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Förord

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Sammanfattning

Syntesgas, också kallat syngas, är en blanding av framförallt CO, CO_2, och H_2 som kan framställas genom förgasning av alla kol-innehållande material. Stora mängder syngas frigörs som industriella avgaser, t.ex. från stålverk. Mikrobiell syngasfermenterin har stor potential för att tillhandahålla hållbara bränslen i ett helt förnybart energisystem. Vissa bakterier, framförallt acetogener inom genusen *Clostridium*, *Sporomusa*, och *Acetobacterium*, kan tillväxa på syngas och samtidigt omvandla den till etanol, andra biobränslen och kemikalier. De kan också använda elektroner som tillförs från elektroder i bioelektrokemiska system. Detta kan förbättra både produktionshastigheten och selektiviteten för de önskade produkterna.

Syngasfermentering kan utnyttja allt kol i biomassa som förgasas, men många vetenskapliga utmaningar återstår. En av dessa är att syngasen innehåller ämnen som inhiberar den mikrobiella metabolismen, vilket kan utmana både den tekniska och ekonomiska genomförbarheten av denna mikrobiella kolinfångningsteknik.

Vi har studerat effekterna av inhibitorer på de mikrobiella cellerna, för att försöka förstå vilka egenskaper som ligger bakom toleransen mot inhibitorerna. Kortvarig adaptering var tillräckligt för att *Clostridium autoethanogenum* skulle klara av att växa i närvaro av bensen, toluen och xylen (BTX) vid koncentrationer som typiskt återfinns i syngas. Ammoniak var hindrade tillväxt vid koncentrationer över 50 mM. *C. autoethanogenum* dominerade till ca 90% en kultur som hade fått växa i upprepade odlingar där CO i gasen var den huvudsakliga kolkällan. Kulturen kunda omsätta CO och CO2 till acetat och etanol när den odlades i en bioelektro-kemisk reaktor med en konstant ström på 10 mA. Dessa resultat visar att

elektrofermentering av CO i ett bioelektrokemiskt system med låg ström kan användas för att omvandla industriella avgaser till användbara kemikalier.

C. ljungdahlii är till 99% identisk med *C. autoethanogenum* på genomnivå. Vi använde ”Adaptive Laboratory Evolution” (ALE) för att försöka förbättra dess förmåga att ta upp elektroner från elektroden. *C. ljungdahlii* fick växa i seriella odlingar under 13 månader, motsvarande ungefär 300 generationer, med Fe(0) som en elektronkälla i en atmosfär som innehöll H_2 and CO_2. Detta gjorde att 6-10 gånger mer acetat bildades och ca 150% av energin i de tillförda elektronerna återfanns i produkterna. Vi genomsekvenserade åtta isolat från den evolverade populationen, och fann att sju mutationer återfanns i alla isolaten. Två av isolaten hade dessutom en mutation i en gen kopplad till bildningen av enzymet hydrogenas. Trots att den evolverade kulturen presterade bättre i slutet av ALE-försöket visade de isolerade mutanterna ingen förbättring jämfört med den ursprungliga stammen när de odlades i serumflaskor och i bioelektriska system.

Produktionen av biobaserade drivmedel och kemikalier kan förbättras med hjälp av genetisk modifiering av cellernas metabolism. Trots detta ledde en ökad bildning av hydrogenas, ett enzym som flyttar elektroner från vätgas till olika elektronmottagare, inte till några förbättrade egenskaper hos *C. ljungdahlii*. Detta berodde sannolikt på att cellernas metabolism pressades för hårt på grund av bildningen av hydrogenas, trots att vi använda en kombination av en relativt svag promotor och en theophylline-inducerbara så kallad ”riboswitch”.

När genen för pyruvat-format-lyas B1A2 (PFL) från *Acetobacterium woodii* uttrycktes i *C. ljungdahlii* med samma system ökade den slutliga cellkoncentrationen med 50% vid odling i ett format-innehållande medium, och de celler som bildade PFL växte både snabbare och tidigare än celler utan PFL-genen.

Våra resultat visar flera möjligheter för hur elektrofermentering av syngas kan utvecklas vidare och erbjuder verktyg för att genomföra dessa förbättringar, med målet att skapa en koldioxidinfångande produktion av förnybara biobränslen.

Summary

Syngas is a mixture of mainly CO, CO_2, and H_2 that can be produced via gasification of literally any carbon-containing material. Large quantities of syngas are released as industrial off-gases, e.g. from steel mills. Microbial syngas fermentation has great potential for providing sustainable fuels in an entirely renewable energy system. Certain bacteria, primarily acetogens of the *Clostridium*, *Sporomusa*, and *Acetobacterium* genuses, not only have the capacity to grow on syngas and convert it to ethanol and other fuels and chemicals. They can also accept electrons provided in a bioelectrochemical system, which may improve the rate and specificity of the biofuel production.

Syngas fermentations can fully exploit the carbon in the gasified biomass, but many scientific challenges persist. One of these is the presence of inhibitors of microbial metabolism, that can challenge the technological and economic feasibility of this microbial carbon capture and utilization technology.

We studied the effects of inhibitors on the production host, in order to understand what features determines the physiological traits behind the tolerance. Short term adaptation was enough for *Clostridium autoethanogenum* to cope with benzene, toluene and xylene (BTX) at concentrations typically found in syngas. Ammonia was inhibitory at concentrations above 50 mM. *C. autoethanogenum* was also the dominant species (~90%) in a mixed population that was serially cultured in a CO-containing atmosphere. The culture could convert CO and CO_2 to acetate and ethanol when cultured in a bioelectrochemical system provided with a current of 10 mA. These results show that CO electro-fermentation at low current in a bioelectrochemical system can be an alternative way of valorizing industrial waste gas.

C. ljungdahlii is 99% similar to *C. autoethanogenum* on the genomic level. We used evolutionary engineering to try to improve its external electron uptake from the electrode. *C. ljungdahlii* was serially cultivated 13 months, corresponding to about 300 generations, with Fe(0) as a solid electron donor with an H_2- and CO_2-containing gas mix. This resulted in 6-10 times higher acetate concentration and a coloumbic efficiency of almost 150%. By whole-genome sequencing, we found seven identical mutations in eight isolates purified from the evolved population. Two of the isolates also had a mutation in a hydrogenase maturation gene. Despite the improved performance in the evolved population, none of the mutants performed better than the original strain when cultured in serum flasks and BES.

The production of biofuels and chemicals can be increased by engineering the cellular metabolism. Expression of different hydrogenases, that channel electrons from hydrogen to various electron acceptors, however, did not lead to any improvement. This was likely due to the metabolic burden of too strong expression, despite using a combination of a relatively weak promoter and a theophylline-responsive riboswitch.

Expression of the *Acetobacterium woodii* pyruvate formate lyase B1A2 (PFL) in *C. ljungdahlii* with the same expression system improved the final biomass concentration in formate-containing media by about 50%. The PFL mutants grew faster and reached steady state earlier than cells expressing the vector without the PFL.

Our results give several leads to how syngas electrofermentation can be further improved, and tools to realize the improvements, to provide a CO_2-capturing production of renewable biofuels.

Introduction and background

Providing society with fuels and commodity chemicals from renewable sources calls for novel and innovative solutions, such as syngas fermentation. Microbial fermentation processes are used to produce many chemicals that are important today, and will be tomorrow. These processes currently use sugars as feedstock, utilizing only about half the available carbon. The use of syngas as feedstock and employing electrochemical methods would make it possible to convert all the carbon in a broad range of feedstocks, such as biomass and industrial effluents, into valuable fuels and commodity chemicals. Microbial fermentation processes thus have great potential in our efforts to create a resource-efficient society and reach an entirely renewable energy system.

Syngas fermentation merges thermochemical and biochemical conversion of carbon based raw material. In the conventional thermochemical route, advanced reactor systems and large production volumes are used, comparable with those of the oil refineries. During this process, all ingoing flows are converted into a homogeneous mixture of simple molecules. This gas mixture is afterwards converted to desired products by advanced chemical catalysts that are often made by rare and costly metals and demand extensive cleaning systems and big investments. On the other hand, biochemical conversions are incorporated into less complex reactor systems and the microbial catalyst can be developed through metabolic engineering to produce more valuable target products. This makes the technology feasible in the scale 20-100 MW fuel input, a scale that fits the available fuel resource in many locations and is typically too small to motivate a traditional chemical catalysis process. However, current biochemical technologies require that the raw material is converted into sugar constituents, a practice that is limiting the feedstock options. This limitation also creates a need for good biomass quality, requires rather complex pretreatment steps with costly enzymes, and inevitably leaves us with the lignin part of the biomass that is often burnt. Heat from lignin can theoretically be used for the distillation process; however, in future energy systems, heat from renewable electricity and geothermal energy can be considered to be less valuable than carbon which would be better utilized as chemicals and storable energy carriers. Therefore, future processes need to optimize utilization of the carbon source, lignin included.

