Technische Universität München – TUM School of Life Sciences

Metabolic engineering of the cellulolytic bacterium *Clostridium cellulovorans* 743B

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Summary

The chemical industry mainly uses fossil resources to produce solvents. On the other hand, the sustainable biotechnological production of solvents can be achieved through the fermentation of carbohydrates. For instance, *Clostridium acetobutylicum* is able to produce acetone, butanol, and ethanol as fermentation products in a process called ABE fermentation. In this context, lignocellulosic biomass, the most abundant feedstock on earth, could be used as a resource for the biotechnological production of valuable solvents, such as butanol. However, lignocellulosic biomass is naturally recalcitrant to microbial degradation. Fortunately, some microorganisms can efficiently break down lignocellulose into fermentable sugars. *C. cellulovorans* is one of the most promising candidates for producing lignocellulosic solvents due to its high cellulolytic capacity. Although *C. cellulovorans* itself cannot naturally produce solvents, it could be used in a synthetic co-culture with a solventogenic clostridia for the continuous production of solvents from lignocellulosic biomass. In such a co-culture, *C. cellulovorans* will decompose polysaccharides and thereby feed the solventogenic clostridia with fermentable sugars, which in turn will produce the desired solvents. However, the establishment of such co-culture is challenging. If both organisms are not metabolically dependent on each other for growth, one organism can overgrow the other, making the utilization of the feedstock and the production of solvents inefficient.

The present study aimed to generate a *C. cellulovorans* strain metabolically dependent on a solventogenic clostridia. The selected strategy was to abolish acetate production and generate a strain that depends on the acetate produced by the solventogenic clostridia to grow. With the deletion of the *pta-ack* operon, the strain should no longer produce acetate because it then lacks the enzymes acetyl-CoA:phosphate acetyltransferase (phosphotransacetylase, Pta) and acetate kinase (Ack), which convert acetyl-CoA into acetate. Additionally, in the co-culture the engineered *C. cellulovorans* strain should provide butyrate, an intermediate in butanol production, to the solventogenic strain. The enzymes butanoyl-CoA:phosphate acetyltransferase (phosphotransbutyrylase, Ptb) and butyrate kinase (Buk) convert butyryl-CoA into butyrate. However, these enzymes can function reversibly and produce butyryl-CoA from butyrate, an undesired feature in the intended co-culture. Therefore, it was intended to replace the *ptb-buk* operon with the *cat1* gene from *C. tyrobutyricum*, encoding a CoA-transferase. This CoA-transferase can convert butyryl-CoA to butyrate linked with the conversion of acetate to acetyl-CoA. This should avoid the formation of butyryl-CoA from butyrate.

Prior to this study there was a lack of reliable genetic tools for *C. cellulovorans*. To facilitate metabolic engineering in this bacterium, a new gene editing system based on the Allele-Coupled Exchange (ACE) technology was established. The ACE technology is based on the replacement of a DNA region through homologous recombination (HR). ACE is a two-step process that functions as follows e. g. for deletion of a gene: during the first HR event, a plasmid containing flanking regions to the gene of interest fused together is integrated into the bacterial chromosome, while in the second HR event the plasmid is excised again, and either the mutant or the wild-type genotype is generated. For the success of this approach, the precise selection of two genetic

elements in the deletion vector—the origin of replication (ori) and the counter-selection marker—is essential. The selection of a replication-defective ori (resulting in a “pseudo suicide” replicon) was extremely important to achieve plasmid integration. In the present study, five different oris (pBP1, pCB102, pUB110, p19, and pAMβ1) were found to be compatible with *C. cellulovorans*. Later, the stability and copy number of plasmids containing the different oris was investigated. The plasmid containing the pUB110 ori (pMTL87151) showed the least stability (which indicates that it is a replication-defective ori) and had the lowest copy number (0.73 ± 0.31 copies per chromosome) in *C. cellulovorans*. On the other hand, the plasmid containing the pCB102 ori (pMTL83151) was found to be the most stable and with the highest copy number (15.41 ± 3.83 copies per chromosome). For the examined heterologous oris, there apparently was a correlation between stability and copy number. Thus, the evaluation of plasmid copy number could be used as a less laborious way to estimate the stability of different plasmids. For counterselection of clones that lost the plasmid vector after chromosomal integration, the *codBA* counterselection technique, using the *codBA* cassette from *C. ljungdahlii* under the control of the P_{clpB} promoter from *Staphylococcus aureus*, in combination with 5-fluorocytosine (5-FC) as the counterselective compound, was established for use in *C. cellulovorans* in this study. CodB represents a transporter for 5-FC while CodA is a cytosine deaminase converting nontoxic 5-FC into toxic 5-fluorouracil. Therefore, in the presence of 5-FC, only cells that lost the *codBA* cassette (those are either wild type cells or the desired mutants) can grow.

As initial deletion targets for chromosomal engineering in *C. cellulovorans*, the restriction/modification (RM) systems were selected in order to remove one important barrier that severely impairs the transfer of the deletion plasmids used for genetic engineering. *In silico* analysis using the bioinformatic tool REBASE revealed the presence of 12 putative RM loci on the *C. cellulovorans* chromosome. However, only four loci encoded both a restriction endonuclease (REase) and a methylase (MTase) in the same operon, which is considered a prerequisite for a functional RM system according to the literature. Thus, deletion plasmids targeting the putative restriction endonuclease ORFs Clocel_1114 (type I), Clocel_4005 (type II), Clocel_4006 (type II), and Clocel_2651 (type III) were constructed. By application of the newly developed gene editing system, it was possible to knock out the ORFs Clocel_1114 and Clocel_2651. With the resulting strains *C. cellulovorans ΔClocel_1114* and *C. cellulovorans ΔClocel_2651* as recipients for plasmid transfer from *Escherichia coli*, conjugation efficiency was improved by two orders of magnitude. The restriction deficient strain *C. cellulovorans ΔClocel_1114* was selected as the base of further metabolic engineering.

