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CRISPR/Cas9n knock-out plasmid construction and transformation into *Clostridium autoethanogenum* for deletion of a formate dehydrogenase gene

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Title: CRISPR/Cas9n knock-out plasmid construction and transformation into *Clostridium autoethanogenum* for deletion of a formate dehydrogenase gene

Abstract:

Gas fermentation using acetogen bacteria is an attractive technology for tackling the problem of the accumulation of solid waste and greenhouse gases while contributing towards a circular economy through sustainable production of chemicals and fuels. Acetogens are the preferred biocatalysts for gas fermentation as they can use gases such as carbon monoxide (CO) and carbon dioxide (CO_2) as their sole carbon source. They fix carbon oxides via the Wood-Ljungdahl pathway (WLP), but specific functionalities of several key enzymes in the WLP are unclear. This thesis constructed a CRISPR/Cas9 nickase (Cas9n) plasmid for targeted deletion of the formate dehydrogenase gene *fdhA* in the model-acetogen *Clostridium autoethanogenum* that can catalyse the reduction of CO_2 to formate in the WLP. The *fdhA* knock-out plasmid was constructed using PCR-based cloning methods and InFusion cloning. The latter was also used to insert homology arms flanking the *fdhA* gene into the knock-out plasmid that are needed to facilitate gene deletion via homologous recombination in *C. autoethanogenum*. The plasmid was successfully transformed into *C. autoethanogenum* but *fdhA* deletion-bearing cells were not identified. Future work includes sub-culturing and plating the transformed cells to isolate *fdhA* knock-out cells, plasmid curing, and growth characterisation of the *fdhA* knock-out strain. This thesis provides both an intermediate strain for further study and valuable information for optimisation of future genetic engineering efforts.

Key words: *Clostridium autoethanogenum*, gas fermentation, genetic engineering, CRISPR/Cas9n

CERCS: T490 Biotechnology

Pealkiri: CRISPR/Cas9n *knock-out* plasmiidi konstrueerimine ja transformatsioon *Clostridium autoethanogenum*'isse formaat dehüdrogenaasi geeni deleteerimiseks

Lühikokkuvõte:

Gaasfermentatsioon atsetogeensete bakterite abil on atraktiivne tehnoloogia tahkete jäätmete ja kasvuhoonegaaside kogunemise probleemi lahendamiseks, andes samal ajal panuse ka ringmajanduse poole liikumisele. Atsetogeenid on gaasfermentatsioonis eelistatud biokatalüsaatorid, kuna nad suudavad kasutada ainsa süsinikuallikana gaase nagu süsinikdioksiid või -monoksiid (CO_2, CO). Süsinikoksiidide sidumiseks kasutavad atsetogeenid Wood-Ljungdahl metaboolset rada (WLP), mis sisaldab mitut ebaselge funktsiooniga ensüümi. Käesolevas töös konstrueeriti esmalt Cas9 *nickase* (Cas9n) plasmiid CO_2 formaadiks redutseeriva ensüümi formaat dehüdrogenaasi *fdhA* geeni deletsiooniks mudelatsetogeenis *Clostridium autoethanogenum*'is. *FdhA* deletsiooni plasmiid konstrueeriti kasutades erinevaid PCR-l põhinevaid kloneerimismeetodeid ning InFusion kloneerimist. Viimast kasutati *fhdA* geeni ümbritsevate homoloogsete järjestuste plasmiidi sisestamiseks, et suunata vastava geeni deletsiooni kasutades homoloogilist rekombinatsiooni *C. autoethanogenum'*i genoomi lõikamisel Cas9n valguga. Konstrueeritud plasmiid transformeeriti edukalt *C. autoethanogenum*'isse, kuid *fdhA* deletsiooniga rakke ei tuvastatud. Tulevane töö hõlmab ümberkülvi ja transformeeritud rakkude plaatimist *fdhA* deletsiooniga rakkude isoleerimiseks, plasmiidi eemaldamist saadud tüvest ning *fdhA* deletsioonitüve iseloomustamist. Käesoleva töö tulemuseks on nii Cas9n plasmiidi kandev bakteritüvi, mis on abiks edasiseks uurimiseks, kui ka väärtuslik teave tulevaste geenitehnoloogia alaste jõupingutuste optimeerimiseks atsetogeenis *C. autoethanogenum*.

Võtmesõnad: *Clostridium autoethanogenum*, gaasfermentatsioon, geneetiline inseneerimine, CRISPR/Cas9n

CERCS: T490 Biotechnology

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Terms, abbreviations and notations

Amp	Ampicillin
C. autoethanogenum	*Clostridium autoethanogenum*
Cas9n	Cas9 nickase
Cla	Clarithromycin
CO	Carbon monoxide
CO_2	Carbon dioxide
CODH/ACS	Carbon monoxide dehydrogenase/Acetyl-CoA synthase enzyme complex
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
gRNA	Guide RNA
H_2	Hydrogen
HA	Homology arm
Hyt/FdhA	[FeFe]-hydrogenase/formate dehydrogenase complex
iPCR	Inverse polymerase chain reaction
LB	Lysogeny Broth
Nfn	Transhydrogenase
OD	Optical density
P/D/L	Polynucleotide kinase, *DpnI*, T4 ligase
Rnf	Oxidoreductase
rpm	revolutions per minute
WLP	The Wood-Ljungdahl pathway
WT	Wild-type

1. Introduction

Our climate is rapidly changing, exemplified by rising sea levels, higher temperatures which cause more extreme weather variations, ice quantity in seas etc. The linear structure of our economy and the relying on burning fossil fuels, wood etc. for power have resulted in catastrophic amounts of accumulated physical waste as well as unprecedented levels of carbon dioxide (CO_2) and other greenhouse gases in our atmosphere. All of this has been proven to contribute to climate change. New technologies are urgently needed to recycle physical waste efficiently and lessen the burden of greenhouse gases on our climate.

Gas fermentation using acetogen bacteria can be utilised at large scales in a way that tackles both of the problems brought out. Acetogens fix carbon from gases such as carbon monoxide (CO) and CO_2 natively via the Wood-Ljungdahl pathway (WLP). Acetogens can produce many industrially relevant chemicals that could be used in the making of fuels and bioplastics among others, recently evidenced by the commercialisation of acetogen gas fermentation by LanzaTech.

Clostridium autoethanogenum is a model acetogen being used in the gas fermentation industry at large scale and being investigated by many research groups. However, many aspects of the bacteria's metabolism, especially regarding the WLP are unknown at the moment. While the energy conservation and biochemistry of the pathway has been investigated quite extensively, roles of even key enzymes in the WLP are not fully clear, one of those being the formate dehydrogenase FdhA that forms a complex with a [FeFe]-hydrogenase Hyt protein.

The aim of this investigation was to characterise the functionality of the *fdhA* gene of *C. autoethanogenum* via genetic engineering to help accelerate the process of using gas fermentation as a means to produce valuable chemicals with a negative carbon footprint. More specifically, the *fdhA* gene was attempted to be deleted from the *C. autoethanogenum*'s genome with a CRISPR/Cas9n plasmid system.

2. Background information

2.1. Carbon crisis and gas fermentation

It is widely known by now that our climate is changing: rising sea levels, higher temperatures which cause more extreme weather variations, ice quantity in seas etc. have been observed for a long time and by a multitude of different research groups (USGCRP, 2017). It is apparent that the changes happening in our climate right now are more rapid and do not match the pattern of climate changes throughout the ages before, indicating that this change is caused by human activity (USGCRP, 2017). One of the main drivers of climate change is the increased amount of greenhouse gases such as CO_2, methane and nitrous oxide in our atmosphere (USGCRP, 2017). According to CO_2 measurements taken from arctic ice and comparison to recent measurements of our atmosphere directly, the CO_2 levels are at least 25% higher than they have ever been over the past 800 000 years (USGCRP, 2017). Evidently, it is crucial to develop and employ a method of removing the load of greenhouse gases from our atmosphere.

Another problem in our current economy is the widely used linear economy structure where a product is made, used and then discarded (Ellen McArthur Foundation, 2012). Waste accumulation due to our consumerist and linear economy structure has already shown to have catastrophic ramifications to our planet as illustrated by the ever growing Great Pacific Garbage Patch (Kottasová, 2023). Additionally, companies mine/produce fossil fuels or other energy dense materials that are then consumed by anything that needs fuel (aeroplanes, cars, some factory apparatus etc.). This consumption usually means burning which releases many gases into the atmosphere. Finding a way of recycling solid waste and reutilising the released gases would shift the economy into a more circular one which would in turn have many benefits to both the economy and the environment (Ellen McArthur Foundation, 2012).

Gas fermentation using acetogens is a particularly attractive technology for tackling both of the challenges of the linear economy and reduction of greenhouse gas emissions from big factories. This process has already been commercialised by LanzaTech where acetogens are cultivated for the production of ethanol from steel-mill waste gas (Köpke & Simpson, 2020). Pilot-scale production of acetone and isopropanol has been also demonstrated (Liew et al., 2022) and more products are in the pipeline, e.g. 2,3-butanediol (*LanzaTech and Sumitomo Riko Partner to Create Substitute for Natural Rubber Production – LanzaTech*, 2022) and more (Liew et al., 2016). LanzaTech's current action plan involves collaborating with big factories and landfills to use their emitted gases and then feeding the gases to acetogens who can produce valuable chemicals and fuels (*About – LanzaTech*, n.d.). In addition to directly emitted gases, synthesis gas that comprises CO, CO_2 and hydrogen (H_2), is often used to feed the acetogens in bioreactors because almost any organic waste can be converted to syngas through gasification (Liew et al., 2016).

2.2. Acetogens as biocatalysts

Acetogens are an anaerobic group of bacteria that, during autotrophic growth, can fix carbon from CO and CO_2 via the WLP (Figure 1) which has been deemed as energetically the most efficient CO_2 fixation pathway (Fast & Papoutsakis, 2012). Importantly, acetogens can use gas as their sole carbon and energy source (Wood, 1991). Acetogens are therefore the main drivers of the gas fermentation technology because of the previously mentioned unique ability to fix carbon from CO and CO_2 gases and the anaerobic nature of acetogens – handling of synthesis gas is much safer when the anaerobic environment does not allow for the H_2 to explode (Liew et al., 2016).

The optimum temperature for the growth of acetogens is 37°C at atmospheric pressure making the cultivation conditions cheaply maintainable compared to the chemical synthesis of similar products which oftentimes requires hundreds of degrees of heat and high pressure to be maintained (Liew et al., 2016). Furthermore, acetogens are particularly diverse for their native metabolic products, being capable of producing acetate, ethanol, lactate, 2,3-butanediol etc. (Liew et al., 2016).

The most notable acetogenic species for their industrial potential are *Clostridium ljungdahlii*, *C. autoethanogenum*, *Clostridium ragsdalei*, and *Cloctridium coskatii*. All of them share a 99-100% similar 16S rRNA sequence, making them "nearly indistinguishable" as highlighted by Bengelsdorf et al. (2016). *C. autoethanogenum* is one of the more prominent of them and has grown to be the model acetogen because of the magnitude of gathered data of the organism as well as its use as a biocatalyst in commercial industrial settings (Liew et al., 2016).

2.2.1. *C. autoethanogenum*: a model acetogen

C. autoethanogenum is a chemolithoautotrophic (Köpke & Simpson, 2020) obligate anaerobe that was first extracted from rabbit faeces using CO as its only carbon and energy source (Abrini et al., 1994). It is a rod-shaped gram-positive bacteria that can also form spores (Abrini et al., 1994). It is known that this organism is capable of using CO, CO_2 and H_2 as substrates via the WLP (Figure 1; Heffernan et al., 2020). However, other substrates such as fructose, arabinose, xylose, pyruvate etc. can be used by the organism as well (Abrini et al., 1994). The optimum growth temperature of *C. autoethanogenum* is 37°C and the optimum pH for growth is 5.8-6.0 (Abrini et al., 1994).

Even though the first extract of the bacteria was found to be growing on CO (Abrini et al., 1994), it is worth noting that some of its key enzymes, particularly the [FeFe]-hydrogenase/formate dehydrogenase complex (Hyt/FdhA, Figure 1) is inhibited by CO already at low concentrations (Wang et al., 2013). It has been elucidated that *C. autoethanogenum* tackles the CO toxicity problem with the carbon monoxide dehydrogenase/Acetyl-CoA synthase enzyme complex (CODH/ACS, Figure 1) which acts as the main CO scavenger in the organism (Wang et al., 2013).