Complete utilization of carbon is a fundamental goal for a resource-efficient society, although we are still far away from being able to implement it. Syngas producing processes offer a unique opportunity for carbon utilization via biochemical conversions, as the carbon in the biomass is thermochemically converted into two simple molecules (CO and CO_2) which can be used to synthesize more complex ones.

Syngas electrofermentation, i.e. supplementing syngas fermentations with redox power in the form of electrical energy, is a groundbreaking concept that is expected to enhance biofuel production rates and yields. Many acetogenic bacteria (e.g. *Clostridium* spp.) can use raw electrical energy as an energy source to

produce reduced products like alcohols [9, 10]. These microbes have a unique ability to utilize raw electricity coming from solid electrodes, which gives us the option to provide them with redox power needed in the form of electricity (Figure 1).

Fairly little knowledge is currently available on the physiology and metabolism of acetogenic bacteria. The efficiency of syngas fermentation is still far below that of sugar-based fermentation processes and the great majority of publications describing syngas fermentation processes are performed using a pure mixture of CO, CO_2 and H_2 in place of real industrial syngas that contains inhibitors which are very toxic to the cells and thus have a great impact on the whole process. Benzene, toluene, xylene, ammonia, cyanides and tars found in raw syngas affect the fermentation process by changing the pH, osmolarity, redox potential and enzymatic functions [1].

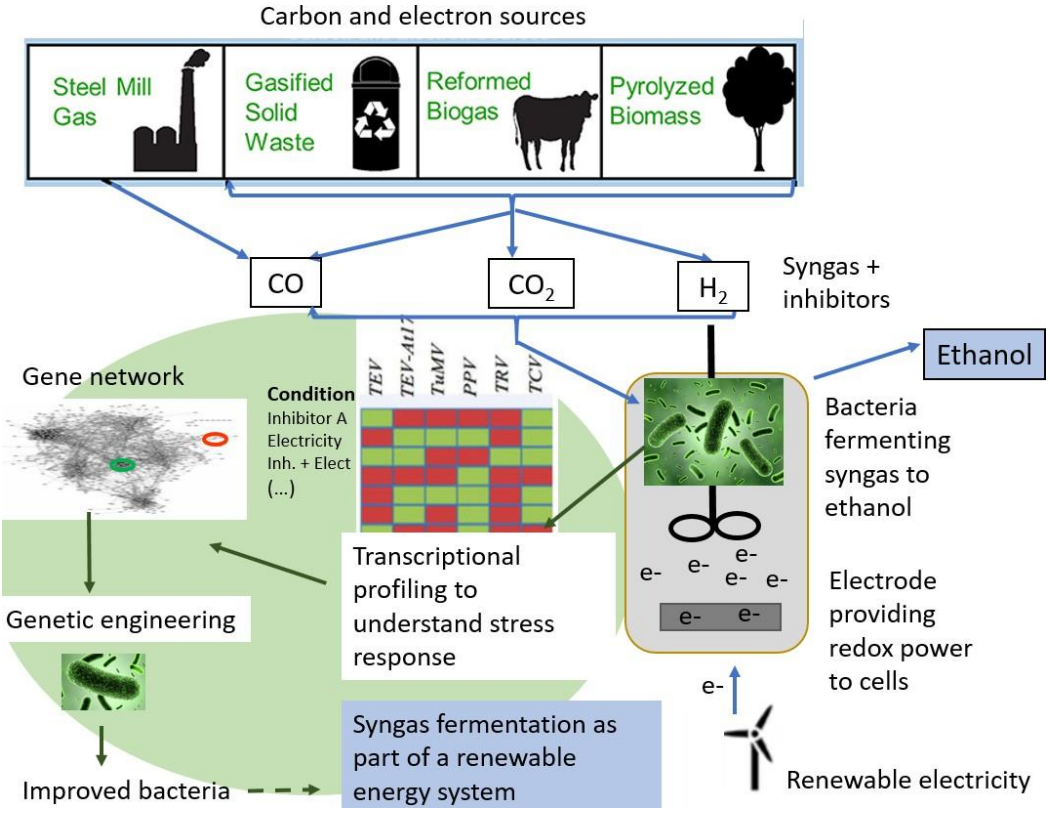

Figure 1. Scope of the project. Any carbon containing gas or material that is gasified can be used as a raw material in syngas fermentation, for production of ethanol for as sustainable energy carrier. The focus of this project, the elucidation of stress responses of cells fermenting syngas and genetic engineering for developing improved syngas fermenting bacteria is highlighted in green.

Genetic engineering

Recent advances in molecular biology have led to significant progress in genetic modifications of industrially relevant, syngas-fermenting bacteria [2]. CRISPR/Cas9 based genome editing has successfully been applied to various *Clostridia* [3, 4], and so far, metabolic engineering examples have focused on expanding product spectra or enhancing production rates [2]. A few genome-scale metabolic networks have been published for acetogens, the first being that of *C. ljungdahlii* [5]. In addition, a few transcriptomic studies of bacteria during syngas fermentation have been published [6-8].

Evolutionary engineering

Adaptive laboratory evolution plays an important role in developing strains for commercial projects. Different strains can display very diverse features, and adaptation experiments can lead to better strains and identification of beneficial mutations to be implemented in production organisms (reverse engineering). Adaptation experiments typically consists of a high number (up to 150) of transfers of cells in medium with high substrate and/or inhibitor concentrations. Many *Clostridia* have been used and adapted for fermentation of lignocellulosic hydrolysates, but only a couple of studies on relatively unknown acetogen species (e.g. *Sporomusa ovata* [16] or *Thermoanaerobacter kivui* [17]) describing adaptation to CO or CO2, have been reported. Ahmed et al., [18] showed that adaptation to tar in biomass-generated gas could improve growth and ethanol production of *C. carboxidivorans*. A broader understanding on stress responses observed in adaptation towards various stress and inhibitors may be useful for understanding general stress behaviour of *Clostridia*, many species of which have high potential for use in consolidated bioprocesses [19].

Acetogens and the Wood-Ljugdahl pathway

Syngas producing processes offer a unique opportunity for carbon utilization via biochemical conversions, as the carbon in the biomass is thermochemically converted into two simple molecules (CO and CO_2) which can be used to synthesize more complex ones.

Acetogens, that generate acetate as a product of anaerobic respiration, have been studied for decades and have recently gained increased attraction as biocatalysts in gas fermentation or carbon capture technologies. The linear Wood–Ljungdahl pathway (see Figure 2) employed by acetogens has been reported to be the most efficient non-photosynthetic carbon fixation mechanism [11]. The native ability of acetogens to synthesize useful products such as ethanol, butanol and 2,3-butanediol and the fact that they are anaerobes make these organisms particularly attractive for commercial applications [2]. *Clostridium* species, such as *C. ljungdahlii*, *C. autoethanogenum* and *C. carboxidivorans* have, to date, gained most attention in academia as well as for commercial syngas applications.

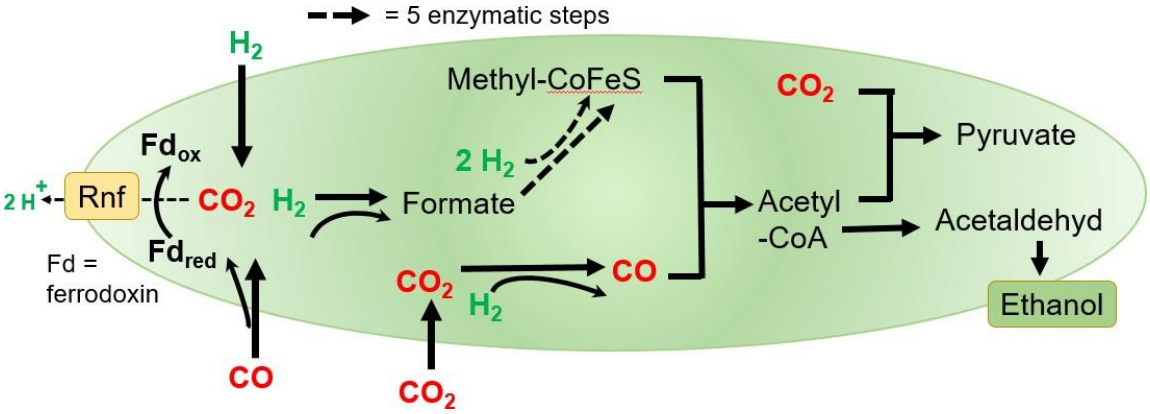

Figure 2. Overview of the Wood-Ljungdahl pathway of *C. autoethanogenum*, an acetogen commonly used in syngas fermentations.