At the same time as this study was being conducted, another gene editing system based on the CRISPR-Cas Nickase was established in our working group. The applicability of this system was also investigated in the present study. Using this system, the strain *C. cellulovorans ΔClocel_1114 Δptb_buk::cat1* was generated. Unfortunately, it was not possible to construct a deletion vector targeting both *pta* and *ack* genes simultaneously, only targeting the *pta* gene. For the construction of strain *C. cellulovorans ΔClocel_1114 Δpta*, both the *codBA*-based and the CRISPR-Cas

Nickase were successful. However, plasmid curing was surprisingly easier using the CRISPR-Cas Nickase system. For the first time, markerless knock-out and knock-in in genes of the central metabolism of *C. cellulovorans* were accomplished.

Additionally, the present study provides new insights into *C. cellulovorans* bacteriology. To investigate its growth on insoluble substrates, it is essential to have a means to quantify its growth other than measuring cell density. Here, a reliable protocol for protein quantification using the Bradford assay was optimized for that purpose. This allowed the precise monitoring of growth of the organism on crystalline and amorphous cellulose. The changes of pH during growth were also monitored. The short-chain fatty acids butyrate and acetate are the main fermentation products in *C. cellulovorans*. The accumulation of acids in the medium can interfere with the organism's fitness. Interestingly, when *C. cellulovorans* was grown on cellobiose, a pH lower than the tolerated limit was observed, while during growth on crystalline or amorphous cellulose, the pH of the culture was never below the minimum pH tolerated by *C. cellulovorans*. The fermentation products were similar under all tested conditions.

C. cellulovorans was described as an endospore-forming bacterium. However, since the description of the species no further studies have investigated the conditions for sporulation and germination. *C. cellulovorans* cells from overnight cultures usually have two distinct cell morphologies under the phase contrast microscope: dark and thin or bright and swollen rods. The bright and swollen rods were described as the sporulating form of *C. cellulovorans*. However, no growth was observed after pasteurization of *C. cellulovorans* cells, despite several attempts to achieve germination. This indicates that *C. cellulovorans* endospores are possibly not heat resistant or that the strain has lost the ability to form endospores. It is also worth mentioning that *C. cellulovorans* growth was frequently unpredictable, making the handling of the organism challenging. Since the induction of prophages may play a role in this regard, *in silico* analysis of the *C. cellulovorans* genome was carried out and revealed the presence of four apparently intact prophage regions. Although it was not possible to obtain phage-free strains using traditional methods (phage induction using mitomycin C or UV light), these prophage regions can be deleted with the genetic tools now available for *C. cellulovorans*.

Zusammenfassung

Die chemische Industrie verwendet hauptsächlich fossile Ressourcen zur Herstellung von Lösungsmitteln. Andererseits kann die nachhaltige, biotechnologische Herstellung von Lösungsmitteln durch die Vergärung von Kohlenhydraten erfolgen. *Clostridium acetobutylicum* ist beispielsweise in der Lage, in einem als ABE-Fermentation bezeichneten Prozess Aceton, Butanol und Ethanol als Fermentationsprodukte herzustellen. In diesem Zusammenhang könnte lignozellulosehaltige Biomasse, der am häufigsten vorkommende Rohstoff auf der Erde, als Substrat für die biotechnologische Herstellung wertvoller Lösungsmittel, wie Butanol, genutzt werden. Lignozellulose ist jedoch von Natur aus widerspenstig gegenüber mikrobiellem Abbau. Glücklicherweise können einige Mikroorganismen Lignozellulose effizient zu vergärbaren Zuckern abbauen. *C. cellulovorans* ist einer der vielversprechendsten Kandidaten für die Herstellung von Lignozellulose-basierten Lösungsmitteln, da dieses mesophile Bakterium hohe zellulolytische Aktivität besitzt. Obwohl *C. cellulovorans* selbst natürlicherweise keine Lösungsmittel produzieren kann, könnte es in einer synthetischen Co-Kultur mit einem solventogenen Clostridium zur kontinuierlichen Produktion von Lösungsmitteln aus lignozellulosehaltiger Biomasse verwendet werden. In einer derartigen Co-Kultur könnte *C. cellulovorans* Polysaccharide abbauen und dadurch die solventogenen Clostridien mit fermentierbaren Zuckern füttern, welche wiederum die gewünschten Lösungsmittel produzieren. Die Etablierung einer solchen Co-Kultur ist jedoch eine Herausforderung. Wenn beide Organismen nicht metabolisch voneinander abhängig sind, kann ein Organismus den anderen überwachsen, was die Nutzung des Ausgangsmaterials und die Produktion von Lösungsmitteln ineffizient macht.

Ziel der vorliegenden Arbeit war es, einen *C. cellulovorans*-Stamm zu erzeugen, der metabolisch von einem solventogenem Clostridium abhängig ist. Die gewählte Strategie bestand darin, die Acetatproduktion zu eliminieren, damit der Stamm für sein Wachstum auf das von dem solventogenem Clostridium gebildete Acetat angewiesen ist. Durch die Deletion des *pta-ack*-Operons sollte der Stamm kein Acetat mehr bilden, da ihm dann die Enzyme Acetyl-CoA:Phosphat Acetyltransferase (Phosphotransacetylase, Pta) und Acetatkinase (Ack) fehlen, welche Acetyl-CoA in Acetat umwandeln. Außerdem sollte der entwickelte Stamm Butyrat, ein Zwischenprodukt der Butanolproduktion, an das solventogene Clostridium liefern. Die Enzyme Butanoyl-CoA:Phosphate Acetyltransferase (Phosphotransbutyrylase, Ptb) und Butyratkinase (Buk) wandeln Butyryl-CoA in Butyrat um. Diese Enzyme können jedoch auch in der Gegenrichtung arbeiten und Butyryl-CoA aus Butyrat herstellen, was in der geplanten Co-Kultur unerwünscht ist. Daher wurde versucht, das *ptb-buk*-Operon durch das *cat1*-Gen aus *C. tyrobutyricum* zu ersetzen, das für eine CoA-Transferase codiert. Diese CoA-Transferase kann Butyryl-CoA zu Butyrat und im Gegenzug Acetat zu Acetyl-CoA umwandeln. So sollte die Umwandlung von Butyrat in Butyryl-CoA vermieden werden.