C. autoethanogenum is capable of natively producing many products from Acetyl-CoA as seen from the branching from that molecule in Figure 1. *C. autoethanogenum* produces acetate (CH_3COOH), ethanol (C_2H_5OH), 2,3-butanediol ($CH_2CHCH_2CH_2OH$), lactate ($CH_3CHCOOH$) and pyruvate (CH_3COCOO^-) from Acetyl-CoA (Liew et al., 2016; Wang et al., 2013). Metabolic engineering has increased the breadth of chemicals that *C. autoethanogenum* can produce through gas fermentation to over 50 such as amino acids (valine, alkaline etc.), polyhydroxybutyrate, ectoine and many more (Köpke & Simpson, 2020).

As mentioned before, *C. autoethanogenum* is attractive as an acetogenic biocatalyst capable of producing zero-emission products with a negative carbon footprint to the biotech industry. The previously mentioned LanzaTech uses this organism as their main biocatalyst in their commercial bioreactors (Humphreys & Minton, 2018).

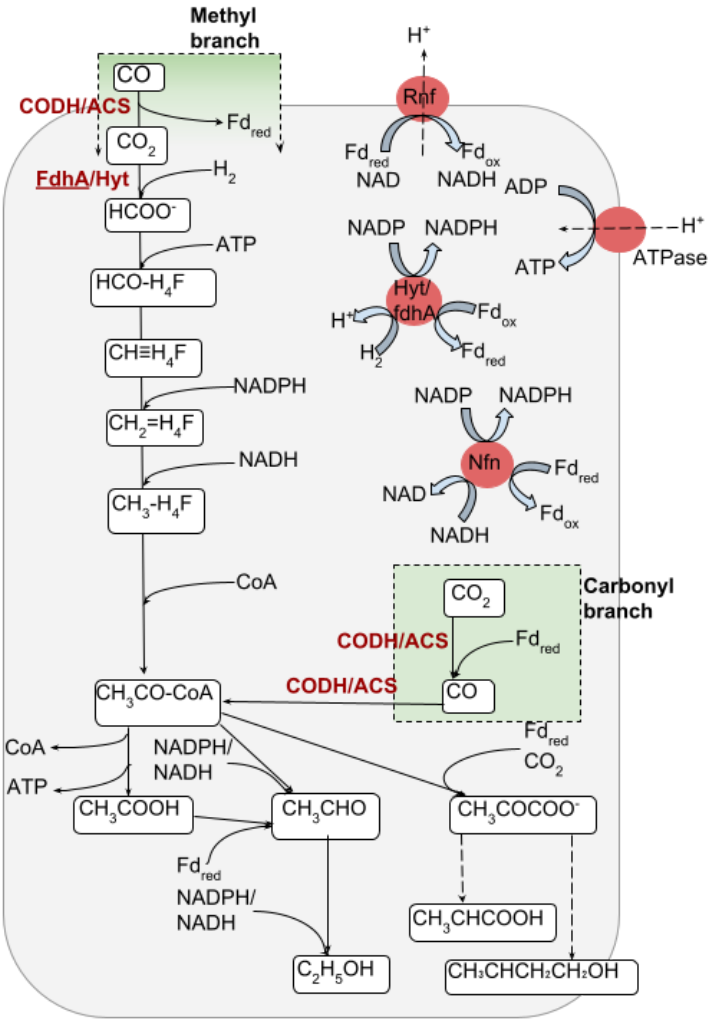

Figure 1. The Wood-Ljungdahl metabolic pathway (WLP) and energy conservation mechanisms in *C. autoethanogenum* when grown on CO. Dotted green areas indicate the methyl and carbonyl branches of the WLP. Dotted arrows from Pyruvate (CH_3COCOO^-) mark the potential products of the WLP. Energy conservation molecules are depicted as red circles and WLP enzymes are shown in bold, red text. The underlined enzyme marks the enzyme concentrated on in this work. FdhA/Hyt - formate dehydrogenase/[FeFe]-hydrogenase complex; Nfn - transhydrogenase; Rnf - reduced ferredoxin and NAD oxidoreductase; CODH/ACS - carbon monoxide dehydrogenase/Acetyl-CoA synthase; CoA - coenzyme A; Fd - ferredoxin. This figure was created on the basis of Liew et al. (2016), Mock et al. (2015), Wang et al. (2013), and Lemaire & Wagner (2020).

2.2.2. Acetogen metabolism

During auxotrophic growth of *C. autoethanogenum* as well as other acetogens, the bacteria uses the WLP (Figure 1) to fix carbon from its growth environment.

Firstly, carbon fixed from the environment is reduced to formate ($HCOO^-$) with the FdhA/Hyt complex (see 2.3. Genes with unclear functionalities) catalysing the reaction

(Wang et al., 2013). If there is no CO_2 present in the environment, the bacteria will make use of the Methyl branch (Liew et al., 2016) of the WLP to oxidise CO with the CODH/ACS complex catalysing the reaction (Lemaire & Wagner, 2020).

The following reaction entails a tetrahydrofolate molecule (H_4F) being added to the formate reduced from CO or CO_2, forming formyl-tetrahydrofolate ($HCO\text{-}H_4F$) which will then be reduced to Methyl-Tetrahydrofolate ($CH_3\text{-}H_4F$) in a series of reducing reactions, using up ATP, tetrahydrofolate, NADPH and NADH molecules. The Methyl-Tetrahydrofolate will be reduced once again to Acetyl-CoA, using Coenzyme-A and CO from the environment (Liew et al., 2016; Mock et al., 2015) and with the CODH/ACS complex catalysing the reaction again (Lemaire & Wagner, 2020). If there is no CO preset in the environment, the bacteria can make use of the Carbonyl branch of the WLP (Liew et al., 2016) to reduce CO_2 to CO with the CODH/ACS complex catalysing the reaction (Lemaire & Wagner, 2020).

As can be seen from Figure 1, when converting all the fixed carbon to acetate, the ATP production is at net zero. Because of that acetogens need the presence of energy conserving enzymes to keep the cell energetically viable. The oxidoreductase (Rnf) and ATPase work in synergy to regenerate ATP necessary for the cell's energy requirements by maintaining a proton gradient together. The Rnf is a membrane bound proton pump that uses reduced ferredoxin to pump protons out of the cell and produce NADH while the ATPase uses the proton gradient generated by the Rnf to synthesise ATP (Mock et al., 2015). Acetogen metabolism, however, needs another mode of energy conservation for operation - electron bifurcation (Schuchmann & Müller, 2014).

Bifurcating enzymes oxidise a hydride donor and deliver the released electrons simultaneously to two different electron acceptors (Schuchmann & Müller, 2014) where the reduction of one acceptor is always exergonic, which drives the endergonic reduction of the second acceptor (Schuchmann & Müller, 2014). The FdhA/Hyt complex and the transhydrogenase (Nfn) are responsible for the above mentioned process of electron bifurcating where H_2 and NADH are used for the hydride donor by the FdhA/Hyt complex and the Nfn, respectively (Buckel & Thauer, 2018). The two electron acceptors both in the FdhA/Hyt complex and Nfn electron bifurcating process are ferredoxin and NADP (Buckel & Thauer, 2018). Thus, the Hyt protein of the FdhA/Hyt complex is an NADP dependent enzyme that generates protons and regenerates reduced ferredoxin (Mock et al., 2015). The Nfn is a reduced ferredoxin dependent enzyme that uses NADH to regenerate NADPH (Mock et al., 2015). These energy conserving enzymes directly provide substrates and cofactors for the WLP, making the redox metabolism in the form of the WLP and the energy metabolism closely related.

2.3. Genes with unclear functionalities

A key enzyme in the WLP is the FdhA that encodes for a formate dehydrogenase which is in complex with the Hyt proteins discussed above (see 2.2.2. Acetogen metabolism). The FdhA's main role in the WLP is to reduce CO_2 to formate. It has been shown that the Hyt and FdhA proteins are energy coupling so that the reduction of ferredoxin and NADP is carried out by using formate as the electron donor (Wang et al., 2013; Buckel & Thauer, 2018). The enzyme complex can also reduce CO_2 to formate directly with H_2 without the use of redox factors with its formate-H_2 lyase activity (Wang et al. 2013). Because CO is toxic for the Hyt already at low concentrations and *C. autoethanogenum* grows natively on CO, the Hyt is in complex with the FdhA so that together they would have a high H_2 formation potential to overcome the CO toxicity problem at the steady state growth phase (Wang et al., 2013). Interestingly, it is not known whether the FdhA could reduce CO_2 to formate without the Hyt component of the complex. This complex forms 6% of all cytoplasmic proteins in *C. autoethanogenum* (Wang et al., 2013).

It has been shown that deleting *fdhA* from the genome of *C. ljungdahlii* greatly reduces its maximum optical density (OD) measurement and the production of ethanol and acetate (Zhang et al., 2020). However, this gene's phenotypic function nor its isoenzymes have not been investigated in *C. autoethanogenum* yet (Mock et al., 2015). Investigation of *fdhA* functionality in *C. autoethanogenum* would be valuable to both the biotech industry and the scientific community because it would offer a better understanding of the organism and of other acetogens. The *fdhA* gene (CAETHG_2790) is the knock-out target in this work.

2.4. Genetic engineering of acetogens

2.4.1. Methods for handling *C. autoethanogenum* and challenges

Transformation of *C. autoethanogenum*

C. autoethanogenum is known for its very low transformation rate (Woods et al., 2019), however, two methods – transformation by electroporation and conjugation – have been shown to work in the past with this particular acetogen (Liew et al., 2017; Nwaokorie et al., 2023).

Transformation by electroporation is a process where DNA is introduced into desired cells using high-voltage electric shocks (Potter & Heller, 2003). Careful and precise preparation of electrocompetent cells is essential for success, including for example that cells mass should be washed multiple times to get rid of any salts that could ignite the electroporation machine (Sigma-Aldrich, n.d.). This kind of transformation is possible due to the properties of the cell membrane - it is an electrical insulator and thus largely acts as a capacitor when subjected to electricity (Potter & Heller, 2003). This in turn causes the temporary formation of pores within the membrane which can be big enough that large molecules (such as plasmid DNA) can enter the cell. If the electroporation mixture is

cooled down, the pores can stay enlarged for a longer period of time, making the electroporation process more effective (Potter & Heller, 2003). However, *C. autoethanogenum* has been shown to have a significantly lower transformation and recombination efficiency than the more commonly used model organisms such as *Escherichia coli* (Köpke & Simpson, 2020). That necessitates the use of another organism as a host for cloning such as *E. coli*. Before transformation of *C. autoethanogenum* with genetic material, the material has to possess the same modification patterns as the transformed organism natively in order to not activate the host's restriction-methylation system and in turn degrade the transformed genetic material (Woods et al., 2019). Thus, all constructed plasmids need to be transformed to the *dcm*$^-$ NEBExpress *E. coli* strain so that the vector would have the *C. autoethanogenum* native methylation pattern (Woods et al., 2019).

Conjugation is a method where a type of bacteria that is easily transformed mediates the mobilisation of a desired DNA vector into another type of bacteria (Woods et al., 2019). In the case of *C. autoethanogenum*, the correspondingly engineered *E. coli* strain sExpress has been used as the vector donor in recent years (Woods et al., 2019). This strain is *dcm*$^-$ meaning that it does not methylate DNA at the Dcm sites, preventing the recipient strain (*C. autoethanogenum*) from activating its restriction-methylation system (Woods et al., 2019). This is important so that *C. autoethanogenum* would not degrade the conjugated vector due to non-native modification patterns of the DNA (Woods et al., 2019).

Genetic modifications in *C. autoethanogenum*

Various approaches have been used in acetogens to modify the genome. In *C. autoethanogenum* specifically, the use of mobile elements (e.g. Clostron (Nagaraju et al., 2016)), homologous recombination (e.g. allele-coupled exchange (Annan et al., 2019), *pyrF* selection (Liew et al., 2017)), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - Cas9 (Nagaraju et al., 2016) and others have been successful in modifying the bacteria's genome (Bourgade et al., 2021).