While CO_2 has no energy value for the microbes, CO is an energy-rich compound and a microbial energy source used for regenerating reducing equivalents (NAD(P)H). The microbial use of CO as a microbial energy source is nonetheless a big challenge because it decreases the carbon-to-product yields to unsustainable levels [12]. Moreover, biochemical production faces considerable hurdles during CO utilization, as the supplementation of overall redox power must be carefully balanced. When the CO partial pressure is high, ferredoxin reduction is promoted, leading to the production of reduced metabolites such as ethanol, while a further increase in the CO supply can result in overflow metabolism, where NAD(P)H is consumed for the production of pyruvate and further compounds such as 2,3-butanediol [13].

CO fermentation is associated with two major problems: **1.** the production of CO_2 as a by- product, which contributes to the energy-less CO_2 already present in the syngas (6 moles CO consumed produce 1 mole of ethanol together with 4 moles of CO_2), and **2.** the inhibition of hydrogenase activity and the accumulation of H_2 and CO_2 as long as CO is present.

Metabolic constraints related to energetics, intracellular redox potential and inhibitory effects could potentially be resolved by application of electric potential on solid-state electron donors and acceptors. Even relatively small amounts of electric charge supplemented through electrodes have recently been shown to trigger regulatory switches in heterotrophic fermentation of syngas using *Clostridia* [14, 15]. The mechanisms responsible for inducing NADH [14] and reducing ferredoxin consumption [15] are nonetheless still speculative.

The metabolic traits responsible for tolerance to syngas or syngas inhibitors have not yet been identified. Physiological mechanisms through which inhibition occurs as well as metabolic pathways, networks and regulation mechanisms activated

during syngas fermentation are poorly characterized. This is particularly true for electrochemically assisted syngas fermentation processes. Our aim is to tighten this knowledge gap.

The purpose of this project was to understand how fermentation of syngas affect bacteria, and to design more efficient and robust syngas-fermenting microorganisms. A serious challenge is that some compounds in raw syngas and the products of microbial fermentation inhibit the growth of microorganisms.

The specific aims of the project were:

1. to develop a thorough understanding of the physiological and transcriptional effects on bacteria, caused by
    a. syngas and inhibitors therein
    b. electrofermentation
2. to improve syngas fermentation and stress tolerance of bacteria through genetic engineering
3. to improve robustness of bacteria through evolutionary engineering

Our long-term vision is to develop sustainable biorefineries at intermediate scales (20-100 MW fuel input) that can make full use of the carbon and energy content of any carbon-rich feedstock to produce renewable chemicals and fuels. In order to realize this ambitious vision, a thorough understanding of how acetogens respond and adapt to syngas and inhibitors therein is needed. The aim of this project is to increase the robustness of syngas fermenting organisms to allow the conversion of real syngas at efficiencies and rates similar to the ones obtained using mixtures of pure gases.

Methods

The project was divided into five activities, each addressing an important development aspect of syngas fermentation and autotrophic growth and product formation from H_2, CO and CO_2. Here, only brief method descriptions are included. For detailed materials and methods we refer to the resulting publications (I-IX).

Activity 1. Syngas fermentation and characterization of acetogen growth and metabolism in presence of syngas inhibitors

Gas fermentations were conducted in serum flasks and in bioelectrochemical reactors. Modified PETC 1754 (1) or DSMZ879 (II) was used as basal medium. Serum flasks (150 ml) were filled with 50 mL medium and sealed with a rubber stopper. The flask headspace was replaced with pure N_2 by vacuum flushing using a gas-exchange system. Flasks were autoclaved and subsequently gas exchanged again with pure N_2 to flush out remaining traces of air. When different CO, CO_2

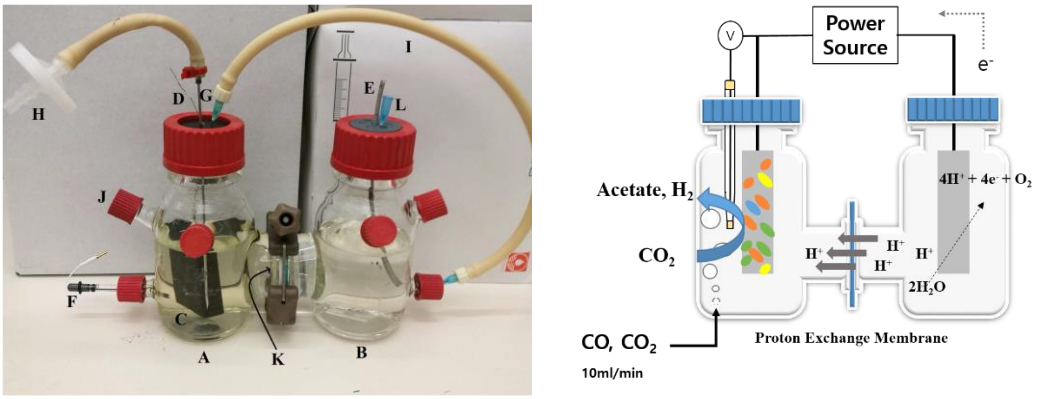

Figure 3. Set-up of the bio-electrochemical system before connecting to CO2 gas and potentiostat. A: Cathode chamber; B: Anode chamber; C: Carbon block used as cathode; D: Titanium wire connected to the cathode; E: Platinised titanium rod used as anode; F: Ag/AgCl reference electrode; G: Needle used for sparging CO2; H: Filter for inlet CO2 gas; I: Tube leading gas from cathode chamber to anode chamber; J: Sampling port; K: Nafion proton exchange membrane between cathode and anode chamber. Photo taken by Fahim Hadi (IX).

and H_2 gas mixtures were used, the flasks were filled to 2 bar overpressure with the gas mixture. During cultivation, the gas in the headspace was replaced with fresh gas when the pressure fell below 1 gauge bar.

Electrofermentation of synthetic syngas mixtures containing different proportions of H_2, CO and CO_2 were carried out in H-type bioelectrochemical reactors (Figure 3). Both constant cathode potentials and constant current modes were applied in the H-type bioelectrochemical reactors.

The bio-electrochemical activity of *C. ljungdahlii* was thus investigated at cathodic potentials between -0.4 and -1.2 mV (II, IX). This was done by running biotic and abiotic bio-electrochemical system experiments at different cathode potentials, as well as serum flask control experiments. The results were analysed in terms of product formation, growth, pH, current consumption, and redox species present in the bio-electrochemical system reactors.

The major inhibitor in syngas electrofermentation is assumed to be CO, one of the major components in syngas. An electrofermenter setup to circumvent the inhibitory effect of CO by separating the CO metabolism from the electrochemical chamber was designed and evaluated. Therefore, the reaction chambers for syngas fermentation and for microbial electrosynthesis were separated into two separate chambers connected by tubing to recirculate the medium (Figure 4). The hypothesis was that the design of the new reactor would avoid the inhibitory effect of CO on extracellular electron transfer.

