whole populations

did not yield discernible mutations, as mixtures of different sequences were found (Figure S2.3). Since *Synechocystis* is known to carry multiple copies of its chromosome [69], this could mean that *(i)* either allelic differences within a single bacterium could be occurring, or *(ii)* that several mutations co-occur in different isolates of the population. The sequence analysis of the isolated colonies revealed that the latter seems to be the case, as different types of fully segregated mutations within the LDH cassette could be found (Figure 2.3C). These mutations were either nucleotide insertions or deletions, leading to a truncated protein because of a premature stop codon, or point mutations, which affected the predicted active sites (Figure 2.3DE, see Supplementary data for the complete sequence alignment). The common ground is that these mutations have a negative impact on the functionality of the LDH cassette, resulting in the impairment of product formation. Among the sequenced single colonies, the single nucleotide insertion that leads to a truncated protein of only 88 amino acids (Figure 2.3E, "Is") was most frequently observed. This is probably due to the fact that this type of mutation not only efficiently eliminates the burden of lactate production, but presumably also reduces the costs associated with heterologous protein synthesis [70]. Another frequent point mutation occurs at the site of NAD and substrate binding (Figure 2.3, "P1"). Since this latter mutation is not as efficient as the previous one in reducing the protein burden, it hints that the added value of the latter is not sufficient enough to totally outcompete mutations that only abolish product formation. Strikingly, in two of the isolated colonies multiple mutations of both types were also found (Figure 2.3E). The detailed mechanism introducing the mutations remains to be fully elucidated. Genetic instability of cyanobacterial cell factories caused by mutations was largely ignored so far and seldom reported, although it is of paramount importance as a major challenge during up-scaling and commercialization [60,71]. Here we report several distinct genetic mutations that affect LDH functionality at different positions. This suggests that there are distinct evolutionary trajectories possible leading toward the impairment of the production cassette.

Genetic engineering has made it possible to use cyanobacteria to convert CO_2 directly into various chemicals. Due to its important positive environmental impact, considerable efforts have been made to accelerate this process as reviewed [72] and exemplified [73], recently. High-throughput cultivation and screening platforms, which enable the screening of large numbers of variants or conditions, play therefore an important role. However, these systems are different from the ones already available for chemoheterotrophs because of the need for an illumination source. High-throughput screening and sorting of single cyanobacterial cells aided by microfluidics has only recently been reported for lactate [74] and ethanol production [75]. With respect to miniaturized

cultivation systems for cyanobacteria, also only a few examples have been reported, though either the description lacks detail to judge the potential [76] or the system appears relatively costly and complicated to operate [77]. In this work, we developed a simple and practical 96-well cultivation system by directly placing an ordinary 96-well microplate on a shaking platform inside a conventional light incubator. The method has been validated, allowing a parallel, miniaturized and cost efficient process, here used to study the effect of AHM on lactate production. Contrary to the commonly used genetic engineering strategies that rely on the modulation of protein expression levels to change the flux distribution through a metabolic pathway, we achieve this flux modulation by dosing allosteric effectors through the addition of a nonmetabolized analogue. Presumably, the addition of exogenous AHM is taken up via a hexose transporter (*e.g. sll1087*), then sequentially converted to AHM-P and AHM-BP through the activities of glucokinase (*sll0593)* and phosphofructokinase (*sll1196*), respectively [63,78,79]. This external supplementation of AHM has led here to the expected effect for both *in vitro* enzyme activities and *in vivo* lactate production. Recently, allosteric regulation has been explored in cyanobacteria to efficiently increase the production of the native compound, cyanophycin [80]. Here, we show that this approach can be used with heterologous pathways as well, allowing the fine-tuning of their flux. As a complementary strategy, allosteric regulation has the benefit of alleviating the burden of protein cost in a relatively simple fashion. In this study, we found that cultures tuned through the addition of an allosteric effector (AHM) to maintain higher productivity, quickly accumulate genetic mutants with a disrupted heterologous cassette that eventually results in a drop in product formation. This phenomenon is regularly observed and acknowledged for chemotrophic microorganisms, but largely neglected in the literature on cyanobacteria and reported rarely [81–84]. Here a detailed analysis is presented of the mutations that fixate in the population, and how these lead to loss of productivity. These results are useful in devising engineering strategies with increased stability [85], a requirement to apply sustainable "green" cell factories on an industrial scale.