Zu Beginn dieser Arbeit bestand ein Mangel an zuverlässigen genetischen Werkzeugen für *C. cellulovorans.* Um das Metabolic Engineering dieses Bakteriums zu erleichtern, wurde ein neues Gene-Editing-System auf der Grundlage der *Allele-*

Coupled Exchange (ACE) Technologie entwickelt. Die ACE-Technologie basiert auf dem Austausch einer DNA-Region durch homologe Rekombination (HR). ACE ist ein zweistufiger Prozess, der folgendermaßen z.B. für die Deletion eines Gens funktioniert: In einem ersten HR-Ereignis wird ein Plasmid, das miteinander fusionierte flankierende Regionen des Gens von Interesse enthält, in das bakterielle Chromosom integriert, während im zweiten HR-Ereignis das Plasmid wieder exzisiert und dadurch entweder der mutierte oder der Wildtyp-Genotyp entsteht. Für den Erfolg dieses Ansatzes sind zwei genetische Elemente im Deletionsvektor entscheidend: der Replikationsursprung (Ori) und der Gegenselektionsmarker. Die Auswahl eines replikationsdefekten Ori (für den Erhalt eines „Pseudo-Selbstmord"-Replikons) war für die Integration des Plasmids äußerst wichtig. In der vorliegenden Arbeit wurden fünf verschiedene mit *C. cellulovorans* kompatible Oris (pBP1, pCB102, pUB110, p19 und pAMβ1) gefunden. Später wurden die Stabilität und die Kopienzahl der Plasmide, die die verschiedenen Oris enthalten, untersucht. Das Plasmid mit dem pUB110-Ori (pMTL87151) erwies sich als das Plasmid mit der niedrigsten Kopienzahl (0,73 ± 0,31 Kopien pro Chromosom) in *C. cellulovorans*. Dagegen erwies sich das Plasmid mit dem pCB102-Ori (pMTL83151) als das stabilste und mit der höchsten Kopienzahl (15,41 ± 3,83 Kopien pro Chromosom). Für die untersuchten heterologen Oris besteht anscheinend eine Korrelation zwischen Stabilität und Kopienzahl. Somit konnte die Auswertung der Plasmid-Kopienzahl als weniger aufwändige Methode zur Einschätzung der Stabilität verschiedener Plasmide verwendet werden. Für die Gegenselektion von Klonen, welche nach vorheriger Integration ins Chromosom das Plasmid wieder verloren hatten, wurde zur Anwendung in *C. cellulovorans* in dieser Arbeit die *codBA*-Kassette aus *C. ljungdahlii* unter der Kontrolle des P_{clpB}-Promotors aus *Staphylococcus aureus,* in Kombination mit 5-Fluorcytosin (5-FC) als gegenselektive Verbindung, etabliert. Bei CodB handelt es sich um einen Transporter für die Aufnahme von 5-FC, während CodA eine Cytosin-Desaminase ist, die das ungiftige 5-FC in das hochgiftige 5-Fluorouracil (5-FU) umwandelt. Daher können in Gegenwart von 5-FC nur Zellen, die die *codBA*-Kassette verloren haben (dies sind entweder Wildtyp-Zellen oder die erwünschten Mutanten), wachsen.

Als erste Deletionsziele wurden die im Chromosom von *C. cellulovorans* codierten Restriktions-/Modifikationssysteme (RM) ausgewählt, um eine wichtige Barriere, die die Einführung der für die gentechnologische Modifizierung verwendeten Deletionsplasmide stark behindert. Die *In-silico*-Analyse mit dem Bioinformatik-Tool REBASE ergab das Vorhandensein von 12 Genloci für mutmaßliche RM-Systeme auf dem Chromosom von *C. cellulovorans*. Allerdings codierten nur vier der Loci für sowohl eine Restriktionsendonuklease (REase) als auch eine Methylase (MTase). Es wurden Deletionsplasmide für die Deletion der für die mutmaßlichen Restriktionsendonukleasen Clocel_1114 (Typ I), Clocel_4005 (Typ II), Clocel_4006 (Typ II) und Clocel_2651 (Typ III) konstruiert. Mit dem neu entwickelten Gene-Editing-System konnten die ORFs Clocel_1114 und Clocel_2651 ausgeschaltet werden. Mit den Stämmen *C. cellulovorans ΔClocel_1114* und *C. cellulovorans ΔClocel_2651* als Empfänger für Plasmidübertragung aus *Escherichia coli* wurde eine um zwei Größenordnungen verbesserte Konjugationseffizienz festgestellt. Der restriktionsdefiziente Stamm *C. cellulovorans ΔClocel_1114* wurde als Grundlage für weiteres metabolisches Engineering verwendet.