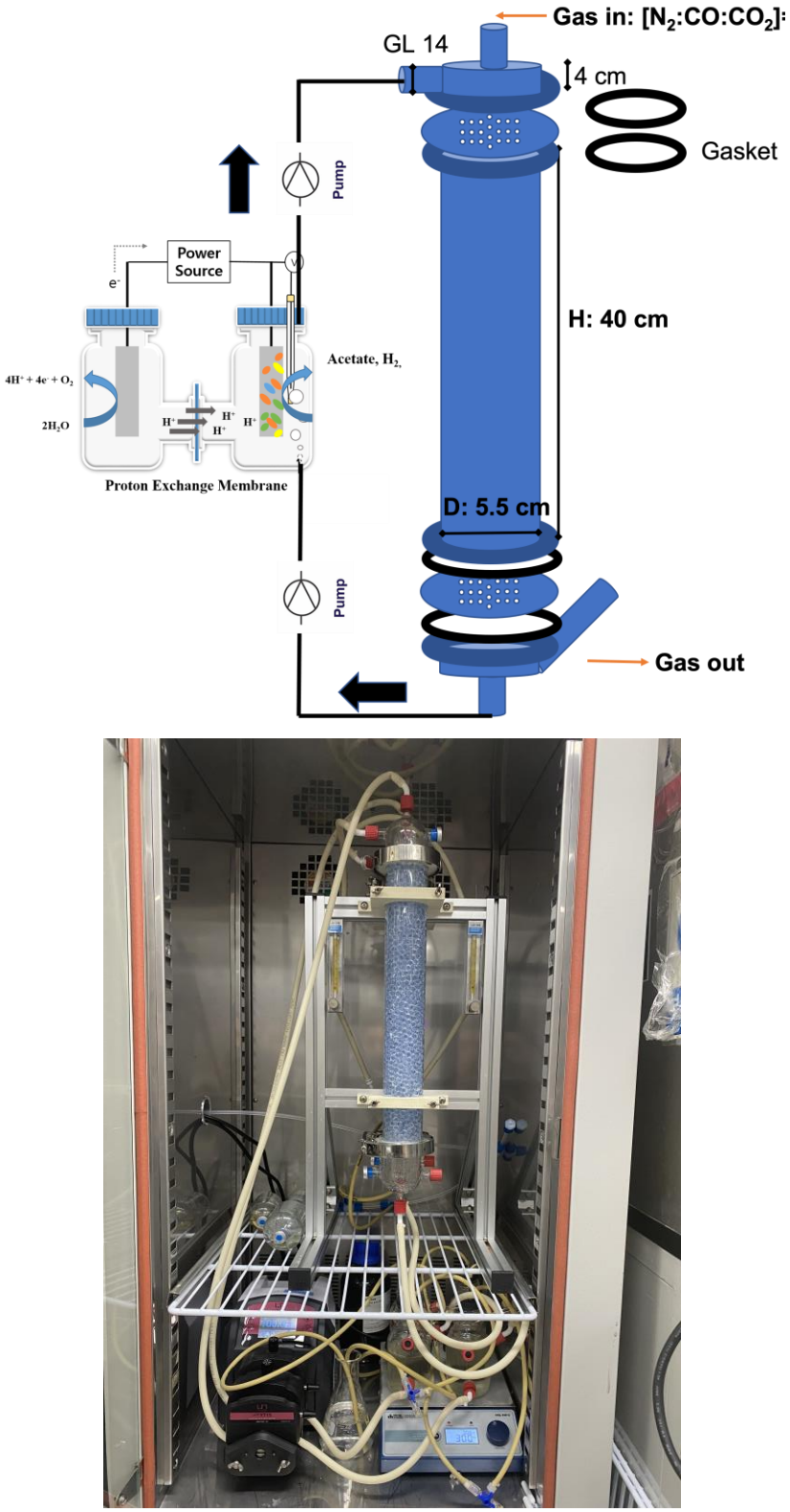

Figure 4. New bioelectrochemical reaction design tested in this project.

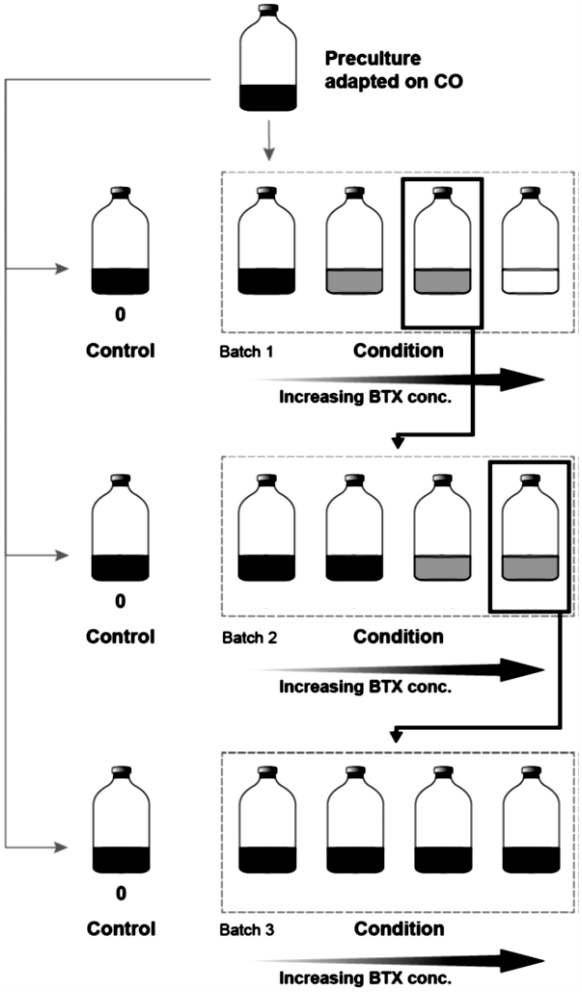

Figure 5. BTX adaptation; *C. autoethanogenum* was first adapted on CO gas until reaching mid-exponential growth phase (dark grey). The preculture was then used to inoculate CO-containing flasks, containing increasing concentrations of a BTX mixture. Cultures that represented tolerance at the highest BTX concentrations (light grey) compared to non-growth (white) were transferred into fresh serum flasks with higher BTX concentrations (light grey flask boxed in black). Adapted from Figure 1 in Piatek et al, 2020 (I) under the CC BY 4.0 license. This work was performed in project TD40010 (Energiforsk – The Swedish Energy Research Centre) and in project P44730 (Swedish Energy Agency).

Activity 2. Adaptive Laboratory Evolution to develop more inhibitor-tolerant strains with higher external electron uptake and higher growth rate.

Effects of BTX inhibitors

Fermentation of syngas mixtures containing increasing of inhibitors (benzene, toluene, xylene, BTX) was done in serum flasks according to the scheme in Figure 5. *C. autoethanogenum* growing in serum bottles was exposed to syngas

mixtures with increasing concentration of inhibitors or to real syngas (Figure 5). However, the low number of generations in that scheme would only allow short term adaptation to the inhibitors, not any evolutionary change on the genomic level. Nevertheless, even short-term adaptation led to significant improvement in the tolerance to BTX (see results below).

Enrichment of mixed culture on CO.

To improve CO tolerance, we adopted a different approach. We had difficulties in estabilishing growth of *C. ljungdahlii* in the Korean lab where the novel electrochemical bioreactor was setup (Figure 4). To look for CO tolerant organisms, a solventogenic acetogen mixed culture was enriched from cow fecal waste under CO gas. The mixed culture was expected to be populated with a lot of *Clostridium* species. Using a mixed culture gives several advantages over testing directly with *C. ljungdahlii*, such as resiliency against oxygen and microbial contamination during operation and faster adaptation to environmental changes.

ALE on iron

We hypthesized that the external electron uptake was limiting the metabolism in the BES. Therefore, we focused on enhancing the bioelectrochemical activity of *Clostridium ljungdahlii* by adaptive laboratory evolution (ALE) while challenging the electron uptake systems, instead of increasing inhibitor concentration.

Several anaerobic bacterium species are capable of metal oxidation of a solid metal in order to obtain reducing equivalents. Assuming that the extra-cellular electron transfer mechanism on the cathode of a bioelectrochemical system is the same as metal oxidation in nature, we did ALE of *Clostridium ljungdahlii* using zero-valent iron and increasing concentrations of CO_2 as selection pressure. The electro-active acetogen was serially cultivated with iron as a sole electron source (IV). Whole genome DNA sequencing was done for eight isolates from the final evolved population, and HPLC was used for analysis of products. 17 mutants were characterized under different growth condition. One mutant was selected based on the results from the whole genome sequencing, and its performance, compared to the original strain, was tested in a H-type BES reactor and by cyclic voltammetry.

Activity 3. Identification of stress responses

Genome wide sequencing of isolated strains obtained in Activity 2.

Originally, we planned to do mRNA sequencing to elucidate the transcriptomic response to inhibitors and to external electron uptake in BES. However, we had to abandon this plan. Since irreproducible results often appeared when BES reactors were operated with a fixed cathodic potential, taking samples for RNA-seq was delayed. Cell growth in BES was very slow, and it was difficult to get good RNA samples. When we obtained reproducible BES results using fixed current mode,

there was not time enough to sequence the mRNA of all necessary samples, and to do a thorough bioinformatic analysis of the results.

Instead, we used 16S rRNA sequencing to identify the species being enriched on CO. Moreover, 8 strains isolated from the population evolved on Fe(0) were genome sequenced to identify the genetic changes obtained during the evolution, and to identify metabolic traits responsible for increased robustness.

Activity 4: Genetic toolbox development

Methodology for engineering syngas fermenting *C. ljungdahlii* was established. This involved both cloning methodology, choice of vectors and promoters, and application of a riboswitch to obtain gene and protein expression levels that were compatible with the low energy flux during gas fermentation. Further details of this development are presented in the Results section below.

Activity 5: Construction of novel strains

Metabolic engineering of Clostridium ljungdahlii for enhanced syngas electro-fermentation

The poor growth of *C. ljungdahlii* will limit its use in industrial scale systems, and the rate of electron or hydrogen uptake will limit the rate of metabolite production. Based on our results and the genome scale metabolic model by Nagarajan et al. [5], the main targets were identified to be pyruvate formate lyase, formate dehydrogenas, and hydrogenase, all related to growth and/or hydrogen metabolism.