CRISPR/Cas genetic modification systems offer many advantages over the others such as being faster and more efficient in constructing and screening mutant strains as well as being specific due to the RNA guided genome editing (Bourgade et al., 2021). However, the CRISPR/Cas system alone is not sufficient to efficiently modify prokaryotic genomes. Just cutting the genome with a Cas protein does not work for knock-out formation because of the inefficiency or total absence of the nonhomologous end-joining repair mechanism that would be able to mediate the repair of single- or double-strand breaks in DNA. Thus, the presence of homology arms (HAs) is needed in the gene knock-out plasmid (Davis & Chen, 2013; McAllister & Sorg, 2019) so that the homologous recombination repair system would activate. Also, the use of a nickase variant of Cas (e.g. Cas9 nickase – from now on Cas9n) is preferred as it makes a single-strand cut, easing the recombination process (Gasiunas et al., 2012).

The knock-out formation process starts with the expression of the gene knock-out plasmid and construction of the Cas9n protein which is guided to a specific locus in *C. autoethanogenum*'s genome with an appropriate guide RNA (gRNA) sequence. The Cas9n protein introduces a single stranded break in that locus, signalling to the bacteria that there is a cut in the genome and the chromosome needs to be repaired (Douglass Wright et al., 2018). As a result, specific DNA reparation enzymes would start searching the bacteria for homologous sequences close to the break locus from the rest of the cell and these enzymes would find the knock-out plasmid with HA sequences that flank the knock-out target gene (Douglass Wright et al., 2018). HAs are then used to repair the bacteria's genome but since the gene between the HAs has been cut out of the knock-out plasmid, the bacteria will repair the genome by cutting the knock-out target gene out (Douglass Wright et al., 2018). Even though the exact mechanism of the homologous recombination repair system hasn't been described in *C. autoethanogenum*, this mechanism has been shown to work in previous genomic engineering works (Nagaraju et al., 2016).

2.4.2. Overview of methods for construction of CRISPR/nCas9 plasmids

Inverse polymerase chain reaction

Inverse polymerase chain reaction (iPCR, Figure 2) is commonly used for site-directed mutagenesis in a circular DNA sequence and uses inversely directed (relative to each other) primers with a mutated region to yield a linearized mutated sequence (Silva et al., 2017). When these primers are used to amplify the plasmid, the mutated sequence in the middle of one of the primers is integrated into the plasmid DNA at a specific site (Silva et al., 2017). This methodology can be used to one's advantage when inserting a short sequence into plasmid DNA is the desired cloning product.

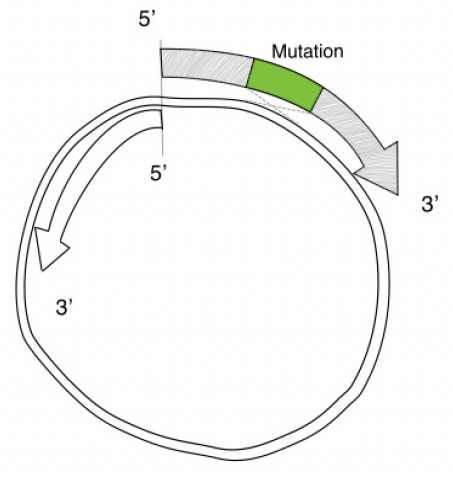

Figure 2. A schematic representation of an inverse PCR reaction. Primers are represented by arrows (arrow size in the figure is not representative of the proportional primer length). The mutated gRNA sequence is shown in green and with the label "Mutation". Both 5' and 3' ends of the primers are shown (Silva et al., 2017).

Restriction cloning

Restriction cloning is a method where both the insert sequence and backbone sequence are digested with the same restriction enzymes to linearise these sequences if needed and make them suitable for the ligase to bind and ligate the desired parts' ends together (Ford, 2016). A schematic of this method can be seen on Figure 3.

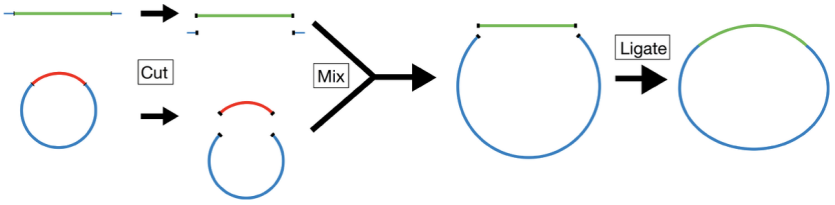

Figure 3. A schematic representation of a restriction cloning technique.The insert sequence is in green and the vector sequence is in blue. Sticky ends created by restriction enzymes are depicted as black dots at the ends of sequences. Figure taken from Ford (2016).

Overlap PCR extension cloning

The overlap PCR extension cloning technique (Figure 4) makes use of PCR and a restriction reaction to insert a sequence into a plasmid. Firstly, the insert sequence is to be amplified with specific primers that contain the old plasmid's sequence in one end (5') and the insert sequence in the other end (3'). After the first PCR of just the insert, another round of PCR is carried out with the amplified insert acting as megaprimers and the old plasmid acting as a template. After a few cycles of the second PCR the insert sequence between plasmid sequences in the mega-primers will be incorporated into the new plasmid.

The PCR product is then digested with the DpnI restriction enzyme to break down the old template plasmid. (Bryksin & Matsumura, 2010)

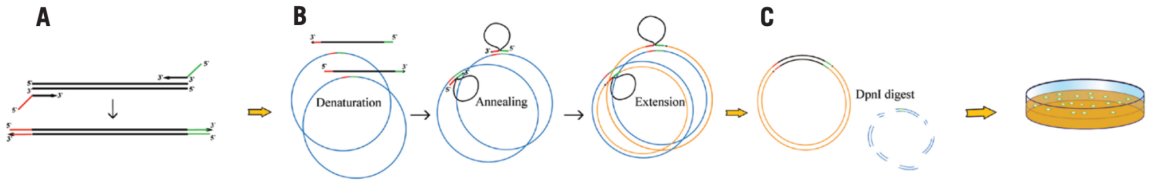

Figure 4. A schematic representation of the overlap PCR cloning method. Insert DNA as megaprimers is depicted in black, the old plasmid vector is depicted in blue and the new modified plasmid is depicted in orange. Figure is taken from Bryksin & Matsumura (2010).

InFusion cloning

The InFusion cloning technique (Figure 5) makes use of the In-Fusion enzyme capable of joining together multiple linear DNA sequences (Takara Bio USA, 2018). Similarly to the overlap PCR (see Overlap PCR extension cloning) this can be done with user designed overlaps between the fragments that are going to be joined together, but unlike the overlap PCR cloning method, InFusion doesn't require lengthy PCRs to be set up nor does it need any restriction enzyme digestion reaction to take place (Takara Bio USA, 2018).

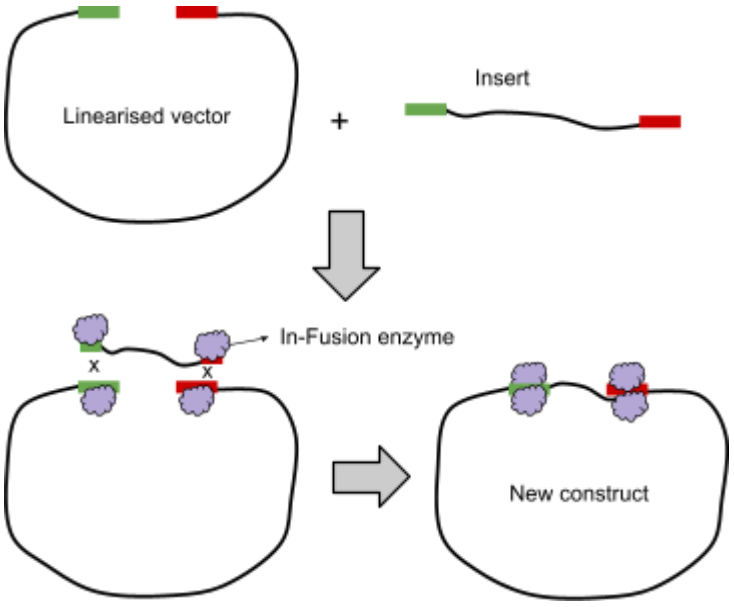

Figure 5. A schematic representation of the InFusion cloning method (Takara Bio USA, 2018). where DNA sequences are depicted as a black line, the In-Fusion enzyme is depicted as a purple cloud and "sticky ends" are depicted as green and red boxes, respectively.

3. Aims of the thesis

The aims and general workflow of the present study was the following:

1. CRISPR/Cas9n plasmid construction for deleting the *fdhA* gene using NEB Turbo and NEBExpress *E. coli* transformation competent strains.
2. *C. autoethanogenum* transformation with the constructed gene knock-out plasmid and confirmation of the transformants.
3. Plasmid curing and characterisation of the *C. autoethanogenum fdhA* knock-out strains

4. Materials and methods

4.1. Primers and plasmids

Table 1 presents all the primers used in this investigation along with a description of their role in the experiments. In general, primers were used to carry out plasmid construction and/or confirmation of PCR reactions. Primers with gRNA sequences were also designed for the Cas9n protein to direct the protein to a specific locus in the *C. autoethanogenum* genome.

Table 1. Sequences of primers (3' → 5') used in this investigation along with a description of their purpose. The upper case bases in gRNA primers signify the mutated region of the iPCR forward primer (see Figure 1). The lower case bases in HA primers signify the overlapping regions for overlap PCR (see Overlap PCR extension cloning).

Name of primer	**Primer sequence**	**Description**	**Designed by**
NickClos-1-F	GTCGTTGTTTAGGTGTGTTGTAATAC	Primer used for Sanger sequencing of HA start sequence	Kristina Reinmets
NickClos-2-R	ATCCGCTTTATCAGTAGAATCTACC	Primer used for Sanger sequencing of HA end sequence	Kristina Reinmets
pNC_gRNA-R	TGAGCTAGCTGTCAACTGCAGC	Universal reverse primer used for iPCR (see 4.5.2. gRNA swapping using inverse PCR)	Kristina Reinmets
fdhA_gRNA 2	gtcctaggtataatactagtCTGTGTTTAAAAGGACACTTgttttagagctagaaatagcaagttaaa	Primer for inserting gRNA #1 for the gene *fdhA* using iPCR (see 4.5.2. gRNA swapping using inverse PCR) with mutated region in uppercase	Pille-Riin Kurrikoff
fdhA_gRNA 3	gtcctaggtataatactagtTGCGCCTACCCACCATTGATgttttagagctagaaatagcaagttaaa	Primer for inserting gRNA #2 for the gene *fdhA* using iPCR (see 4.5.2. gRNA swapping using inverse PCR) with mutated region in uppercase	Pille-Riin Kurrikoff

fdhA_KO_ha 1000up-F	gaaaaagtggcaccgagtcggt gctttttgcggccgcGGCAA CCTTTCAAAGACTC AAT	Forward primer for amplifying the upstream 1kb HA of the gene *fdhA*	Pille-Riin Kurrikoff
fdhA_KO_ha 1000up-R	ctcttaatcaaggtctttttagccat cctgcagCCCCCCAATA ACTTTATATATTAAAT T	Reverse primer for amplifying the upstream 1kb HA of the gene *fdhA*	Pille-Riin Kurrikoff
fdhA_KO_ha 1000dn-F	taaatttaatatataaagttattggg gggctgcagGATGGCTA AAAAGACCTTGATT AAG	Forward primer for amplifying the downstream 1kb HA of the gene *fdhA*	Pille-Riin Kurrikoff
fdhA_KO_ha 1000dn-R	tattattatttttatcaatatattttgtt aaaaactcgagGCGGATT TCCTGGAAGTCC	Reverse primer for amplifying the downstream 1kb HA of the gene *fdhA*	Pille-Riin Kurrikoff
acsB-F	GGTGCATTAGATATA GTAAGAAGTTTG	*C. autoethanogenum* genome specific primer for species confirmation	Kristina Reinmets
acsB-R	ACTTCTCCCTGATCT GCACAC	*C. autoethanogenum* genome specific primer for species confirmation	Kristina Reinmets
pGFT-oriT_tr aJ-F	AGTCTAACACACTA GACTTATTTACTTCG TAATTAAGAGGCGG GGTCATTATAGCGAT TT	pGFT (See Table 2) specific primer for plasmid presence confirmation	Kristina Reinmets
pGFT-oriT_tr aJ-R	TTAAAGGTTTTATAC TTTTGGTCGTAGAGC ACACGGTTAGGGTA CTTCACCAGCTCCGC G	pGFT (See Table 2) specific primer for plasmid presence confirmation	Kristina Reinmets
fdhA_up_F	TCAGATATTATATGTG CTTCCATAAAGA	*fdhA* gene knock-out confirmation primer	Pille-Riin Kurrikoff

All plasmids constructed and used during this thesis work (Table 2) were based on a pGFT012 plasmid (Figure 6) previously created in the GasFermTech group. The pGFT012 plasmid which is a derivative of the pNICKclos2.0 plasmid (Li et al., 2016) contains a sequence for the Cas9n protein, a gRNA for the Cas9n and a HA region – all necessary for successfully modifying *C.autoethanogenum*'s genome. In addition to that antibiotic resistance and origin of replication regions for both gram-negative and gram-positive bacteria are present.