Während der Arbeiten zur vorliegenden Dissertation wurde in unserer Arbeitsgruppe ein weiteres Gen-Editierungssystem auf der Grundlage der CRISPR-Cas-Nickase entwickelt. Die Anwendbarkeit dieses Systems wurde auch in der vorliegenden Arbeit untersucht. Mit diesem System wurde der Stamm *C. cellulovorans ΔClocel_1114 Δptb_buk::cat1* erzeugt. Für die Herstellung des Stammes *C. cellulovorans ΔClocel_1114 Δpta* waren sowohl die codBA-basierte als auch die CRISPR-Cas-Nickase Methoden erfolgreich. Allerdings war unerwarteterweise das Plasmid-*curing* mit dem CRISPR-Cas-Nickase-System einfacher. Erstmalig wurden markerlose Knock-outs und Knock-ins in Genen des zentralen Stoffwechsels von *C. cellulovorans* erreicht.

Darüber hinaus gewährt die vorliegende Studie neue Einblicke in die Bakteriologie von *C. cellulovorans*. Um das Wachstum auf unlöslichen Substraten zu untersuchen, ist es entscheidend, ein anderes Mittel zur Quantifizierung des Wachstums als die Messung der Zelldichte zu haben. Für diesen Zweck wurde ein zuverlässiges Protokoll für die Proteinquantifizierung mit Hilfe des Bradford-Tests optimiert. Dies ermöglichte die genaue Verfolgung des Wachstums von *C. cellulovorans* auf kristalliner und amorpher Zellulose. Auch die pH-Veränderungen während des Wachstums wurden untersucht. Die kurzkettigen Fettsäuren Butyrat und Acetat sind die wichtigsten Fermentationsprodukte in *C. cellulovorans*. Die Anhäufung von Säuren im Medium kann die Fitness der Zellen beeinträchtigen. Interessanter Weise wurde beobachtet, dass bei der Kultivierung von *C. cellulovorans* auf Cellobiose ein pH-Wert erreicht wurde, der unter dem tolerierten Grenzwert lag., wohingegen beim Wachstum auf kristalliner oder amorpher Cellulose der pH-Wert der Kultur nie unter dem niedrigsten von *C. cellulovorans* tolerierten pH-Wert lag. Die Fermentationsprodukte waren jedoch unter allen getesteten Bedingungen ähnlich.

C. cellulovorans ist als endosporenbildendes Bakterium beschrieben worden. Es gibt jedoch seit dessen Beschreibung keine weitere Studien, die die Bedingungen für die Sporenbildung und Keimung untersucht hätten. Übernachtkulturen von *C. cellulovorans* zeigen unter dem Phasenkontrastmikroskop in der Regel zwei unterschiedliche Zellmorphologien: dunkle und dünne oder helle und geschwollene Stäbchen. Die hellen und geschwollenen Stäbchen wurden als die sporulierende Form von C. cellulovorans beschrieben. Trotz mehrerer Versuche, *C. cellulovorans* zum Keimen zu bringen, wurde nach der Pasteurisierung jedoch kein Wachstum beobachtet. Dies deutet darauf hin, dass die Endosporen von *C. cellulovorans* möglicherweise nicht hitzeresistent sind oder dass der Stamm die Fähigkeit zur Endosporenbildung verloren hat. Erwähnenswert ist auch, dass das Wachstum von *C. cellulovorans* häufig unvorhersehbar war, was den Umgang mit dem Organismus schwierig macht. Da die Induktion von Prophagen hierbei eine Rolle spielen könnte, wurde das *C. cellulovorans* Genom *in silico* analysiert und dabei vier anscheinend intakte Prophagenregionen aufgefunden. Obwohl es nicht möglich war, mit herkömmlichen Methoden (Phageninduktion mit Mitomycin C oder UV-Licht) phagenfreie Stämme zu erhalten, können diese Prophagen künftig mit den jetzt für *C. cellulovorans* verfügbaren genetischen Werkzeugen entfernt werden.

Publications

Peer-reviewed journals

Almeida, L., Schöllkopf, A., Edelmann, H., Ehrenreich, A. & Liebl, W. Markerless deletion of the putative type I and III restriction-modification systems in the cellulolytic bacterium *Clostridium cellulovorans* using a *codBA*-based counterselection technique. Journal of Biotechnology. Submitted.

Schöllkopf, A., **Almeida, L.**, Krammer, K., Rivero, C., Liebl, W. & Ehrenreich, A. Deletion of atypical type II restriction genes in *Clostridium cellulovorans* using a Cas9 based gene editing system. Applied Microbiology and Biotechnology. Submitted.

Contributions in conferences

Almeida, L., Schöllkopf, A., Edelmann, H., Liebl, W. & Ehrenreich, A. (2024). Establishment of marker-less genomic modification system for metabolic engineering of the cellulolytic bacterium *Clostridium cellulovorans*. 17th International Conference on the Genetics, Physiology, and Synthetic Biology of Solvent- and Acid-Forming Clostridia, Qingdao, China.

Schöllkopf, A., **Almeida, L.**, Liebl, W. & Ehrenreich, A. (2024). Development of a CRISPR/Cas9 system for the genetic engineering of *Clostridium cellulovorans*. 17th International Conference on the Genetics, Physiology, and Synthetic Biology of Solvent- and Acid-Forming Clostridia, Qingdao, China.

Abstreiter, A., Burgmaier, V., Schneider, M., **Almeida, L.**, Weuster-Botz, D., Liebl, W. & Ehrenreich, A. (2024). Markerless deletion of a type II restriction/modification system in *Clostridium kluyveri* using the CodAB counterselection system. 17th International Conference on the Genetics, Physiology, and Synthetic Biology of Solvent- and Acid-Forming Clostridia, Qingdao, China.

Almeida, L., Schöllkopf, A., Edelmann, H., Liebl, W. & Ehrenreich, A. (2024). Markerless genetic modification in *Clostridium cellulovorans* 743B based on the *codBA* counterselection system. 7th Joint Microbiology & Infection Conference of the German Society for Hygiene and Microbiology (DGHM) and the Association of General and Applied Microbiology (VAAM), Würzburg, Germany.