There are many interesting target genes from *C. ljungdahlii* to investigate since development of genetic toolbox of *Clostridia* happened quite recently [20]. First, heterologous expression of hydrogenase. This is because hydrogenase is assumed to play a major role in extracellular electron transfer of *C. ljungdahlii*. If hydrogen mediated electron transfer is the mechanism of electron transfer of *C. ljungdahlii*, high affinity hydrogenase expression would be able to improve its electro-activity. *Acetobacterium woodii* and *Sporomusa ovata* are known autotrophic acetogen that can grow well under low hydrogen partial pressure. Therefore, hydrogenases from those two autotrophic acetogen were evaluated for potential expression in *C. ljungdahlii*. The group A3 and Group A4 hydrogenases from *Acetobacterium woodii* were chosen for overexpression using the P_{fdx} promoter and a theophylline-responsive riboswitch [21]. The performance of the mutant strains were assessed in serum flasks in PETC medium at pH 5.0, in 2 bar of 20% CO_2 and 80% H_2.

In addition to the energy provided by the hydrogenase activity, improved incorporation of acetyl-CoA in biomass was hypothesized to promote growth. Heterologous expression of formate dehydrogenase and pyruvate-formate lyase

may promote growth, because formate is produced by an electrochemical side-reaction during bioelectrochemical system operation. Moreover, pyruvate-formate lyase (*pflAB*) is upregulated in *Acetobacterium woodii* and *Eubacterium limosum*, autotrophic acetogens that are able to grow under formate condition. Therefore, several combinations of the two *pflA* and three *pflB* subunits from *A. woodii* were expressed in *C. ljungdahlii*. However, it was difficult to get positive clones using pflA1. Therefore, combinations of *pflA2* and *pflB1*, *pflB2*, and *pflB3* were evaluated in serum flasks at different formate concentrations with 2 bar 20% CO_2 and 80% H_2. The *pflB1A2* combination was also assessed in 20 mM formate BES in at constant current of 10 mA, sparged with pure CO_2.

Resultat

Dealing with inhibitors of syngas fermentation

BTX

Benzene, toluene and xylene (BTX) are a group of compounds detected in many crude syngas mixtures. However, BTX have been identified to negatively affect microorganisms, including acetogenic species that are capable of fermenting syngas into valuable biocommodities. In order to overcome BTX inhibitory effects, we performed a stepwise adaptation of *Clostridium autoethanogenum* as described in Figure 5. The adaptation led to tolerance to up to 0.5 mM benzene, 0.21 mM toluene and 0.07 mM xylene (Table 1), which is equivalent to eightfold of that which is found in the syngas stream from the wood gasification pilot plant at Chalmers. The fully adapted cultures matched the growth, acetate and ethanol product concentrations, and the CO consumption to those of the control. The results demonstrate an efficient route towards producing a highly tolerant, industrially relevant acetogenic strain for syngas fermentation.

Table 1. Growth of *C. autoethanogenum* in the presence of varying amounts of BTX compounds, compared against the control without BTX inhibitor addition. "Similar" refers to growth similar to the control.

Con-dition	Benzene	Toluene (mM)	Xylenes (mM)	Growth on **CO without BTX** adaptation	Growth on **CO after 1st** adaptation, cells from condition 7	Growth on **CO after 2nd** adaptation, cells from condition 10	Growth on **CO after 2nd** adaptation, cells from condition 10
0 [a]	0	0	0	Control	Control	Control	Control
1	0.05	0	0	Similar	–	–	–
2	0.1	0	0	Similar	–	–	–
3	0.5	0	0	Similar	–	–	–
4	1	0	0	Similar	–	–	–
5	2	0	0	No growth	–	–	–
6	5	0	0	No growth	–	–	–
7	0.05	0.021	0.007	Similar	Similar	–	–
8	0.1	0.042	0.014	–	Delayed	Similar	–
9	0.2	0.085	0.028	–	Delayed	Delayed	Similar
10	0.3	0.13	0.042	–	–	Delayed	Similar
11	0.4	0.19	0.056	–	No growth	No growth	Similar
12	0.5	0.21	0.07	Similar	–	–	Similar
13	1	0.43	0.14§	No growth	–	–	–
14	2	0.85	0.28	No growth	–	–	–

Dash (–) represents no experiments performed using this condition.
[a] Condition 0 served as control.

Figure 6. Anaerobic serum flasks with A, *C. autoethanogenum*; B, *C. carboxidivorans*; and C, *C. ljungdahlii*, after 10 days in PETC medium with, from right to left, 100, 100, 50 , 50, 35, 35, 20, 20, 0 and 0 mM ammonia. Adapted from (VIII).

Ammonia

In serum flask cultivations, *C. autoethanogenum*, *C. carboxidivorans*, and *C. ljungdahlii* were able to grow in ammonia concentrations below 50 mM (Figure 6). This means that ammonia would not pose a significant problem as long as its concentration is kept below this level. However, the level of ammonia affected the distribution of acetate and ethanol production (Figure 7).

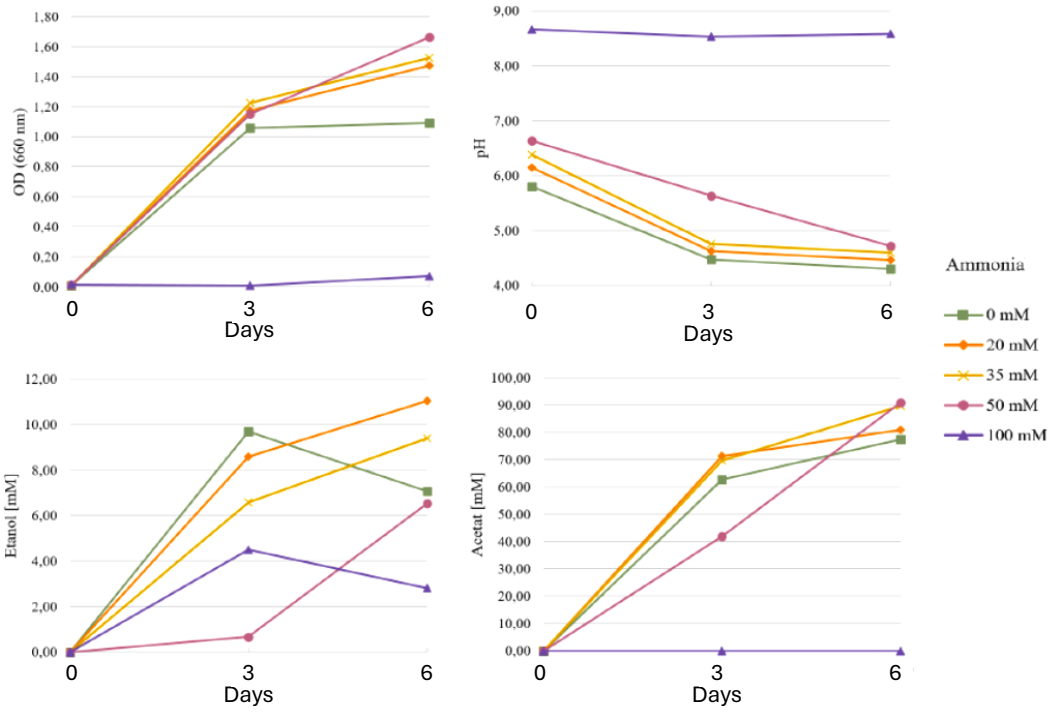

Figure 7. OD, pH, ethanol and acetate measured in anaerobic serum flasks with *C. ljungdahlii*, in PETC medium at diffferent ammonia concentrations. From Frithiofson et al. (2018) (VIII).

Carbon monoxide

We have systematically tested different cathodic potentials for operating BES reactors using *C. ljungdahlii* with CO_2 to assess the feasibility of electrochemically assisted gas fermentation (II). A cathodic potential of -0.8 V to -1.0 V was found optimal for reproducible BES reactor operation using *C. ljungdahlii*. From these experiments we could draw the following conclusions (II)

- Lower initial pH improved acetate production of *C. ljungdahlii* from H_2:CO_2 gas mix.
- Yeast extract in the medium increased reproducibility of BES experiments.

- When cultivated in bioelectrochemical systems, the optimal coulombic efficiency (i.e. close to 100 %) was observed at a cathode potential between −0.8 V and −1.0 V, while the highest productivity was reached at −1.0 V.
- H_2-mediated extracellular electron transfer was predominant *for C. ljungdahlii*.

BES reactor operation with a fixed cathodic potential often causes irreproducible results. Provided that the extracellular electron transfer mechanism of *C. ljungdahlii* is hydrogen-mediated, applying a fixed current to BES reactors would solve the irreproducibility problem.