Table 2. All plasmids that have been constructed and/or used during this work along with a brief description of their composition and the author of the plasmid.

Plasmid name	**Description**	**Author**
pGFT012	Template *C. autoethanogenum* transformation plasmid	Kristina Reinmets
pGFT017	pGFT012 + fdhA_gRNA2	Pille-Riin Kurrikoff
pGFT031	pGFT012 + fdhA_gRNA3	Pille-Riin Kurrikoff
pGFT097	pGFT031 + 1 kb *fdhA* HAs	Pille-Riin Kurrikoff

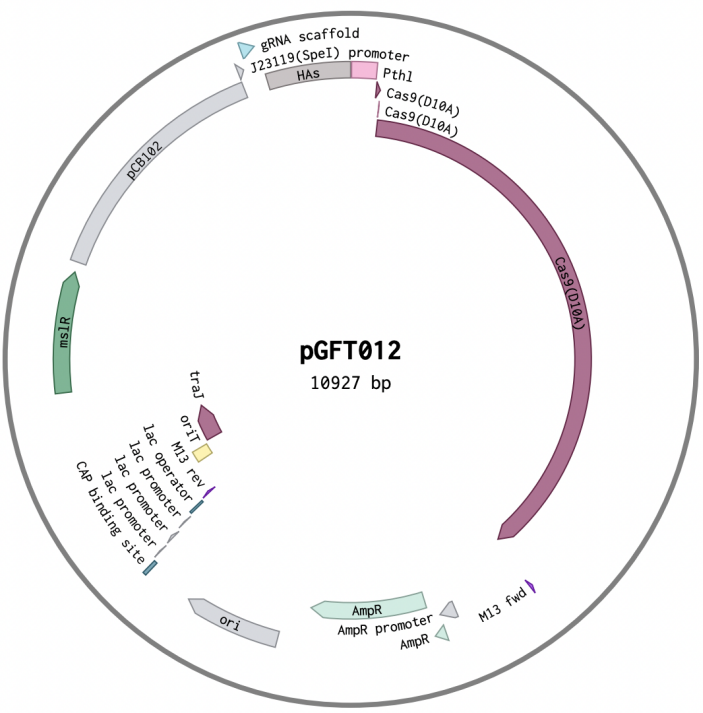

Figure 6. The schematic representation of pGFT012 plasmid. AmpR cluster - ampicillin resistance, mslR - macrolide lincosamide streptogramin B class antibiotic resistance (Tenson et al., 2003), HAs - homology arms, Cas9(D10A) cluster - *Cas9n* sequence, ori - origin of replication for gram-negative bacteria, gRNA scaffold - gRNA sequence for *Cas9n*, Pthl - strong promoter of the thiolase gene of *Clostridium acetobutylicum*, J23119 promoter - synthetic promoter for the expression of gRNA, pCB102 - pCB102 origin of replication for gram-positive bacteria from *Clostridium butyricum* (Zhang et al., 2015; Li et al., 2016).

4.2. Strains

NEB Turbo Competent *E. coli* strain was the transformation competent strain used for constructing and replicating the knock-out plasmids (pGFT097, Table 2) and their predecessor gRNA plasmids (See Table 2). The NEBExpress competent *E. coli* strain is a *dcm*$^{-}$ strain and thus will not methylate at DCM sites. This methylation pattern is important to overcome the restriction-methylation system of *C. autoethanogenum* (Woods et al., 2019). LAbrini is the *C. autoethanogenum* strain used in this work for constructing knock-out strains (Ingelman et al., n.d.). All described strains can be seen in Table 3.

Table 3. List of strains used in this investigation along with the stain name, species and a brief description of the role in experiments.

Strain name	Strain species	Description
NEB Turbo Competent *E. coli*	*E. coli*	Transformation competent cells used for plasmid propagation.
NEBExpress Competent *E. coli*	*E. coli*	*Dcm*$^-$ NEB express cells used to get efficient methylation pattern of knock-out plasmid before transforming into *C. autoethanogenum*
LAbrini	*C. autoethanogenum*	*C. autoethanogenum* base strain (Ingelman et al., 2023)

4.3. Media and cultivation conditions

All liquid *E. coli* cultures were grown in Lysogeny Broth (LB) (Bertani, 2004) media (10 g/l Tryptone, 5 g/l Yeast extract, 10 g/l NaCl) put on a shaker at 210 rpm, 37°C and supplemented with 100 µg/ml ampicillin (Amp) unless stated otherwise. Plated *E. coli* cultures were grown on selective LB agar plates containing 100 µg/ml Amp at 37°C. Incubation time for both liquid and plated *E. coli* cultures ranged between 16-20 h.

C. autoethanogenum cultures were grown on anaerobically prepared YTF (16 g/l Bacto Tryptone, 10 g/l Yeast extract, 200 mg/l NaCl, 5 g/l Fructose, 500 µg/l Resazurin, pH 5.8) media. Exposed *C. autoethanogenum* cells were handled in an anaerobic chamber, liquid cultures were grown in anaerobic Balch tubes and serum/Schott bottles of different volumes, YTF agar (1.5% Formedium agar) plates were stored in anaerobic jars during incubation outside the anaerobic chamber. *C. autoethanogenum* liquid and plated cultures were incubated at 37°C. Liquid cultures were kept shaking horizontally at 120 rpm in case of serum/Schott bottles and tilted at 210 rpm (revolutions per minute) in case of Balch tubes.

4.4. *E. coli* transformation procedure

Depending on the transformation procedure, *E. coli* competent cells were mixed with either ~1 µg of genetic material or <10 µl of a reaction mixture (exact volume was selected based on literature/manufacturer's suggestions). This mixture was then incubated on ice for 30 min and heat shocked at 42°C for 20 or 30 sec for NEBExpress and Turbo strains,

respectively (See Table 3). The mixture was incubated on ice again for 5 min. This mixture was then inoculated into 1 ml of non-selective LB media and shaken at 150 rpm and 37°C for 1 h as a recovery step. 100 µl of the recovery culture was plated onto a selective LB agar plate, the rest of the recovery culture was spun down in an Eppendorf tube, 100 µl of the supernatant was left in the tube. The mixture was resuspended and 100 µl of the concentrated recovery culture was plated onto a selective LB agar plate.

4.5. Primer design and plasmid construction

4.5.1. Primer design

Firstly, the target gene sequences (see 2.3. Genes with unclear functionalities) along with approximately 1500 bp from each side of the gene were downloaded from the *C. autoethanogenum* genome assembly version NC_022592.1 from the NCBI online database.

For traditional primers like the knock-out confirmation primer fdhA_up_F (Table 1), 20-30 bp of DNA was selected around the region where the primer was needed (e.g. for the forward upstream 1 kb HA primer, the primer sequence was selected around 1000 bp upstream of the target gene). Next, the NetPrimer website was used to evaluate the quality of the primer sequence. The following values were monitored:

- Primer melting temperature around 56-61°C
- Dimer value of >-5 kcal/mol
- Hairpin value of >-3 kcal/mol

A primer's melting temperature was also matched with other primers' that would be run in the same PCR so that the annealing temperature would allow all primers to anneal to the DNA.

Overhangs that were homologous to either the plasmid sequence or the neighbouring HA sequence were added to primers used for overlap PCR extension cloning and InFusion cloning (see Overlap PCR extension cloning, InFusion cloning, Table 1) such that the final length of the primer was 60 bp.

gRNA sequences were designed for iPCR (see 3.5.2. gRNA swapping using inverse PCR) primers with the web-based tool CRISPR RGEN Tools (BAE Lab et al., 2015). The PAM-type of SpCas9 from *Streptococcus pyogenes* (Cas9n protein used in this study) and the target genome of *C. autoethanogenum* DSM10061 (strain LAbrini, see Table 3) were selected. These parameters were screened against ~100 bp from the start of the knock-out target gene. gRNAs were deemed appropriate if the mismatch number was 0 and the out-of-frame score was above 66 (recommended number by CRISPR RGEN Tools). The obtained Cas9n gRNA sequences were screened against the *C. autoethanogenum* genome

with the NCBI Standard Nucleotide BLAST tool (National Library of Medicine, n.d.) to make sure the gRNA leads to unique cuts in the *C. autoethanogenum* genome.

4.5.2. gRNA swapping using inverse PCR

Firstly, the pGFT012 was amplified with gRNA primers (fdhA_gRNA2, fdhA_gRNA3) with the mutated sequence in the middle being incorporated into the amplified plasmid during the PCR reaction. The reverse primer used was the universal pNC_gRNA-R primer which does not contain any mutated sequences. The iPCR product was run on 1% Atlas agarose gel, gel images were evaluated and the product was extracted from the gel using the FavorPrep kit in order to yield a product exempt of incomplete PCR fragments.

Secondly, a P/D/L (Polynucleotide kinase, DpnI, T4 ligase) reaction (Table 4) was performed with the amplified and extracted product to circularise the iPCR product and digest the template plasmid without the desired gRNA mutation.

Table 4. Volumes of reagents used in the Polynucleotide kinase, DpnI, T4 DNA ligase (P/D/L) reaction after performing the inverse PCR.

Volume	Reagent	→	Volume	Reagent
8 µl	iPCR gel-extracted product	Incubate at 37°C for 30 min	2.5 µl	PEG 400
2 µl	T4 DNA Ligase buffer		1 µl	T4 DNA ligase
1 µl	Polynucleotide kinase			
1 µl	DpnI			
8 µl	mQ deionised water			

The P/D/L reaction was transformed (See 1. *E. coli* transformation procedure) into *E. coli* Turbo cells and the obtained colonies were inoculated into LB media supplemented with Amp and incubated at 37°C for 6 hours while shaking at 210 rpm. Plasmids were extracted using the FavorGen plasmid extraction kit. Extracted plasmids were run on 1% Atlas agarose gel for extraction confirmation, and the desired gRNA insertion was confirmed by Sanger sequencing. This method yielded 2 plasmids: pGFT017 and pGFT031 (see Table 2) – plasmids that have a gRNA sequence for the Cas9n protein and that are ready for HA insertion.

4.5.3. Insertion/replacement of the HAs

HAs needed for successful genomic editing of *C. autoethanogenum* were amplified from the genomic DNA of the LAbrini strain (Table 3) using the 1 kb HA primers (fdhA_KO_ha1000dn-R, fdhA_KO_ha1000dn-F etc.) using the PCR program seen in Table 5. The PCR product was run on 1% Atlas agarose gel and extracted using the FavorGen Gel extraction mini-kit. Since the used primers contain overlapping homologous regions, after amplification with these primers, the HA PCR fragments also contain the same overlapping regions. This can be used to fuse the HAs together: another PCR with the same programme was run with the gel extracted HAs and no primers were needed due to the aforementioned overlapping regions.

Table 5. PCR program for amplification and fusion of HAs. * - the annealing temperature was adjusted based on the melting temperatures of primers used in the reactions. The annealing temperature was set approximately 2°C higher than the melting temperature of the primers.

Initial denaturation	98°C	1 min	
Denaturation	95°C	45 sec	|
Annealing*	60°C	30 sec	|
Extension	72°C	40 sec	| 30 cycles
Final extension	72°C	7 min	
Hold	15°C	∞	

Restriction cloning

The gRNA swapped backbone plasmid was linearized and digested at either ends of the old HA sequence with the restriction enzymes XhoI and NotI. The HA's ends were digested with the same restriction enzyme (XhoI and NotI) to make them "sticky". T4 DNA ligase was mixed together with the T4 DNA ligase buffer and both the backbone and insert sequences in a ratio of 3:7 respectively and then incubated at room temperature for 4 hours. The product was then transformed into *E. coli* Turbo competent cells, the transformation mixture was plated onto LB agar plates supplemented with Amp and resulting colonies were screened.