Abstreiter, A., Ehrenreich, A., Liebl, W., Burgmaier, V., Weuster-Botz, D., **Almeida, L.** (2024). Construction of a restriction deficient *Clostridium kluyveri* strain using the *codAB* counterselection system. 7th Joint Microbiology & Infection Conference of the German Society for Hygiene and Microbiology (DGHM) and the Association of General and Applied Microbiology (VAAM), Würzburg, Germany.

Almeida, L., Schöllkopf, A., Edelmann, H., Liebl, W. & Ehrenreich, A. (2023). Determination of plasmid stability and copy number in *C. cellulovorans* 743B. Annual Conference of the Association for General and Applied Microbiology (VAAM), Göttingen, Germany.

Schöllkopf, A., **Almeida, L.**, Rivero, C. G., Liebl, W. & Ehrenreich, A. (2023). The FAST way to assess promoter strength, riboswitches and inducibility in *Clostridium cellulovorans*. Annual Conference of the Association for General and Applied Microbiology (VAAM), Göttingen, Germany.

Almeida, L., Schöllkopf, A., Schneider, M., Liebl, W. & Ehrenreich, A. (2022). The influence of the different origins of replication on *Clostridium cellulovorans* transconjugation efficiency. 16th International Conference on the Genetics, Physiology, and Synthetic Biology of Solvent- and Acid-Forming Clostridia, Toulouse, France.

Schöllkopf, A., **Almeida, L.**, Liebl, W. & Ehrenreich, A. (2022). Expanding the molecular toolkit for *Clostridium cellulovorans*. 16th International Conference on the Genetics, Physiology, and Synthetic Biology of Solvent- and Acid-Forming Clostridia, Toulouse, France.

Schöllkopf, A., **Almeida, L**., Liebl, W. & Ehrenreich, A. (2022). Development of tools for genetic engineering of *Clostridium cellulovorans*. Annual Conference of the Association for General and Applied Microbiology (VAAM), Dusseldorf, Germany.

Co-supervision of students

Anna Kimadze, 2024, Bachelor thesis: Einsatz von CRISPR/Cas9-Plasmiden zur genetischen Modifikation von *Clostridium cellulovorans*.

Daniel Köhler, 2024, Research internship: Konstruktion eines doppeltrestriktionslosen Stammes von *Clostridium cellulovorans*.

Tzu-An Chong, 2024, Research internship: Construction of a citrate biosensor *Bacillus licheniformis*.

Yuliia Ulianova, 2024, Master thesis: Investigation of citrate secretion by arbuscular mycorrhiza fungi.

Selina Zenz, 2023, Bachelor thesis: Konstruktion Deletionsplasmiden für das zellulolytische Bakterium *Clostridium cellulovorans* 743B.

Selina Zenz, 2023, Research internship: Development of a markerless deletion system for *Clostridium cellulovorans*.

Paul Walker, 2023, Research internship: Entwicklung eines markerlosen Deletionssystems für *Clostridium cellulovorans*.

Maria Fernanda Frutos Marquez, 2023, Research internship: Development of a markerless deletion system for *Clostridium cellulovorans*.

Jana Baumgartner, 2023, Bachelor thesis: Konstruktion von Deletionsplasmiden für *Clostridium cellulovorans*.

Table of Contents

List of abbreviations

5-FC	5-fluorocytosine
5-FU	5-Fluorouracil
ABE	Acetone–butanol–ethanol
ACE	Allele-Coupled Exchange
ATCC	American Type Culture Collection
BLAST	Basic Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
Cas	CRISPR-associated (protein)
CBP	Consolidated bioprocessing
CFE	Cell free extract
CFU	Colony forming units
CM	Chloramphenicol
CT	Cycle threshold
CRISPR	Clustered regularly interspaced short palindromic repeats
ddH_2O	Double-distilled water
DMM	Defined minimal media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
EDTA	Ethylenediaminetetraacetic acid
EtBR	Ethydium bromide
EtOH	Ethanol
FD	Fast digest
Fdx	Ferredoxin

g	Gram
GC	Gas chromatography
GOI	Gene of interest
h	Hour
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HiFi	High Fidelity
HR	Homologous recombination
Indels	insertions/deletions
kb	Kilobase pair
kV	Kilo volt
L	Liter
LB	Lignocellulosic biomass
LB medium	Luria-Bertani (LB) medium
Ll.ltrB	*ltrB* gene of *Lactococcus lactis*
log	Logarithm
M	Molar (mol/l)
McF	McFarland Unit
MCS	Multiple cloning site
Min	Minute
mL	Milliliter
MMC	Mitomycin C
MTase	Methyltransferases
n	Nano (10^{-9})
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NCBI	National Center for Biotechnology Information
NCS	Native cellulolytic strategies
ng	Nanogram

NGS	Next Generation Sequencing
NTC	Non-template control
OD	Optical density
Ori	Origin of replication
P	Promoter
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFOR	Pyruvate:ferredoxin oxidoreductase
pH	Potential of hydrogen
qPCR	Real-time quantitative PCR
RCS	Recombinant cellulolytic strategies
REase	Restriction endonucleases
RM	Restrictionmodification
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
SNP	Single nucleotide polymorphisms
SNV	Single nucleotide variations
SOC	Super optimal broth with catabolite repression medium
Ta	Annealing temperature
TE buffer	Tris-EDTA buffer
TET	Tetracycline
TM	Thiamphenicol
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume

WT	Wild Type
µ	Micro (10^{-6})
µF	Mikro Farad
Ω	Ohm
%	Percentage
°C	Degree Celsius

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1. Introduction

1.1. History and current progress in biofuel production

Biofuels have been used for domestic purposes by humans since prehistoric times. Biofuel refers to fuel that comes directly or indirectly from plant biomass. They can be classified into three groups: solid biofuels (e.g. fuelwood, wood pellets, animal waste, etc.), liquid biofuels (e.g. biogasoline, biodiesel, bioethanol, etc.), and biogases (e.g. syngas produced by anaerobic fermentation or thermal processes) (Guo et al., 2015). Prior to the 19th century, wood and plant oil were the main fuel used for cooking, heating, and lighting. Even though the bioenergy available from biomass is 3–4 times greater than humans' energy demand, fossil fuels are still the dominant energy resource nowadays (Guo et al., 2015).