CO is a well-known inhibitor of hydrogenases and our results allows us to hypothesize that CO itself is the major inhibitor of the microorganisms during syngas/CO fermentation. Based on the results of Task 1.2, hydrogen-mediated electron transfer is assumed to be the major mechanism of electron transfer of *C. ljungdahlii*. Therefore, it is crucial to prevent high levels of CO in the chamber where the actual syngas fermentation is happening. We are attempted to develop of a novel syngas electrofermenter design to circumvent inhibitory effects of CO on hydrogenases, by having separate chambers for syngas fermentation and electrofermentation. A lab-scale prototype was designed and was tested using CO and CO_2 gas mix. This was done at the University of Pusan, South Korea, with support of a STINT mobility grant. Unfortunately, the prototype reactor did not improve the electrofermentation, partly due to challenges in maintainting anaerobic conditions, partly due to poor growth of *C. ljungdahlii* in the Korean lab.

Instead, we investigated if short term adaptation and enrichment of a mixed population in a CO-containing atmosphere (III). Fresh cow fecal waste was taken from a cow farm and enriched under an atmosphere of 50% CO and 20% CO_2 in N_2 using serial cultivation. By 16S rRNA sequencing, we showed that the CO-enriched culture was dominated by *Clostridium autoethanogenum*. The CO-enriched culture showed electro-activity in a BES reactor with CO_2 sparging. When 50% CO was included in the 20% CO_2 gas with 10 mA applied current, acetate and ethanol were produced up to 12.9 ± 2.7 mM and 2.7 ± 1.1 mM, respectively. The coulombic efficiency was estimated to 148.1 ± 7.5 % without an electron mediator. At 25 mA, the culture showed faster initial growth and acetate production but no ethanol production, and only at 85.9 ± 4.2 % coulombic efficiency. The maximum OD of 10 mA and 25 mA reactors were 0.29 ± 0.07 and 0.41 ± 0.03, respectively, whereas it was 0.77 ± 0.19 without electric current.

The CO-enriched population was highly dominated by *Clostridium autoethanogenum* (~90%), which has very high similarity (99%) with *C. ljungdahlii* on the genomic level. Also, the mixed population showed lower hydrogen production than abiotic experiment, which means the extracellular electron transfer was possible in the presence of CO in a BES reactor. Here, the

Table 2. Acetate production rate comparison between each round of repeated batch experiment flasks, F1 and F2, over the long-term adaptive laboratory evolution on zero-valent iron. 11M and 12M indicate 11th month and 12th month, respectively. Adapted from (III).

Batch no.	1st	2nd	3rd	…	7th	…	11M 1st	11M 2nd	11M 3rd	12M 1st	12M 2nd
Acetate production rate of F1 [mM/d]	0.20	0.12	0.10	…	0.32	…	0.51	1.49	1.37	1.14	2.01
Acetate production rate of F2 [mM/d]	0.38	0.20	0.27	…	0.30	…	0.59	1.13	1.22	1.09	2.46

extracellular electron transfer is speculated to be hydrogen-mediated, based on cyclic voltammetry results (III). These results show that CO electro-fermentation at low current can be an alternative way of valorizing industrial waste gas using a bioelectrochemical system.

Adaptive Evolutionary Engineering of external electron uptake

Adaptive laboratory evolution of *C. ljungdahlii* on zero-valent iron was performed for 13 months, corresponding to approximately 300 generations (III). Such long-term adaptive evolution was expected to cause genetic mutations which may lead to better metal oxidation ability, indicating an improved external electron uptake. After about 300 generations in two parallel repeated batch experiments, the acetate production rate of the evolved population had increased by 6-10 times, from 0.20 mM/d and 0.38 mM/d in the initial cultivations to 2.01 mM/d and 2.46 mM/d in the final ones (Table 2). From the evolved population, single colonies were picked and cultivated to identify potentially mutated strains.

In this way, 17 isolates were selected and were tested under different growth conditions and compared to the wild-type *C. ljungdahlii*. Despite the significant improvement in acetate productivity of the evolved population, the isolated mutants grew slower than the wild-type under both hetero- and autotrophic conditions. Whole genome sequencing of wild-type, isolated mutants, and evolved population was done. Based on the genome sequencing results, one mutant (#8) was chosen for further studies in BES because the isolated mutant #8 has a mutation on hydrogenase maturation gene, which might affect the performance in a bioelectrochemical system. However, the performance of the isolated strain #8 in a bioelectrochemical system was also worse than the wild-type.

Eight of the isolates from the evolved population were whole genome sequenced to identify potentially significant mutations (III). Mutations in genes encoding mannose 6-phosphate isomerase (CLJU_RS02610), methyl-accepting chemotaxis

protein (CLJU_RS06805), high-affinity proline permease (putP, CLJU_RS10075), APC family permease (CLJU_RS11965), cobalamin adenosyltransferase (cobO, CLJU_RS15680), a transcriptional repressor (CLJU_RS17505), and amino transferase (gatA, CLJU_RS18180), were found in all eight of the sequenced isolates. In addition, a gene encoding a hydrogenase maturation protein (hypF, CLJU_RS11360) was mutated in two of the evolved isolates, and CLJU_RS07225 in a single isolate. In addition, the starting strain and all isolates had mutations in hemA (CLJU_RS02190), codY (CLJU_RS06385), CLJU_RS07315, rrf (CLJU_RS07325), CLJU_RS10055, uraA (CLJU_RS11595), CLJU_RS22935, CLJU_RS14955, and CLJU_RS20060, compared to the reference *C. ljungdahlii* genome sequence. We have not yet been able to elucidate the exact consequence of these mutations. However, based on the observations described above, it appears that a mix of the mutants performed better than each individual mutant. This may be because enzymatic activities in some of the mutants may contribute positively to the overall metabolism of the population. When tested in isolation, such interaction is no longer present.

Hydrogen-mediated electron transfer is thought to be the major electron transfer mechanism of *C. ljungdahlii* in a BES reactor, but also direct electron mediators are thought to be possible. Therefore, iron, which can act as an electron donor due to its spontaneous corrosion in aqueous solution, was used for adaptive laboratory evolution instead of setting up a BES reactor. We originally planned to do RNAseq to compare the transcriptional profile of BES-grown cells and hydrogen-grown cells to obtain further evidence regarding the nature of the electron transfer, as well as the effect of inhibitors. Unfortunately, we could not follow through on this plan, because of difficulties in obtaining RNA samples of high enough quality due to the poor cell growth in BES.

Tools for genetic modification of C. ljungdahlii

Previous results have shown that two processes are likely limiting the rate of electrofermentation in *C. ljungdahlii*, namely cell growth and hydrogen uptake at low hydrogen concentrations. We set out to improve growth by expression of heterologous genes coding for pyruvate formate lyase (pfl) and formate dehydrogenase (fdh), and hydrogen uptake by expression of hydrogenases with high affinity for hydrogen. To facilitate this, a transformation procedure had to be established in our lab.

Transformation using conjugation has previously been established in *C. ljungdahlii* [20, 22]. Unlike other *Clostridium* species, *C. ljungdahlii* requires a specific helper plasmid, pRK2013, for conjugal transformation between *E. coli* and *C. ljungdahlii* [22].

First we attempted to use the pRK2013 system using the P_{thl} promoter but this led to mutations during cloning and poor growth. We assume this was due to the

promoter being constitutive and too powerful, causing high expression of the *pfl* gene and toxic metabolic burden to the cells. To avoid mutations during cloning by avoiding expression during cloning step, we added the inducible *lacO* operator, which natively expressed lacI bind to and inhibit the transcription of the gene without inducer in *E. coli* cloning strain, to form a P_{thl}+*LacO* operator. However, this was also not successful. We assumed that natively expressed LacI could not cover all the copies of the plasmid. All the unsuccessful cloning showed mutations on the start codon. We then turned to a more recently developed system using the P_{fdx} promoter together with a theophylline-responsive riboswitch to control translation [21]. The riboswitch is induced by theophylline, allowing translation of the desired transcripts by conformational change of mRNA hairpin site that consist of the ribosome binding site and the start codon. This expression system led to successful cloning in *E. coli* and conjugal transformation to *C. ljungdahlii*. The mutant *C. ljungdahlii* strain carrying the plasmid showed improved cell growth, meaning apparent transcription and translation of the desired genes. Although we have no definite proof of the desired proteins being produced, the cultivation results (see next section) indicate successful gene expression and translation.