2.1   Methods

Cultivation of *Synechocytis* for Cell Free Extract Preparation. The *Synechocystis* strains under investigation were recovered from −80 °C 5% (v/v) DMSO stocks on BG-11 (Cyanobacteria BG-11 Freshwater Solution, Sigma) containing plates supplemented with 1.5% (w/v) agar, 10 mM TES-KOH (pH = 8), 0.3% (w/v) sodium thiosulfate, and with 50 μg mL^{-1} kanamycin added if appropriate (Table 2.1). Liquid cultures were started with cell material originating from a single colony inoculated into BG-11 media supplemented with 10 mM TES-KOH (pH = 8) and antibiotics if appropriate. The glass flasks were shaking at 120 rpm at 30 °C in a temperature controlled incubator (Innova

43, New Brunswick Scientific) and were illuminated with 35 µE m^{-2} s^{-1} of white-light. Cells were harvested in mid log phase for cell free extract preparation.

Table 2.1 List of Strains and Plasmids Used in This Study

Strain/Plasmid	Genetic background	Reference
Wild type	Glucose tolerant derivative of *Synechocystis* sp. PCC6803	D. Bhaya (Stanford)
SAA015 (FBP-)	*Synechocystis Δslr0169::P$_{trc}$::ldh$_{Bs}$::tt::Kan^R*	(60)
SAA023 (FBP+)	*Synechocystis Δslr0169::P$_{trc}$::ldh$_{Ll}$::tt::Kan^R*	(55)
pHKH005	Used to make strain SAA015; control for PCR/sequencing in this study	(60)
pHKH020	Used to make strain SAA023; control for PCR/sequencing in this study	(55)

Cell Free Extract Preparation. Cell free extracts of *Synechocystis* cells were prepared essentially as reported earlier [60]. Briefly, cell suspensions were harvested by centrifugation at 4000 rpm for 10 min at 4 °C in a benchtop centrifuge. The cell pellets were resuspended in the corresponding (see below) prechilled assay buffer. Cells were disrupted with glass beads with a Precellys24 bead beater (Bertin Technologies) operating at 6000 rpm beating three times for 30 s in 2 min intervals in which samples were cooled on ice. Cell debris was removed by centrifugation at 12 500 rpm for 30 min at 4 °C in a benchtop centrifuge. Protein content was determined with a BCA protein assay kit (Pierce).

Preparation of 2,5-Anhydro-D-mannitol-1,6-diphosphate. In the *in vivo* experiments we made use of the ability of cells to take up and convert 2,5-anhydromannitol (AHM) into the nonmetabolizable FBP analogue 2,5-anhydromannitol-1,6-bisphosphate (AHM-BP). For the *in vitro* enzymatic activity assays, we followed the following protocol to make AHM-BP. In a total reaction volume of 2 mL, 30 mM 2,5-anhydro-D-mannitol (Santa Cruz Biotechnology), dissolved in 100 mM HEPES-KOH (pH = 7.0) were mixed with Hexokinase (H4502, Sigma) and Fructose-6-phosphate Kinase (F0137, Sigma) both to a final concentration of 10 U mL^{-1}. 60 mM ATP were then added and the reaction was filled up with 100 mM HEPES-KOH and incubated for 30 min at 30 °C. After a pH drop the mixture was adjusted to pH 7.0 with KOH and incubated again for 10 min and finally stored at −20 °C until further usage. Assuming a complete conversion of AHM to AHM-BP the reaction mixture was considered as a 30 mM AHM-BP solution for the enzymatic activity assays.