Overlap PCR extension cloning

The HA sequences were amplified as described earlier (see 4.5.3. Insertion/replacement of the HAs) with appropriate primers. A second PCR reaction was set up with both the fused HA product and the plasmid template, the exact reaction parameters can be seen in Table 6. The PCR product was run on 1% Atlas agarose gel to confirm correct amplification. 1 µl of DpnI was added directly to the PCR product mixture and incubated at 37°C for 1 h. A portion of the mixture was then directly transformed into *E. coli* Turbo cells, the transformation mixture was plated onto LB agar plates supplemented with Amp and resulting colonies were screened.

Table 6. PCR program for overlap PCR cloning method. * - the annealing temperature was adjusted based on the melting temperatures of primers used in the reactions. The annealing temperature was set approximately 2°C higher than the melting temperature of the primers. The extension time was set to 20 sec per kb of extension (e.g. if there is a 4 kb desired product, the extension time would be 1 min 20 sec).

Initial denaturation	98°C	3 min	
Denaturation	98°C	10 sec	|
Annealing*	60°C	30 sec	|
Extension	72°C	20 sec/kb	| 18 cycles
Final extension	72°C	5 min	
Hold	15°C	∞	

InFusion cloning

The InFusion reaction was carried out using the InFusion HD Cloning kit. The recommended amount of InFusion kit reaction mixture was mixed with the maximum total volume of linearised vector and insert. The latter were mixed in a 3:5 ratio, respectively. The total 10 µl mixture was incubated at 50°C for 15 min. A portion of the reaction mixture was transformed into commercial NEB Turbo competent *E. coli* cells, the transformation mixture was plated onto LB agar plates supplemented with Amp and resulting colonies were screened. This method yielded a new plasmid: pGFT097 (Table 2).

4.5.4. *C. autoethanogenum* electroporation and gene knock-out confirmation

NEBExpress *E. coli* transformation

Before moving on to transforming the constructed plasmids into *C. autoethanogenum*, the plasmids were transformed into NEBExpress competent *E. coli* cells to remove the Dcm methyltransferase methylation pattern in order to reduce the targeting of the plasmids by the *C. autoethanogenum* restriction-methylation system (Woods et al., 2019). The transformation mixture was plated onto LB agar plates supplemented with Amp and resulting colonies were screened for the best plasmid concentrations (at least 80 ng/µl) with NanoDrop for optimal transformation of *C. autoethanogenum*.

Prepration of *C. autoethanogenum* electrocompetent cells

For *C. autoethanogenum* electrocompetent cells' preparation, a LAbrini strain stock was revived in 50 mL anoxic YTF media and subcultured two times into the same conditions. The final culture was inoculated into 200 mL YTF media supplemented with 40 mM DL-threonine. This culture was grown to the optical density (OD) at 600 nm of 0.2-0.3 and then cooled on ice-cold metal beads. The entire threonine culture was spun down and the pellet was washed twice with ice-cold SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, 7 mM Na_3PO_4, pH 6.0). The final cell pellet was resuspended in a mixture of 200 µl of SMP buffer and 10% of DMSO, and then distributed as 25 µl aliquots into Eppendorf tubes. The electrocompetent cells were stored at -80°C until electroporation.

C. autoethanogenum electroporation

25 µl of electrocompetent cells were mixed with 2.5 µg of the knock-out plasmid DNA and then transferred to a chilled electroporation cuvette. The voltage was set to 1.25 kV, capacitance to 25 µF. The process was always repeated in two replicates, one with the resistance of 300Ω and the other with 600Ω. 1 ml of YTF media was added to the electroporation mixture right after the pulse to prevent any sudden pH changes on the electrodes from shocking the cells (Potter & Heller, 2003). The resulting mixture was inoculated into YTF and incubated at 37°C for 24 hours as a recovery. After the incubation 2.5 and 5 ml of the recovery culture was mixed with anoxic YTF 1.5% Formedium agar with 4 µg/ml of clarithromycin (Cla) cooled to 43-45°C and poured onto petri dishes. The plates were incubated at 37°C for up to a week until colonies were visible in the agar.

Knock-out confirmation

The obtained *C. autoethanogenum* colonies were inoculated into YTF liquid cultures supplemented with 6 µg/ml Cla and incubated at 37°C while shaking at 120 rpm for 18-24 h. The knock-out confirmation was done by extracting the DNA of *C. autoethanogenum* from liquid cultures with OD between 0.01-1 using the Link mini-extraction kit. The extracted DNA was then used in three simultaneous PCRs (Table 7) to confirm successful electroporation:

1. PCR to confirm the identity of the cells with *C. autoethanogenum* specific primers AcsB-F and -R.
2. PCR to confirm the presence of the electroporated plasmid inside the cells with pGFT specific primers OriT-traJ-F and -R.
3. PCR to confirm the genomic knock-out of the target gene in the cells with the target specific primers (fdhA_up-F, Table 1)

Table 7. The setup of *C. autoethanogenum*, plasmid and knock-out confirmation PCRs. * - the annealing temperature was adjusted based on the melting temperatures of primers used in the reactions. The annealing temperature was set approximately 2°C higher than the melting temperature of the primers. The extension time was set to 30 sec per kb of extension (e.g. if there is a 4 kb desired product, the extension time would be 2 min).

Initial denaturation	98°C	1 min	
Denaturation	95°C	45 sec	|
Annealing*	59°C	30 sec	| 30 cycles
Extension	72°C	30 sec/kb	|
Final extension	72°C	7 min	
Hold	15°C	∞	

The colony cultures that were confirmed by PCR to be *C. autoethanogenum* and possess the knock-out plasmid were subcultured twice in the same medium before plating on YTF agar containing 4 µg/ml Cla.

5. Results and discussion

5.1. Insertion of Cas9n gRNA into gene knock-out plasmid

In order to delete the *fdhA* gene from the *C. autoethanogenum*'s genome, the Cas9n protein would need to be guided to the respective locus in the genome by gRNA. Four CRISPR/Cas9n gRNA primers were designed and the two with the best parameters (mismatch number and out-of-frame score, see 4.5.1. Primer design) were ordered. These were used to replace the gRNA sequence in the parent pGFT012 plasmid with a gRNA that would guide the Cas9n protein to the *fdhA* gene in *C. autoethanogenum*'s genome.

Two plasmids with an inserted gRNA sequence for the Cas9n protein (pGFT017 and pGFT031, see 4.1. Primers and plasmids) were obtained by the iPCR methodology (see 4.5.2. gRNA swapping using inverse PCR). The extracted plasmids were analysed on a gel (Figure 7A), and confirmed by restriction enzyme digest using EcoRI and XhoI (Figures 7B and 8) to ensure that no significant mutations had taken place during the cloning. As seen on Figure 7, the replicates pGFT031_C and pGFT031_D matched the expected results the best with both a uniform run of the circular plasmid and the presence of both 7.9 kb and 1.5 kb bands from the restriction reaction. All four plasmid copies were analysed by Sanger sequencing and the successful insertion of the gRNA was confirmed in the case of pGFT017_D and pGFT031_A.

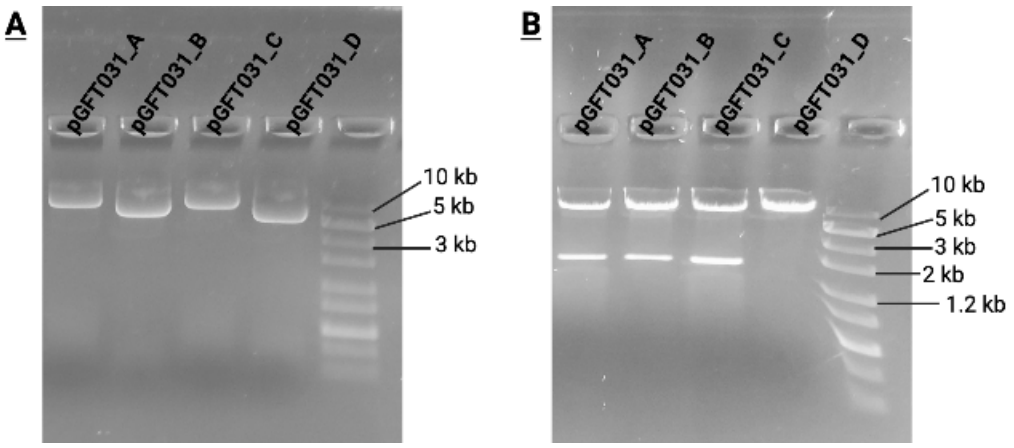

Figure 7. Analysis of the fdhA CRISPR/Cas9n gRNA plasmid pGFT031 replicates on 1% agarose gel. (A) Circular pGFT031_A-D plasmids obtained by iPCR. (B) Double-digest restriction analysis of pGFT031_A - D with EcoRI and XhoI with expected band sizes of 7.9 kb and 1.5 kb. 1 kb – a thousand base pairs. This figure was created with BioRender.

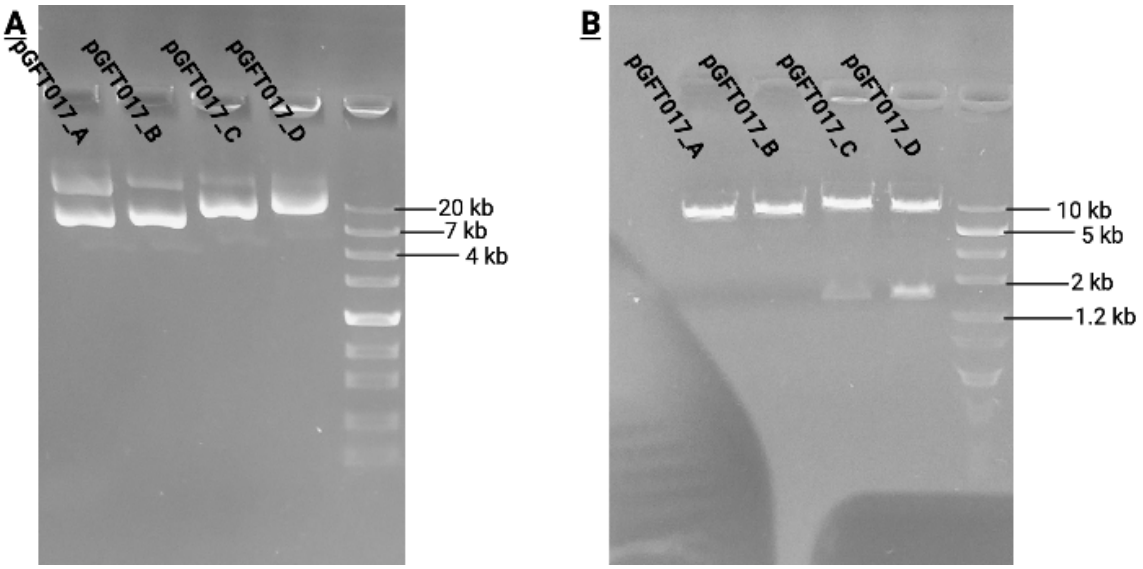

Figure 8. Analysis of the *fdhA* CRISPR/Cas9n gRNA plasmid pGFT017 replicates on 1% agarose gel. (A) Circular pGFT017_A - D plasmid obtained by iPCR. (B) Double-digest restriction analysis of pGFT017_A - D with EcoRI and XhoI with expected band sizes of 7.9 kb and 1.5 kb. 1 kb – a thousand base pairs. This figure was created with BioRender.

5.2. HAs insertion to gene knock-out plasmid

HAs had to be inserted into the gene knock-out plasmid to provide a DNA repair template following the Cas9n-induced nick of the *fdhA* gene. The length for the HAs was chosen to be 1 kb for each (2 kb in total) for more effective repair of the genome via homologous recombination (McAllister & Sorg, 2019).

It was difficult to insert the 1 kb HAs necessary for successful knock-out strain construction into the already 11 kb long backbone plasmid probably because of the low transformation efficiency of such a large plasmid (Hanahan, 1983). Thus, several methods such as overlap PCR extension cloning, Gibson assembly or restriction cloning were tested and failed. In the end, the InFusion method was the method that ended up yielding the correctly constructed plasmid pGFT097 (Table 2, Figure 9).