Heterologous hydrogenase expression in C. ljungdahlii

C. ljungdahlii seems to utilize hydrogen-mediated electron transfer. The initial harvesting of the free energy in H_2 occurs via the enzyme hydrogenase. Hydrogenases are classified primarily according to their preferred electron acceptors, that receive electrons from H_2 [23]. [NiFe] hydrogenase usually have high affinity for hydrogen. *Sporomusa ovata*, which is known for high performance in a BES reactor, has six genes encoding hydrogenases, while *C. ljungdahlii* has only one [FeFe] hydrogenase-encoding gene. Therefore, heterologous expression of high affinity hydrogenases in *C. ljungdahlii* was done. A set of target hydrogenases and connected cytochromes were identified in *Sporomusa* and in evolutionary closely related acetogenic *Clostridium* strains. *Acetobacterium woodii*, which also shows high performance in a BES reactor, has only two [FeFe] Group A3 and A4 hydrogenases. The [FeFe] Group 4 hydrogenase of *A. woodii* has very high affinity to hydrogen as well as CO tolerance [23, 24]. *A. woodii* is also very closely related with acetogenic *Clostridium* strains. We used the P_{fdx}-theophylline riboswitch system to express Group A3- and Group A4-type hydrogenases from *A. woodii* in *C. ljungdahlii* and tested the effect on biomass growth both in the presence and absence of the riboswitch inducer theophylline (Figure 8). Surprisingly, the expression of the hydrogenases led to poorer growth in serum flasks using PETC medium at pH 5.0 with a 2 bar overpressure atmosphere containing 20% CO_2 and 80% H_2 (Figure 8).

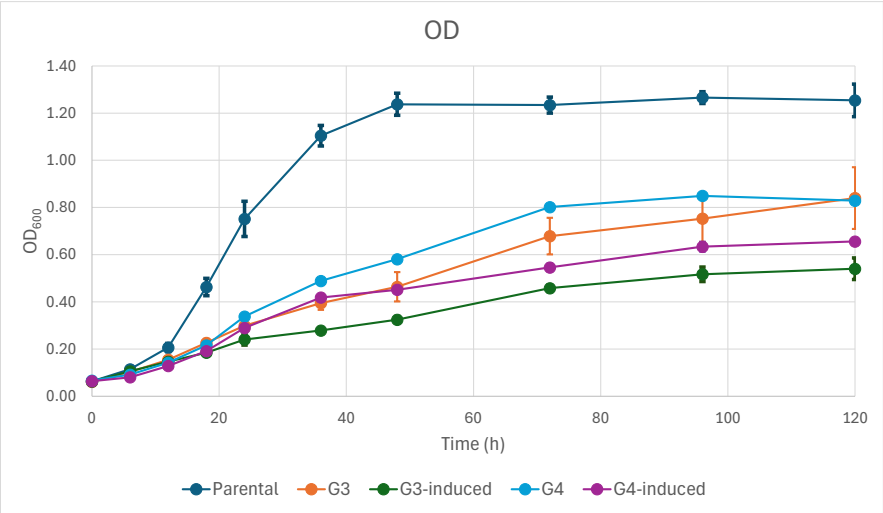

Figure 8. Growth curves (OD) of *C. ljungdahlii* (Parental) transformed with the *A. woodii* Group A3 and Group A4 hydrogenase in the absence (G3, G4) and presence (G3 induced, G4 induced) of theophylline. Average values ± standard error of biological duplicates.

We assume these negative results depend on toxic overexpression of the desired genes. The two chosen hydrogenases have 4 and 6 subunits, respectively, but when expressed together on a single mRNA, only the first of the enzymes was controlled by the riboswitch (unless the riboswitch is placed before each gene). Thus, the subunits may also have been expressed at non-stoichiometric ratios. This would lead to a metabolic burden without achieving the extra benefit of an active heterologous hydrogenase. The hydrogenases may still be beneficial at the low hydrogen concentrations being produced at the cathode in a BES, but due to the poor growth in serum flasks we did not try this. Instead, we prioritised BES experiments using strains expressing pyruvate formate lyase.

Heterologous expression of pyruvate formate lyase in C. ljungdahlii

CO and CO_2 reduction in acetogens lead to the formation of formate. Formate is a potent inhibitor of microbial growth, but it may also be incorporated as a carbon source. Pyruvate formate lyase catalyzes the reversible reaction

Acetyl-CoA + Formate $\leftrightarrow$ Pyruvate + CoA

Hence, overexpression of PFL may lead to increased incorporation of formate, produced by reduction of CO_2, into pyruvate for further conversion in metabolism. The active enzyme is a heterodimer of the PFL activating enzyme (subunit A) and the catalytic enzyme (subunit B).

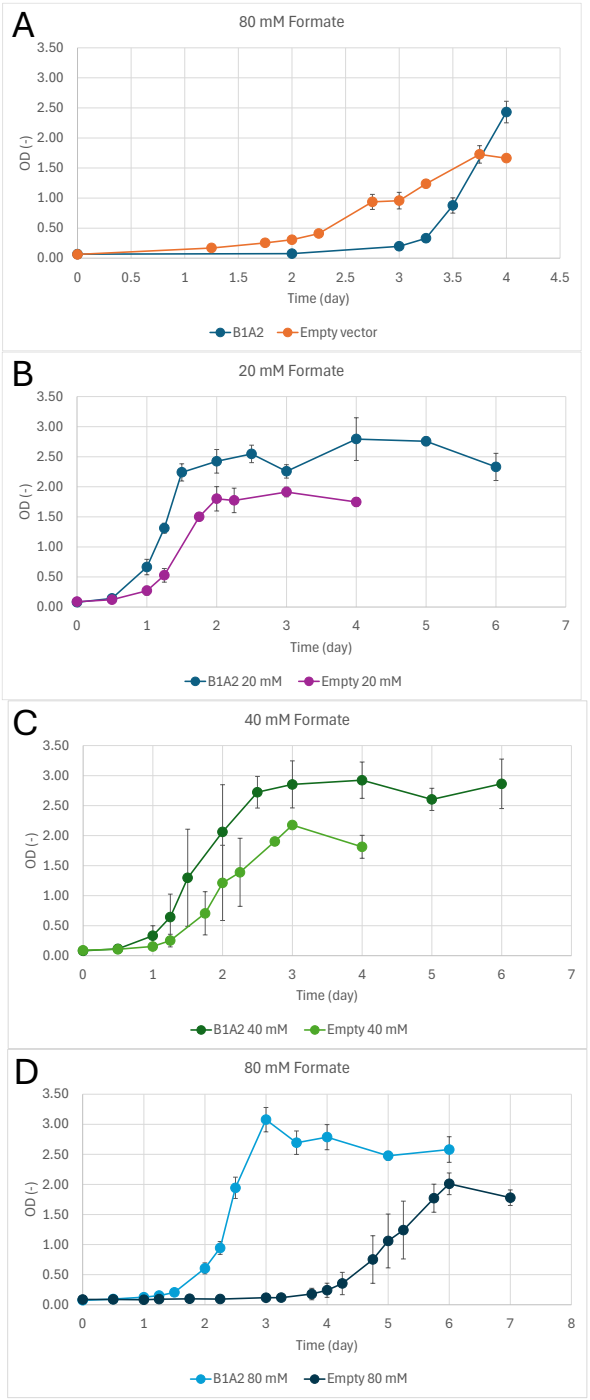

Figure 9. Growth curves (OD) of *C. ljungdahlii* transformed with an empty vector (Empty) and with the vector containing the B1A2 genes (B1A2) in serum flasks. Cells were precultured and adaptated in 80 mM formate (A) and transferred to serum flasks contianing (B) 20 mM, (C) 40 mM and (D) 80 mM formate. Gas was added to serum flasks to 2 bar overpressure of 20% CO_2 and 80% H_2. The initial pH was 5.7. Average values ± standard error of biological duplicates (Except A, average values ± standard deviation, n=3).

We expressed different combinations of subunits A and B from *Acetobacterium woodii pfl* and investigated the effect on growth in serum flasks with PETC medium (including 1 g/l yeast extract) plus 80 mM formate, in 20% CO_2 and 80% H_2 (Figure 9). Cells were first grown for four days in 80 mM to adapt to the medium. In the subsequent experiments, the lag phase was significantly shorter and the final OD was higher for the B1A2-expressing strains than for the strains with an empty vector. Hence, expression of PFL significantly improves cell growth on formate compared to the empty vector.

The performance of the PFL-expressing strain was also tested in BES on PETC medium (including 1 g/l YE) plus 20 mM formate with 100% CO_2 gas (Figure 10). In this case, H_2 is only provided via the cathode, using fixed current mode at 10 mA. The direct control of the strain expressing the empty vector has not yet been done. However, the untransformed *C. ljungdahlii* reached a maximum OD of 0.08 and then stopped growing when cultured in a BES at -1.0 V and in the absence of format (Fig 3G in Im et al 2022 (II)). The observed results with the PFL-expressing mutant were significantly better, and represented not only a threefold improvement in maximum OD, but also sustained growth throughout the 9 days of the experiment (Figure 10).