The use of fossil fuels marked the beginning of the Industrial Revolution and changed human society. However, fossil fuel reserves are limited. Over the last decades, the production of fuel from renewable resources has become a priority of our society. This can be attributed to several factors, such as the dwindling supply of fossil fuels, new legislation limiting the use of non-renewable energy sources, the increase in concerns about global warming and climate change, the rise in crude oil prices, and the high energy demand caused by the human population growth (García et al., 2011; Razali et al., 2018). Consequently, the production of biogenic fuels is considered a promising alternative to meet the energy demand in an eco-friendly manner (Ibrahim et al., 2017).

The first-generation biofuels, also known as conventional biofuels, are produced from edible crops such as sugarcane, sugar beet, sorghum corn, wheat, sunflower, and canola. These fuels were initially seen as a promising alternative to fossil fuels, with biodiesel and bioethanol being the most common types. However, the production of first-generation biofuels raised concerns about food security and supply (Alalwan et al., 2019). To address this issue, second-generation biofuels, also known as advanced biofuels, were developed.

Second-generation biofuels focus on using non-food feedstocks, such as lignocellulosic biomass derived from energy crops, agricultural waste, and industrial waste (Alalwan et al., 2019). The process of producing second-generation biofuels

requires pretreatment of the complex biomass, hydrolysis, followed by fermentation and product distillation (Jönsson & Martín, 2016). Bioethanol, biobutanol, and biodiesel are the main types of second-generation biofuels. Although bioethanol is the most commercial biofuel produced worldwide (Ibrahim et al., 2017), this fuel shows some disadvantages compared to gasoline, due to its lower energy content, high volatility, and high corrosivity to metal and rubber when blended with gasoline (Jin et al., 2011). On the other hand, biobutanol displays significant advantages due to its similarity to gasoline. For instance, n-butanol (butanol) can be used directly or blended with gasoline, its use does not require modification of existing car engines, and it is safer than ethanol to handle due to its low vapor pressure (Dürre, 2007). Diesel fuel is an important petroleum fuel used in transportation. Biodiesel can also be produced from second-generation feedstocks. However, its low performance at low temperatures limits its ability to replace the petroleum used for transport (Elgharbawy et al., 2021).

The third-generation feedstock is derived from algal biomass. This type of biomass has a low content of hemicelluloses and a negligible content of lignin, a short harvesting cycle, and can grow in harsh conditions. On the other hand, the high cost associated with cultivation and downstream processing are considerable limitations (Behera et al., 2015). The fourth-generation feedstock is synthesis gas (or syngas). Syngas is a mixture consisting predominantly of hydrogen (H_2), carbon monoxide (CO), and carbon dioxide (CO_2). Through syngas fermentation, acetogens such as *Clostridium ljungdahlii* (Klasson et al., 1992), *C. autoethanogenum* (Abrini et al., 1994), *Eubacterium limosum* (Chang et al., 2001), and *C. carboxidivorans* (Liou et al., 2005) can convert it through the Wood–Ljungdahl pathway into valuable hydrocarbon fuels, such as methanol. However, it is still a relatively new technology, and several key parameters are still unknown (Guo et al., 2022).

1.2. Consolidated bioprocessing for biobutanol production from lignocellulosic biomass

Many nations now produce biofuels from biomass feedstocks. The USA and Brazil dominate global biofuel production, accounting for 69 % of all biofuels in 2018, followed by Europe with 9 % (Jeswani et al., 2020). However, the market and technologies for the production of first-generation biofuels are still better established

than for the production of second-generation biofuels (Balan, 2014). The large-scale utilization of lignocellulose material is still a challenge due to the complexity of lignocellulosic biomass. Its natural recalcitrance to biodegradation obligates the use of multi-step treatments in the transformation process, increasing costs, and the release of CO_2 (Procentese et al., 2017). Fortunately, despite cellulose being highly resistant to microbial degradation, many microorganisms have been reported to have cellulolytic activities including many bacterial and fungal strains. However, no microorganisms isolated to date can efficiently utilize cellulosic biomass to produce high-value products (e.g., platform chemicals, fuels) in a single-step process (Singhania et al., 2022).

To overcome this limitation, consolidated bioprocessing (CBP) has been considered a promising strategy. CBP refers to the approach of combining the saccharification of lignocellulose material and the production of valuable products in one step. In CBP, the production of saccharolytic enzymes, hydrolysis of the polysaccharides, fermentation of hexose sugars, and fermentation of pentose sugars are all performed by a microbial chassis or by microbial consortia. The feedstock, the microbial host(s), and the desired products are key aspects of the CBP and will be discussed in the following paragraphs.

1.2.1. Lignocellulosic biomass as a carbon source

Lignocellulosic biomass (LB) is the most abundant renewable material on Earth. Approximately 200 billion tons are produced annually, out of which 10 % are available for utilization in the production of biotechnologically important products (Ilić et al., 2023). It can be derived from energy crops (highly lignocellulosic plants such as *Panicum virgatum*, commonly known as switchgrass), agricultural wastes (e.g. corn stover, sugarcane bagasse, rice straw, wheat straw, etc.), forestry or wood waste and cellulose waste (e.g. from the paper industry) (Bharathiraja et al., 2017).