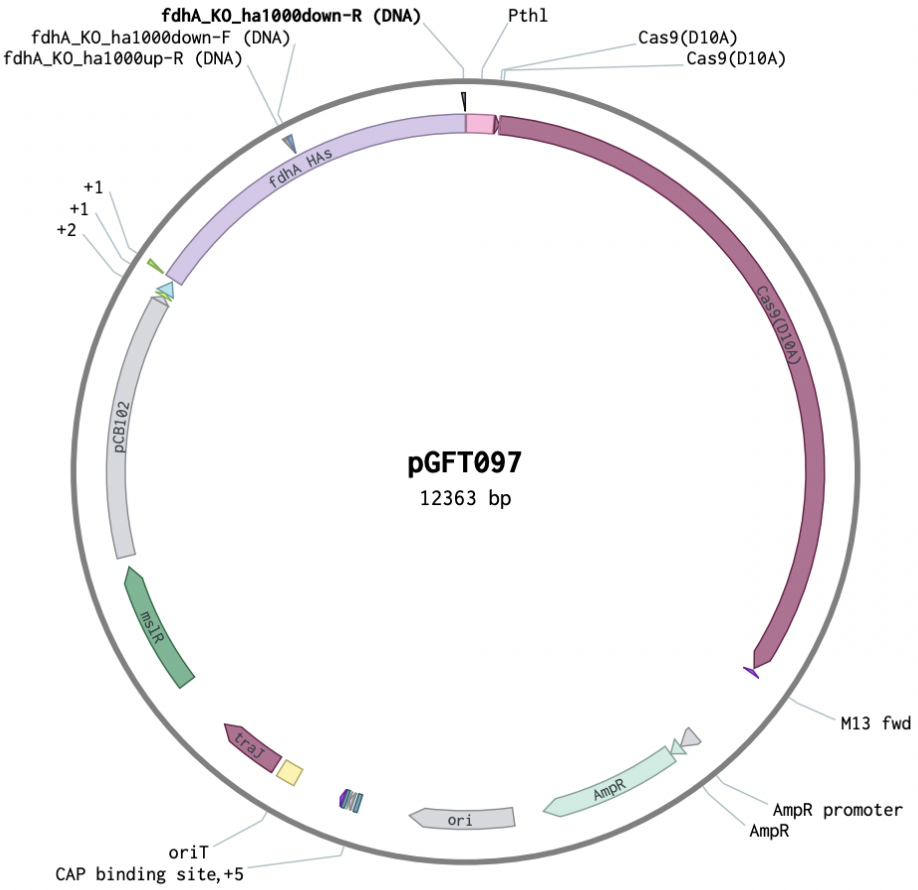

Figure 9. The schematic representation of pGFT097 plasmid with a swapped gRNA and *fdhA* HAs. AmpR cluster - ampicillin resistance, mslR - macrolide lincosamide streptogramin B class antibiotic resistance (Tenson et al., 2003), *fdhA* HAs - homology armsflanking the *fdhA* gene of *C. autoethanogenum*, Cas9(D10A) cluster - Cas9n sequence, ori - origin of replication for gram-negative bacteria, gRNA scaffold - gRNA sequence for Cas9n, Pthl - strong promoter of the thiolase gene of *C. acetobutylicum*, J23119 promoter - synthetic promoter for the expression of gRNA, pCB102 - pCB102 origin of replication for gram-positive bacteria from *C. butyricum* (Zhang et al., 2015; Li et al., 2016).

For HA insertion confirmation, a PCR with the primers NickClos-1-F and NickClos-2-R was performed and run on 1% agarose gel (Figure 10). As can be seen on Figure 10, several replicates match the positive control shown by the expected 3 kb band size. Even though most of the replicates' concentration were too low for sequencing, three samples

(pGFT097_11, pGFT097_15 and pGFT097_20) with sufficient concentrations (276.8, 80.5 and 80 ng/µL, respectively) were sent to sequencing and the correct HA insertion was confirmed with the replicate pGFT097_20 (from now on pGFT097, Figure 9).

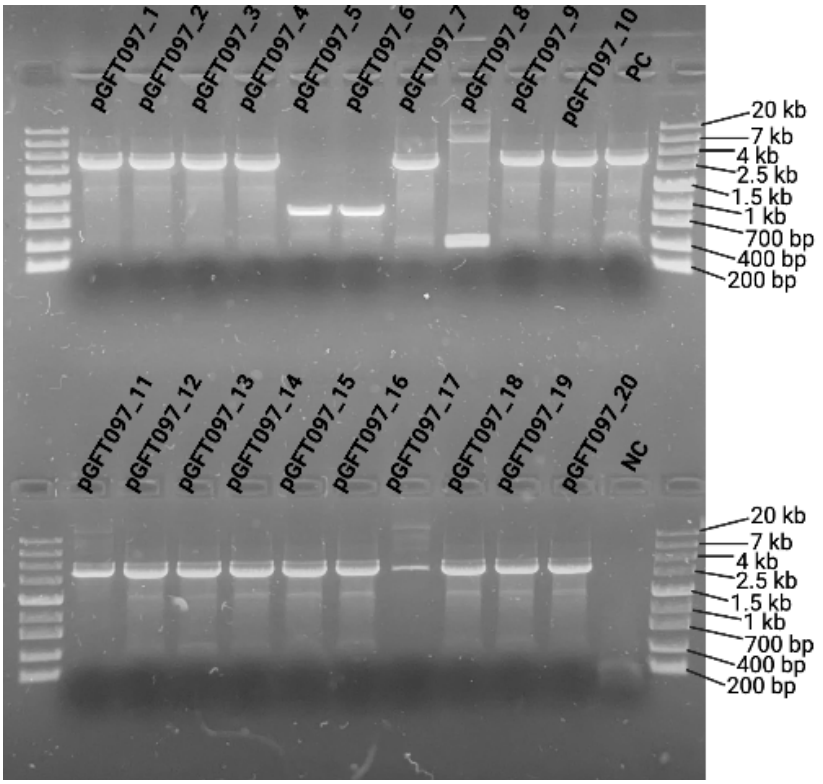

Figure 10. Analysis of the HA insertion plasmid confirmation PCR replicates with the primers NickClos-1-F and NickClos-2-R on 1% agarose gel. 20 colonies – pGFT097_1 - 20 – were tested. PC - positive control and example of the expected band size of 3 kb; NC - negative control; 1 kb – a thousand base pairs. This figure was created with BioRender.

Ultimately, the obtained pGFT097 plasmid contained the basic required components - antibiotic resistance genes for selection of both *E. coli* and *C. autoethanogenum* strains and origins of replication for both gram-negative and gram-positive bacteria. To mediate genetic modification, a Cas9n gene sequence that could be translated into a Cas9n protein that is capable of introducing specific single-strand breaks in the native genome of *C. autoethanogenum* (Gasiunas et al., 2012) was present. Furthermore, so that the Cas9n mediated genetic modification could be successful in *C. autoethanogenum*, a gRNA sequence to direct the Cas9n to the target knock-out gene's locus in the *C. autoethanogenum* genome and a HA sequence to mediate repair of the genome and formation of the knock-out in process were successfully inserted (Nagaraju et al., 2016).

5.3. NEBExpress *E. coli* transformation

In order to remove the *E. coli* native Dcm methylation pattern which would trigger the restriction-methylation system of *C. autoethanogenum* upon transformation, pGFT097 was transformed into NEBExpress (see 4.2. Strains; Woods et al., 2019). After transforming the obtained pGFT097 (Figure 9) plasmid into the *dcm*$^-$ NEBExpress *E. coli*, many satellite colonies formed, and restriction enzyme digest with previously used EcoRI and XhoI showed an unexpected digestion pattern when run on 1% agarose gel. Furthermore, colonies and liquid cultures needed more incubation time to grow than usual (~24 vs 48 h). NCBI Standard Nucleotide BLAST tool suggested potential off-target sites for the fdhA_gRNA3 in the *E. coli* genome that had previously gone unnoticed which could explain the slower growth of the transformed cells. Eventually two plasmids were found that gave a positive result with the HA confirmation PCR with primers NickClos-1-F and NickClos-2-R and also looked as expected when the full circular plasmid was run on gel (Figure 11). Plasmids extracted from NEBExpress were not sequenced to save material for more transformation efforts.

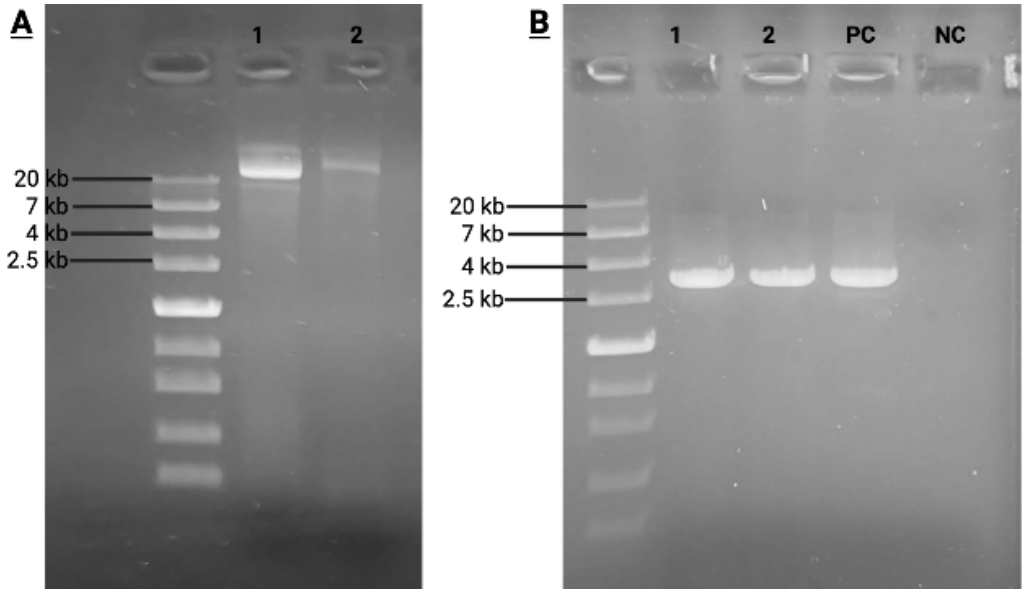

Figure 11. Analysis of the pGFT097 plasmids extracted from NEBExpress *E. coli* strain. (A) Two circular pGFT097 plasmids labelled as "1" and "2", respectively. (B) HA confirmation PCR-s of the two pGFT097 plasmids extracted from NEBExpress *E. coli* cells labelled as "1" and "2", respectively, with the primers NickClos-1-F and NickClos-2-R. PC - positive control and example of the expected band size of 3 kb; NC - negative control; 1 kb – a thousand base pairs. This figure was created with BioRender.

5.4. Electroporation and knock-out confirmation

The previously established NEBExpress extracted pGFT097 (Figure 9) was electroporated into electrocompetent *C. autoethanogenum* strain LAbrini used in this work as the base

strain (Ingelman et al., 2023). Transformation of the LAbrini strain with the pGFT097 plasmid yielded several colonies. In total, 3 rounds of colonies were screened (10 colonies in total) and three colonies carrying the gene knock-out plasmid pGFT097 were found (from now on colonies #1, #3 and #4).

5.4.1. Colony #1

PCR analysis of colony #1 (Figure 12) demonstrated the presence of the plasmid, yet, no knock-out band was observed. According to the gel run with knock-out confirmation PCRs (Figure 13), the colony was a *C. autoethanogenum* colony, possessed the gene knock-out plasmid pGFT097 and was a wild-type (WT) – no gene knock-out had happened yet. Even though the colony was a WT, if it possessed the plasmid, knock-out formation was possible. The colony was subsequently subcultured twice into selective YTF to induce the knock-out formation. The growth of both cultures was monitored and DNA was extracted and knock-out confirmation PCRs were performed twice from both the first and second subsequent subculture.