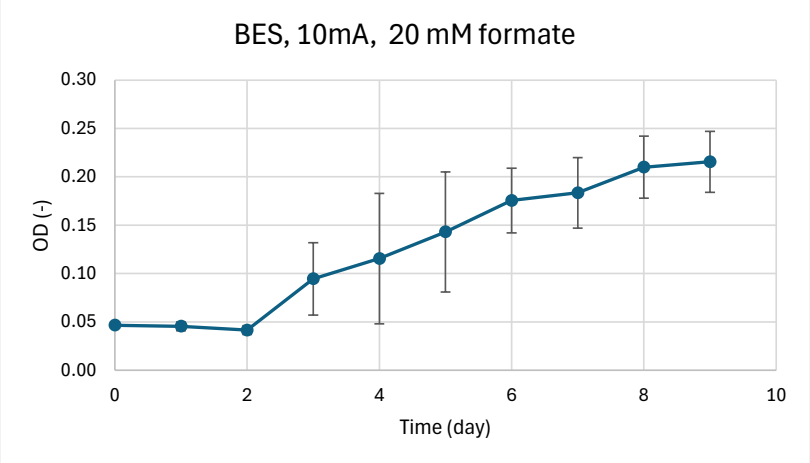

Figure 10. Growth curves (OD) of *C. ljungdahlii* expressing the B1A2 PFL genes (B1A2) in BES at 20 mM formate and constant current of 10 mA. Pure CO_2 was sparged at 10 ml / min. The initial pH was 5.0. Average values ± standard error of biological duplicates.

Diskussion

Dealing with inhibitors of syngas fermentation

The original aims of this project, to do RNA-seq to elucidate the trancriptional response to inhibitors and extracellular electron uptake, could unfortunately not be accomplished. However, by investigating the use of short-term adaptation during precultures, and long-term evolution in ALE, we could establish that these methods can be used to enable acetogens to cope with inhibitor concentrations typically found in real syngas. BTX tolerance could be developed within only a few sequential batch cultivations, so adding an increasing amount of such inhibitors during the cell propagation steps should enable cell growth and electrofermentation in their presence.

Adaptive Evolutionary Engineering of external electron uptake

The experimental analysis and whole-genome sequencing of the isolates purified after the ALE revealed several interesting results. First of all, it was surprising that none of the isolates performed better than the evolved population. This could possibly be due to a positive interaction between differently evolved mutants. However, the genome sequencing revealed that the eight selected isolates were predominantly mutated in the same sites. This means both that these sites should be important for the survival in the used conditions, and that the diversity that would enable positive interaction was not actually present. It is possible, that the eight isolates only represented a fraction of the whole diversity of the population.

The metabolic importance of the identified mutations will be further evaluated.

Tools for genetic modification of C. ljungdahlii

The gene expression system established in this work was adapted from published work [20-22]. Successful expression of heterologous genes depended on the use of a relatively weaker promoter (P_{fdx}) and a theophylline-inducible riboswitch. The expressed genes caused improvement of the cellular performance compared to when only the empty vector, without the gene of interest, was expressed. However, only expression of pyruvate formate lyase led to an improvement compared to the plasmid free parental strain. This indicates that minimizing the metabolic burden of the gene expression will become an important future development. Plasmid-free expression, such as using the CRISPR-Cas9 system, might be a better approach.

Heterologous hydrogenase and PFL expression in C. ljungdahlii

A putative electron transfer mechanism of *C. ljungdahlii* was identified in Activity 1. *C. ljungdahlii* seems to utilize hydrogen-mediated electron transfer in a BES reactor. Therefore, a set of target hydrogenases and connected cytochromes have been identified in *Sporomusa* and evolutionary closely related acetogenic *Clostridium* and *Acetobacterium* strains, and protocols for expressing them in *C.*

ljungdahlii are being developed. We hypothesized that expression of high affinity hydrogenases would improve electron transfer of *C. ljungdahlii* in a BES.

Contrary to our hypothesis, overexpression of *A. woodii* hydrogenases actually reduced cell growth. We interpret this that there is a sensitive balance between the metabolic burden of overexpressing multi-subunit proteins and the energetic benefit these may provide. Hence, a delicate expression system must be developed before successful application can be expected. The expression system must allow for fine tuning of expression levels of all parts of the active proteins. This may involve a developed riboswitch, or a completely different expression system.

Hydrogen metabolism and electron uptake are key elements of syngas electro-fermentation and metabolism of electro-active acetogens. There should still be potential for improvement, especially in view of the inhibition of hydrogenase by CO. However, tunable expression of the desired genes appears to be an important prerequisite for successful enhancement of the hydrogenase activity.

We also struggled to obtain successful expression of the pyruvate formate lyase in *C. ljungdahlii*. Since moderation of the expression system by both using a weaker promoter (P_{fdx}) and a theophylline-inducible riboswitch led to successful expression, we hypthesize that the earlier lack of success was due to the metabolic burden of the P_{thl} promoter system. PFL has only two subunits, which while the *A. woodii* Gr A3 and Gr A4 hydrogenases have four and six subunits, respectively. This offers an explaination why the PFL expression was less toxic to the cells than the hydrogenase expression.

Future work

The significantly improved growth of the PFL-expressing mutant in both serum flasks and BES indicates that his approach may lead to major improvements also in the production of biofuels. Moreover, establishing the P_{fdx} and theophylline-inducible riboswitch system for expression of heterologous genes will enable more efficient overexpression studies in the future. Additional growth-stimulating genes may be included, and combined with direct modification of the electron uptake systems. However, fine-tuning of expression levels and coordinated expression of enzyme subunits must be further developed.

Effects on the energy system

LanzaTech is already employing gas fermentation commercially. Recently, production of acetone and isopropanol has been established at industrial pilot scale, and the life cycle analysis showed that the production led to a net negative carbon footprint of the products [25]. The final titers and productivities that we have obtained in this project are still far too low to motivate an industrial scale-up. However, many interesting leads for further development of syngas electro-fermentation and has been identified, and will be further investigated in future project. Successful utilization and development of the metabolic capacities of acetogens such as *C. ljungdahlii* can lead to sustainable production of several

platform chemicals and biofuels while at the same time mitigating the CO_2 accumulation in the atmosphere.

Publikationslista

The following publications have resulted from the work performed in this project.

(I) Pawel Piatek, Lisbeth Olsson, Yvonne Nygård (2020) Adaptation during propagation improves *Clostridium autoethanogenum* tolerance towards benzene, toluene and xylenes during gas fermentation. Bioresource Technology Reports 12 (2020) 100564. https://doi.org/10.1016/j.biteb.2020.100564

(II) Chaeho Im, Kaspar Valgepea, Oskar Modin, Yvonne Nygård (2022): *Clostridium ljungdahlii* as a biocatalyst in microbial electrosynthesis – Effect of culture conditions on product formation. *Bioresource Technology Reports* 19:101156. https://doi.org/10.1016/j.biteb.2022.101156

(III) Chaeho Im, Minsoo Kim, Jung Rae Kim, Kaspar Valgepea, Oskar Modin, Yvonne Nygård, Carl Johan Franzén (2024): Low electric current in a bioelectrochemical system facilitates ethanol production from CO using CO-enriched mixed culture. *Submitted.*

(IV) Chaeho Im, Oskar Modin, Kaspar Valgepea, Carl Johan Franzén, Yvonne Nygård: Adaptive laboratory evolution on iron as a strategy for obtaining *Clostridium ljungdahlii* mutants with better performance during microbial electrosynthesis. *Manuscript.*

(V) Chaeho Im, Oskar Modin, Kaspar Valgepea, Yvonne Nygård, Carl Johan Franzén: Heterologous expression of pyruvate formate lyase from *Acetobacterium woodii* enhances cell growth of *Clostridium ljungdahlii* in a bioelectrochemical system. *Manuscript.*

(VI) Chaeho Im**,** Oskar Modin, and Yvonne Nygård (2022). CO_2 fixation for value-added chemical production using *Clostridium ljungdahlii* as a host strain in a bioelectrochemical system. *Poster presentation*, ISMET8 (Crete, Greece, September, 2022)

(VII) Chaeho Im**,** Oskar Modin, Carl Johan Franzén, and Yvonne Nygård (2023): Long-term cultivation of *Clostridium ljungdahlii* on iron. *Poster presentation*, EU-ISMET 2023 (Wageningen, the Netherlands, September 2023)

(VIII) Emil Frithiofson, Fahim Hadi, Carl-Johan Landström, Karl Larsson, Sara Mårtenson, Emma Stavås (2018) Etanolbildande acetogener för syngasfermentering – en jämförande studie. BSc thesis. Industrial Biotechnology, Chalmers University of Technology.

(IX) Fahim Hadi (2021) Bio-electrochemical activity of *Clostridium ljungdahlii* at different electric potentials. MSc thesis. Industrial Biotechnology, Chalmers University of Technology.

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