LB is composed of tightly spaced and interlinked layers of polysaccharides such as cellulose (40–50 %), hemicellulose, and pectin (25–30 %) covered with a protective polyphenol layer of lignin (15–20 %) (Tayyab et al., 2018). Cellulose is the major saccharification target. It is a homopolysaccharide consisting of glucose monomers linked by β-1,4-glycosidic bonds (Fig. 1.1). The recalcitrance of cellulose is influenced

by physical characteristics such as crystallinity, accessible surface area, degree of polymerization, particle size, and pore volume (Ilić et al., 2023). Cellobiose typically is the main product of the hydrolysis of cellulose by cellulases. β-Glucosidase converts cellobiose to two molecules of glucose, which can be further metabolized by microorganisms (Banner et al., 2021). Glucose is a widely accepted carbon source for microorganisms. Therefore, the abundant release of glucose molecules makes lignocellulose a rich feedstock (Banner et al., 2021).

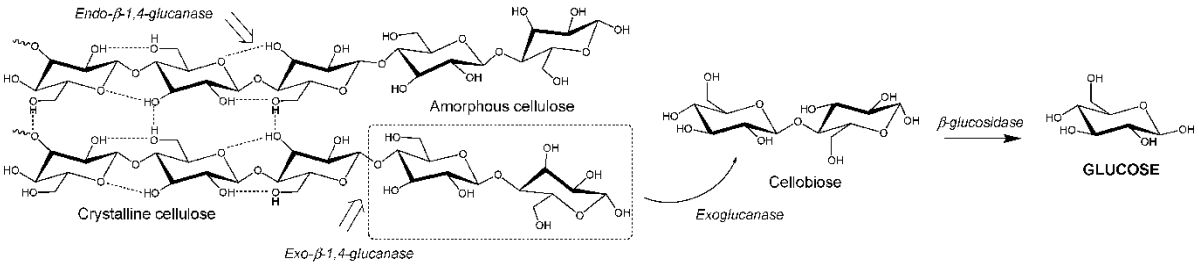

Figure 1.1 | Cellulose structure and representation of the enzymatic degradation of cellulose to glucose. Adapted from Banner et al. (2021).

The hemicellulose portion is a heterogeneous polymer composed of various different sugars such as pentoses (xylose and arabinose) and hexoses (mannose, galactose, and glucose) linked via β-1,4- and β-1,3-glycosidic bonds. Hemicellulose is considered less valuable as a carbon source compared to cellulose due to the presence of C5 sugars, which are less preferred by microorganisms (Banner et al., 2021). The hemicellulose and pectin are closely associated with the cellulose fibers forming a rigid matrix. Lignin is a complex heterogeneous polymer derived mainly from aromatic alcohol monomers (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) (Del Río et al., 2020). The lignin binds to cellulose and hemicelluloses with covalent bonds increasing mechanical strength, hydrophobicity, and protection from microbial attack (Inamori et al., 2016).

1.2.2. Cellulolytic microorganisms

Several pre-treatment approaches have been employed to break down the lignocellulosic biomass, such as chemical methods (e.g. acid hydrolysis, alkali

hydrolysis, ozonolysis), physical methods (e.g. irradiation, ultrasonic treatment, moist heat, and grinding), physicochemical methods (e.g. ammonia fiber expansion, steam explosion, CO_2 explosion), and biological methods (e.g. the use of enzymes or microorganisms) (Singhvi & Kim, 2020). Besides the cost associated with the pre-treatment of the lignocellulosic biomass, toxic compounds can be produced during the reaction (Singhvi & Kim, 2020). To avoid the need for pre-treatments of the lignocellulose before saccharification, the use of cellulolytic microorganisms for biomass degradation is considered a promising strategy (Kaur et al., 2020).

Some of the reported fungal species responsible for breaking down cellulosic biomass include *Chaetomium* spp., *Fusarium* spp., *Myrothecium* spp., *Trichoderma* spp., *Penicillium* spp., and *Aspergillus* spp (Milala et al., 2005). The cellulolytic bacteria include *Clostridium* spp., *Bacteroides cellulosolvens*, *Butyrivibrio fibrisolvens,* and *Ruminococcus albus* (Schwarz, 2001). The microbial host for CBP can be either a naturally cellulolytic or a recombinant cellulolytic microorganism. Native cellulolytic strategies (NCSs) aim to introduce or improve desired biosynthetic pathways into cellulolytic strains. In contrast, recombinant cellulolytic strategies (RCSs) aim to introduce cellulolytic ability in microorganisms that already exhibit high product yields (Mazzoli, 2012).

One of the major challenges of NCSs is the poor characterization and the lack of tools for the genetic manipulation of most of the cellulolytic strains. *C. thermocellum* and *C. cellulolyticum* are among the most extensively studied cellulolytic microorganisms, but still further research on the metabolism of natural cellulolytic microorganisms could provide valuable insights for enhancing the performance of recombinant strains (Desvaux, 2005; Dumitrache et al., 2013). Besides, the production of solvents can lead to chaotropic effects on biological membranes, protein and RNA unfolding and degradation of DNA (Mazzoli, 2012). Therefore, it could be an option to develop solvent-tolerant strains. The overexpression of heat-shock proteins, efflux pumps, and enzymes that change the membrane lipid composition are possible strategies to overcome this problem (Costa et al., 2021; Dunlop et al., 2011; Tomas et al., 2003; Zhao et al., 2003). Regarding the RCSs, the two main challenges are the design of artificial cellulase systems and the efficient secretion in the host strains. The heterologous expression of cellulases may also require the expression of a scaffolding

protein that consists of at least two cohesins for proper function (Lynd et al., 2002). Additionally, the introduction of cellodextrin membrane transporters can be used to obtain a better uptake of cellulose breakdown products (Mazzoli, 2012). However, from the biotechnological point of view, the coordination of the expression of multiple heterologous genes is a challenge (Mazzoli, 2012).