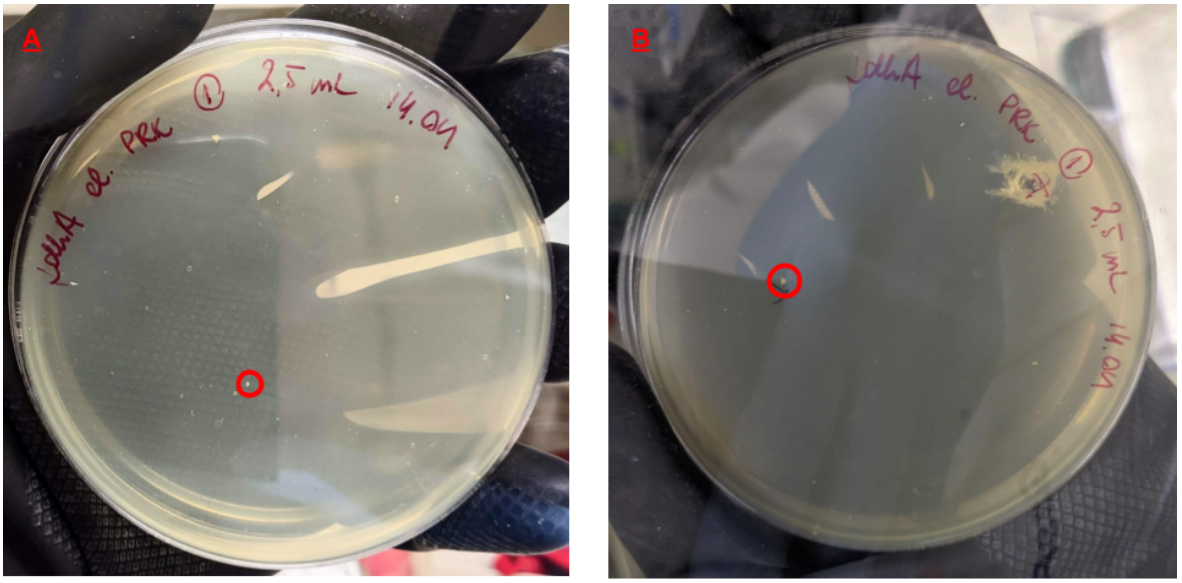

Figure 12. Images of first replicate of the 2.5 ml pGFT097 electroporation culture plate with the red circle indicating the WT *C. autoethanogenum* colony #1 that possesses the gene knock-out plasmid pGFT097. Picture (A) was taken 10 days after the plating and picture (B) was taken 18 days after plating. Picture (B) was taken just before inoculating the colony into YTF liquid media.

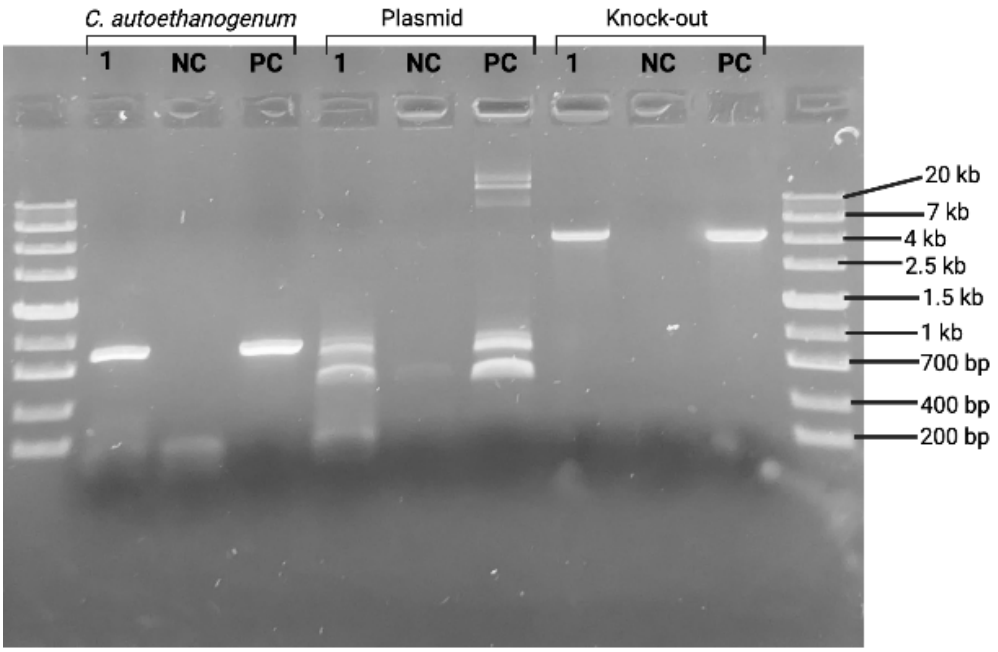

Figure 13. Analysis of knock-out confirmation PCR products of colony #1 run on 1% agarose gel. Lanes labelled as "1" mark the PCR product DNA extracted from the screened colonies. Lanes marked as "PC" and "NC" mark the positive control and negative control for the PCR, respectively. Lanes labelled as "*C. autoethanogenum*" mark the lanes for species confirmation PCR. Lanes labelled as "Plasmid" mark the lanes for plasmid presence PCR. Lanes labelled as "Knock-out" mark the lanes for gene knock-out confirmation PCR. This figure was created with BioRender.

After plasmid confirmation of colony #1, it was subcultured into the selective medium (culture 1.1). Upon ~86 h of incubation (Figure 14A), DNA was extracted and plasmid confirmation PCRs were performed (Figure 15A) which was negative for the plasmid PCR. The plasmid PCR was repeated (Figure 15B) because it was unexpected that a plasmid-free culture could grow in an antibiotic supplemented growth medium. The repeated round of plasmid PCR showed a plasmid band, but a significantly fainter one, compared to the band in Figure 13. Plasmid presence in culture 1.1 was deemed inconclusive and culture 1.1 was further subcultured into the selective medium (culture 1.2) and knock-out confirmation PCR was repeated at ~115 h and ~43 h for culture 1.1 and 1.2, respectively (Figure 16). This PCR once again showed a negative result for the plasmid presence in both subcultures.

Even though the first subculture had inconclusive plasmid PCR results, the second subculture was being monitored for growth. The knock-out confirmation PCR was repeated for culture 1.2 when growth was confirmed at ~160 h of incubation (Figure 14B) at which point it was also subcultured a third time (culture 1.3) to continue experimentation. This PCR (Figure 17) confirmed a plasmid presence in culture 1.2, but as before with culture 1.1, the band was significantly fainter than on Figure 13. Culture 1.3

was confirmed to carry the knock-out plasmid as well (Figure 17) and showed a growth profile similar to the base strain.

The problem of the faintness of the plasmid band from PCRs might have arisen because the PureLink mini-extraction kit used for extracting the DNA for knock-out confirmation PCRs is meant for extracting genomic DNA of cells. Thus the pore size of the columns of the kit might not be that effective in binding smaller DNA molecules than the genomic DNA of cells. Thus a very small amount of plasmid DNA might have been extracted from the *C. autoethanogenum* transformants, making the PCR less effective as well.

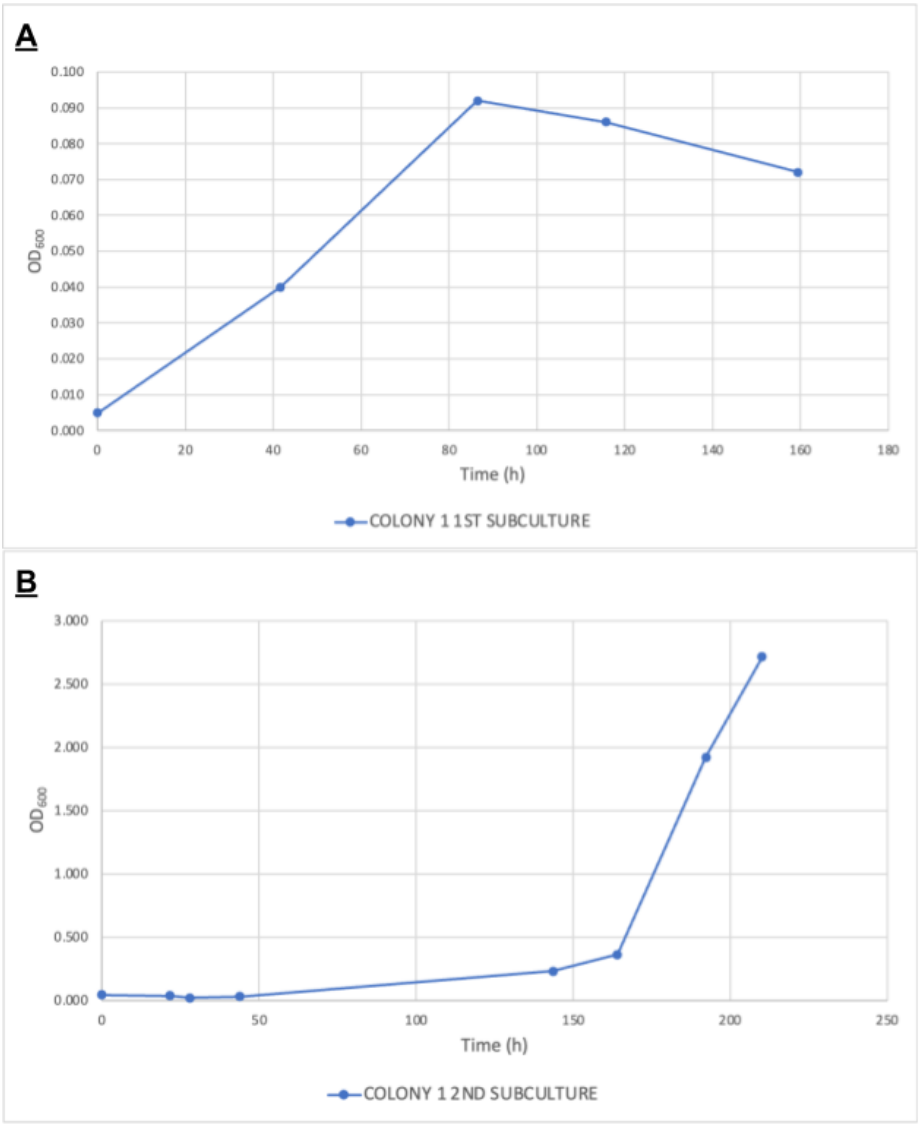

Figure 14. Growth curves of two subcultures of the colony #1 carrying the pGFT097 plasmid. X-axis – Time in hours. Y-axis – OD measurement of the culture at 600 nm. (A) Growth curve of the first subculture of the colony. (B) Growth curve of the second subculture of the colony.

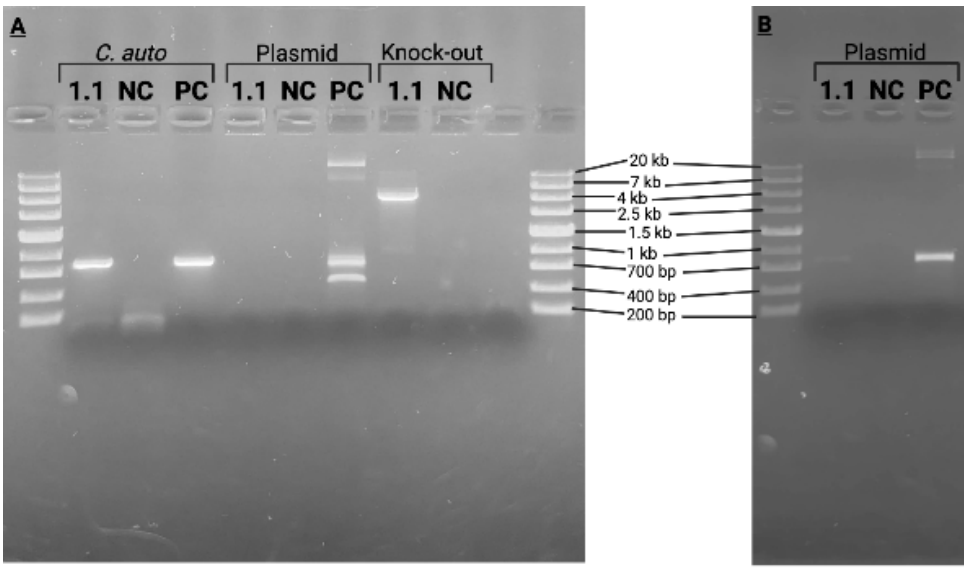

Figure 15. Analysis of knock-out confirmation PCR products of the first subculture of colony #1 at ~86 h of incubation run on 1% agarose gel. (A) First PCR of the subculture with DNA extracted at ~86 h of incubation. (B) Second PCR of the subculture with DNA extracted at ~86 h of incubation. Lanes labelled as "1" mark the PCR product DNA extracted from the subculture. Lanes marked as "PC" and "NC" mark the positive control and negative control for the PCR, respectively. Lanes labelled as "*C. autoethanogenum*" mark the lanes for species confirmation PCR. Lanes labelled as "Plasmid" mark the lanes for plasmid presence PCR. Lanes labelled as "Knock-out" mark the lanes for gene knock-out confirmation PCR. This figure was created with BioRender.