Enzymatic Activity Assays. Activity assays with cell free extracts of *Synechocystis* containing the L-LDH of *B. subtilis* or the L-LDH of *L. lactis* were performed essentially as reported earlier [55,60], respectively. Briefly, here the basic reaction mixture for the *B. subtilis* LDH consisted of 50 mM sodium phosphate (pH = 6.5), 300 µM NADH, and 2.5 mM MgCl$_2$. For the *L. lactis* LDH the basic reaction mixture here consisted of 100 mM Tris-HCl (pH = 7.2), and as before 300 µM NADH, and 2.5 mM MgCl$_2$. The reaction

mixtures were further supplemented either with 3 mM FBP, 3 mM AHM-BP or left without an allosteric regulator. Cell free extracts of *Synechocystis* wild type strain, SAA015 (FBP−), and SAA023 (FBP+) were added to a final concentration of 1 mg mL^{-1} protein content. The reaction was started through the addition of 30 mM sodium pyruvate (Sigma). The oxidation rate of NADH was monitored at 340 nm in a 96-well plate reader at 30 °C (Multiscan FC Microplate Photometer, Thermo Scientific). The activity is reported in nmol mg^{-1} min^{-1} for NADH oxidation.

96-Well Plate Cultivation and Screening System. Precultures grown into exponential phase were used for inoculation at an initial optical density at 730 nm (OD$_{730}$) of approximately 0.05 in a volume of 150 µL in each well of the transparent 96-well plate with a flat bottom (CELLSTAR, greiner bio-one). Cultures were supplemented with 50 mM NaHCO$_3$ (Sigma) and covered with a Breathe-Easy sealing membrane (Diversified Biotech). The plate was placed on a shaking platform (MTS 2, IKA) set to a shaking speed of 600 rpm. That platform was placed inside a temperature controlled light incubator (Innova 43, New Brunswick Scientific) at 30 °C under constant light illumination at ~50 µE m^{-2} s^{-1} and supplied with ambient air. Growth was monitored by taking out the 96-well plate and recording OD$_{730}$ readings in a microplate reader (FLUOstar Optima) every 2 h. We placed a liquid-containing and pre-warmed microplate on the top of the culture plate any time this latter plate was out of the incubator to avoid condensation of moisture on the membrane, which would affect OD$_{730}$ readings. For lactate measurements wells were emptied with a syringe that was also used to pierce the membrane. Multiple wells of the same experimental condition allowed us to follow lactate production over longer time periods. Supernatants of the cultures were collected after centrifugation at 15 000 rpm for 1 min in a benchtop centrifuge and stored in −20 °C until further lactate concentration measurement (L-Lactic Acid Assay Kit Megazyme).

Strains Cultivated in Multi-Cultivator. Precultures from the glass flasks at OD$_{730}$ around 1 to 1.5 were used for inoculation to the Muti-Cultivator MC1000 (PSI, Czech Republic), at a starting OD$_{730}$ of approximately 0.05. Continuous low light (30 µmol photons m^{-2} s^{-1}) was given overnight, and then light was switched to 120 µmol photons m^{-2} s^{-1} until the end of the cultivation. During the experiment, 1% CO$_2$ and 99% N$_2$ was provided to the cultures through a gas mixing system GMS 150 (PSI, Czech Republic). The growth curves were monitored by following OD$_{730}$ (Spectrophotometer Lightwave II, Biochrom), while supernatants of the cultures were collected after centrifugation at 15 000 rpm for 1 min in a benchtop centrifuge and stored at −20 °C for lactate measurement (L-Lactic Acid Assay Kit Megazyme).

Sequencing of the *ldh* Cassette. For the samples taken for the sequencing of the *ldh* cassette, single colonies were first isolated from the population sample by restreaking onto a new plate. After 1 week, 5 colonies were randomly selected from each replicate culture and their genomic DNA was released through 5 cycles of freezing in liquid N$_2$ and thawing in hot water, for 2 min, respectively. One µL of the supernatant containing genomic DNA was taken as a template, using a high-fidelity Herculase II Fusion DNA Polymerase (Agilent Technologies) to amplify the whole *ldh* cassette. After gel purification (ISOLATE II PCR and Gel Kit, Bioline), fragments were sent for sequencing (Macrogen) using the primers listed in Table S2.1.

Validation of the Results from 96-Well Plate Cultivation System. See Supporting Information for detail.

2.2   Acknowledgement

2.3   Supplementary data

Supplementary data (supporting information) to this article can be found online at *http://pubs.acs.org/doi/abs/10.1021/acssynbio.6b00235*