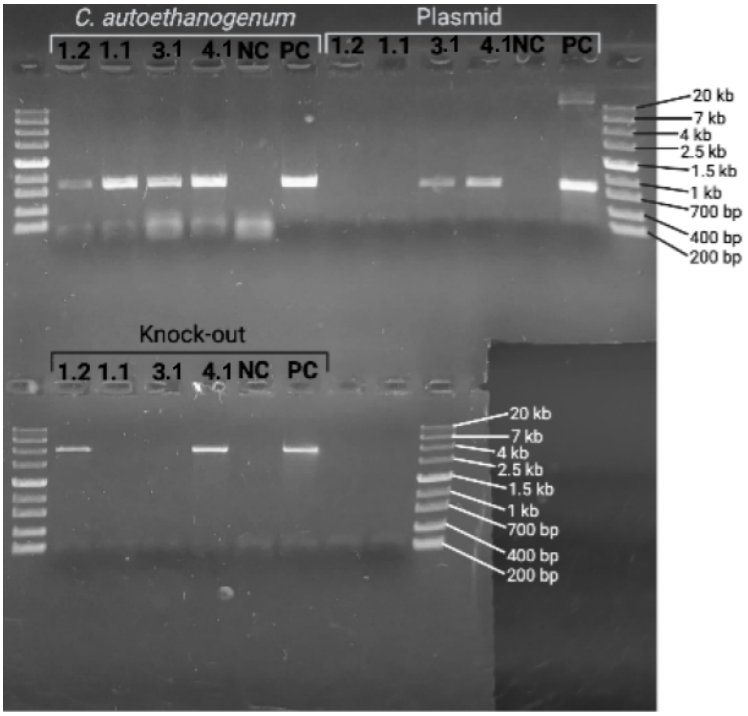

Figure 16. Analysis of knock-out confirmation PCR products from subcultures run on agarose gel. Lanes labelled as "1.1" mark the PCR product of DNA extracted from the first subculture of colony #1 carrying pGFT097. Lanes labelled as "1.2" mark the PCR product of DNA extracted from the second subculture of colony #1 carrying pGFT097. Lanes labelled as "3.1" and "4.1" mark the PCR product of DNA extracted from the first subculture of colonies #3 and #4 carrying pGFT097. Lanes marked as "PC" and "NC" mark the positive control and negative control for the PCR, respectively. Lanes labelled as "*C. autoethanogenum*" mark the lanes for species confirmation PCR. Lanes labelled as "Plasmid" mark the lanes for plasmid presence PCR. Lanes labelled as "Knock-out" mark the lanes for gene knock-out confirmation PCR. This figure was created with BioRender.

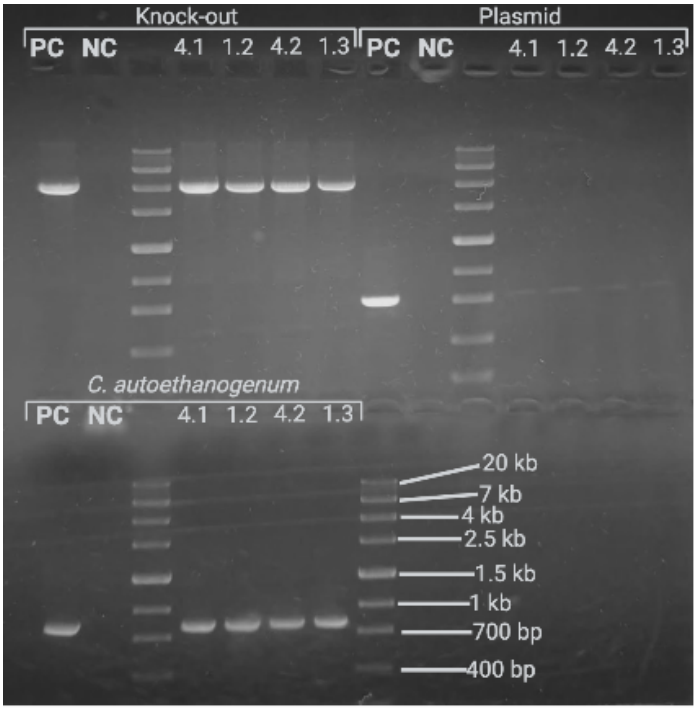

Figure 17. Analysis of knock-out confirmation PCR products of subcultures of colony #1 and #4 run on 1% agarose gel. Lanes labelled as "1.2" and "1.3" mark the PCR product DNA extracted from the second and third subculture of colony #1. Lanes labelled as "4.1" and "4.2" mark the PCR product DNA extracted from the first and second subculture of colony #4. Lanes marked as "PC" and "NC" mark the positive control and negative control for the PCR, respectively. Lanes labelled as "*C. autoethanogenum*" mark the lanes for species confirmation PCR. Lanes labelled as "Plasmid" mark the lanes for plasmid presence PCR. Lanes labelled as "Knock-out" mark the lanes for gene knock-out confirmation PCR. This figure was created with BioRender.

5.4.2. Colonies #3 and #4

From the third round of colony screening (Figure 18), two additional colonies (colonies #3 and #4) with plasmids were found – as can be seen from Figure 18, PCR analysis confirmed that colonies #3 and #4 were *C. autoethanogenum*, carried the gene knock-out plasmid pGFT097 but were still WT colonies in terms of *fdhA*. Similar to colony #1, these colonies were subcultured into selective YTF (culture 3.1 and 4.1) to hopefully facilitate the formation of a knock-out strain. At 48 h of incubation (Figure 19) DNA was extracted from the subcultures and knock-out confirmation PCR was performed (3.1 and 4.1 in Figure 16) and plasmid presence for both cultures was confirmed.

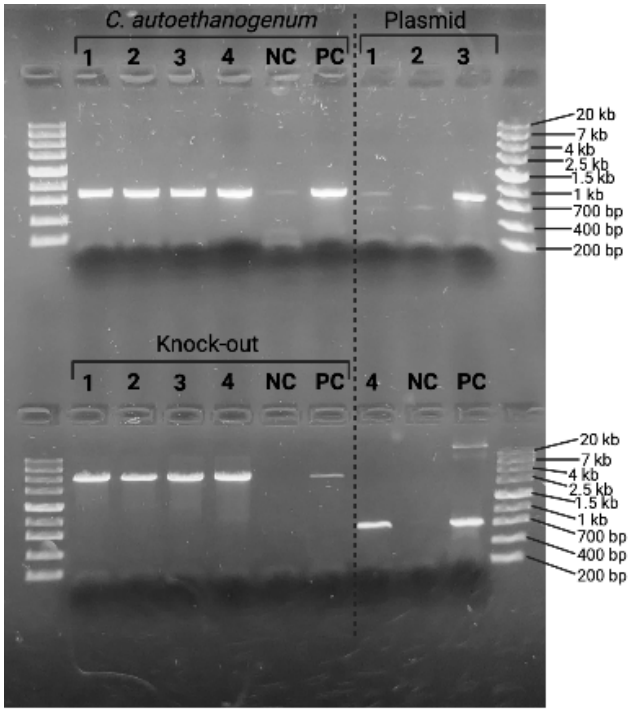

Figure 18. Analysis of knock-out confirmation PCR products from the third round of colony screening run on 1% Atlas agarose gel. Lanes labelled as "1"..."4" mark the PCR product DNA extracted from the screened colonies. Lanes marked as "PC" and "NC" mark the positive control and negative control for the PCR, respectively. Lanes labelled as "*C. autoethanogenum*" mark the lanes for species confirmation PCR. Lanes labelled as "Plasmid" mark the lanes for plasmid presence PCR. Lanes labelled as "Knock-out" mark the lanes for gene knock-out confirmation PCR. This figure was created with BioRender.

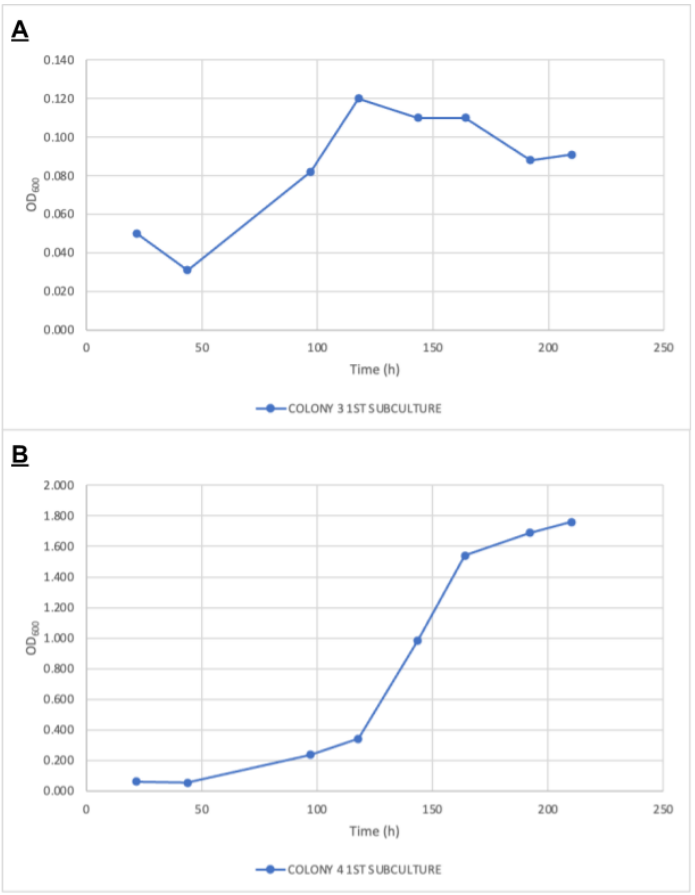

Figure 19. Growth curves of the first subcultures of colonies #3 and #4. X-axis – Time in hours. Y-axis – OD measurement of the culture at 600 nm. (A) Growth curve of the first subculture of colony #3. (B) Growth curve of the first subculture of colony #4.

Culture 4.1 was subcultured (from now on culture 4.2) into selective medium the second time at ~145 h of incubation (Figure 19B). DNA was extracted from cultures 4.1 and 4.2 after ~164 h and ~24 h of incubation, respectively, and knock-out control PCR still showed a WT genotype (Figure 17). The presence of the plasmid was confirmed with both colonies even though similarly to cultures of colony #1, the plasmid band was significantly fainter than in initial colony screenings.

5.4.3. Future plans

Since cultivation of the *fdhA* knock-out plasmid carrying cells in liquid medium did not yield KO events at this time, future work with cultures 1.3 and 4.2 will involve plating them on selective agar medium and the screening of resulting colonies for *fdhA* deletion. Following that, the knock-out strain will be cured of the plasmid by growing on nonselective media and monitoring plasmid loss by testing the growth on selective media and PCR. When a clean knock-out strain has been isolated, it will be grown on synthesis gas and its growth profile and metabolic products will be characterised.

Even though not all of the aims of the thesis were achieved, this work is valuable because an intermediary strain for investigating the *fdhA* gene was successfully engineered. Additionally, the workflow was tested for future optimisation of genetic engineering projects of *C. autoethanogenum*. Furthermore, according to literature, the *fdhA* gene knock-out could be lethal for *C. autoethanogenum* under autotrophic conditions (Woods et al., 2022). Upon confirmation of these results in our strain, a knock-down system available in our lab could be applied for investigating the effects of modifying expression levels of the *fdhA* gene.

6. Conclusion

The industrial and scientific potential of *C. autoethanogenum* is very significant. However, its key metabolic pathway needed for autotrophic growth – the WLP – and particularly the enzymes involved in the WLP are not yet understood comprehensively. One of the key enzymes of the pathway is the FdhA that forms a complex with the Hyt protein and is involved at the beginning of the Methyl branch of the WLP. Even though this enzyme has been characterised before in *C. ljungdahlii*, the full functionalities of this gene in *C. autoethanogenum* have remained unclear.

This work focused on the gene knock-out CRISPR/Cas9n plasmid construction for the deletion of the *fdhA* gene from *C. autoethanogenum*'s genome and transformation via electroporation into the bacterium. A knock-out plasmid for the *fdhA* gene was successfully constructed and transformed into *C. autoethanogenum*. Time did not permit, however, to obtain a strain with deletion of *fdhA*. The aims of the work also included the characterisation of the *fdhA* knock-out strain. However, the latter was not accomplished due to low transformation rate of large plasmids into *E. coli*, the unexpected toxicity of the Cas9n gRNA in *E. coli*, the burden of the knock-out plasmid on *C. autoethanogenum* and the resulting significant amount of time and number of repeated experiments to yield a clean knock-out strain at any of these points of the experiment. Even though there was not enough time to facilitate the formation of a clean knock-out strain and characterising it, the work done will serve as a convenient starting point for future experiments to further investigate the *fdhA* gene.

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