1.2.3. Biobutanol production from lignocellulosic biomass

Butanol, also called butyl alcohol, can be produced by fermentation or petrochemically. In addition to the environmental advantages, its biotechnological production can be economically viable (Dürre, 2007; Zhang et al., 2018). The process of producing butanol through fermentation using a solvent-producing bacterium, like *C. acetobutylicum*, is known as ABE fermentation (acetone, butanol, ethanol fermentation) or Weizmann process. *C. beijerincki*, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* are also well-known ABE fermenters (Dürre, 2008; Zheng et al., 2009). Although ABE fermentation for butanol production using *Clostridium* spp. has been established for over 100 years, there are still several challenges to the industrial production of biobutanol, such as the cost of the substrates and the development of strains with greater butanol tolerance (Moon et al., 2016).

The first industrial-scale ABE fermentation began in 1916. The rapid progress in fermentation research contributed to the use of cheaper carbon sources, lower fermentation temperatures, and improved utilization of residual sugars (Moon et al., 2016). Until the 1950s, ABE fermentation supplied approximately 66 % of the world's supply of butanol, but the price of the carbon sources made it less attractive than the petrochemical industry (Dürre, 2008). However, the oil crisis in the 1970s renewed interest in ABE fermentation.

Selecting the right substrate has a significant impact on fermentation performance and production costs. Initially, the industrial ABE fermentation was based on cooked maize mash as the fermentation medium at 37 ºC (Moon et al., 2016). In the attempt to find cheaper and more sustainable substrates, lignocellulose biomass was considered a cost-effective carbon resource. For instance, bagasse and rice straw were used by the cellulolytic fungi *T. reesei* and *A. wentii* to obtain fermentable sugars (Soni et al., 1982). The produced sugars were available to be fermented by

C. saccharoperbutylacetonicum that produces butanol (Soni et al., 1982). *C. beijerinckii* was able to use banana crop residue as substrate for ABE fermentation (Reddy et al., 2020). However, the need for complex pretreatment and detoxification is still a drawback for producing butanol from biomass (Guo et al., 2015).

1.2.4. Microbial consortia

Lignocellulose degradation and butanol metabolism can be a significant burden for single strains in lignocellulosic butanol fermentation. However, it has been observed in nature that microbial communities containing fungi and cellulose-degrading bacteria can completely degrade and consume lignocellulosic biomass. By mimicking nature, the co-cultivation of cellulolytic microorganisms and solventogenic microorganisms can be an interesting strategy for the production of lignocellulosic biobutanol.

It has already been demonstrated that synthetic microbial communities have advantages over monoculture approaches in terms of substrate utilization and solvent yield. Wen and co-workers (2014) obtained butanol from alkali extracted corn cobs (AECC) by co-cultivating the cellulolytic *C. thermocellum*, and the solvetogenic *C. beijerinckii* (Wen et al., 2014). Wang and co-workers (2015) used a mesophilic co-culture of *C. celevecrescens*, and *C. acetobutylicum* to obtain the direct conversion of cellulose to butanol (Wang et al., 2015). Wen and co-workers (2017) obtained an enhanced butanol yield using a genetically engineered twin-clostridial consortium composed of *C. cellulovorans* and *C. beijerinckii* (Wen et al., 2017). Nevertheless, the effectiveness of a synthetic microbial consortia depends on its stability. Different growth requirements (e.g. temperature, pH, medium composition), growth rates, inoculum ratio, and tolerance to the solvents can interfere with the stability of the consortia (Du et al., 2020). For instance, a population that grows quickly can extinguish a population that grows slowly (Duncker et al., 2021). In addition to optimizing the fermentation conditions, adaptive evolutionary engineering, the use of immobilized cells (since they are less affected by changes in medium), and creating metabolic-dependent strains are strategies to improve stability (Du et al., 2020).

1.3. Cellulolytic clostridia

1.3.1. The genus *Clostridium* spp.

Clostridia is a polyphyletic class of microorganisms belonging to the phylum *Bacillota* (previously named *Firmicutes*) (Oren & Garrity, 2021). Within the order *Clostridiales* and family *Clostridiaceae*, *Clostridium* is a large genus of microorganisms encompassing 231 species (Brasca et al., 2021). *Clostridium* spp. are rod-shaped, spore-forming, mostly strictly anaerobic, and generally Gram-positive microorganisms. The first observation of an anaerobic bacterial form of life goes back to 1861 by Louis Pasteur. He named this microorganism *Vibrion butyrique* due to the production of butyrate as the main fermentation product, and to the fact that it was a mobile rod (Popoff, 2015). He also introduced the words aérobies (aerobes) or anaérobies (anaerobes) to distinguish microorganisms capable of growing in the presence or absence of oxygen, respectively (Sebald & Hauser, 1995). In 1867, A. Trecul used for the first time the word "*Clostridium*" (from Greek klōstēr-ster, small spindle) to designate the shape of *V. butyrique* in its sporulating form (Popoff, 2015).

Almost 20 years later, in 1880, Adam Prazmowski renamed *V. boutique* as *C. butyricum*. Besides, A. Prazmowski described straight rods as "*Clostridium*", while the curved rods were called "*Vibrio*". However, Van Tieghem also named this species *Bacillus amylobacter*. This type of misclassification was not uncommon at that time since morphology was the main criterion used in the nomenclature of bacteria. The physiological distinctions started to be taken into account in 1922. The Committee on Classification of the Society of American Bacteriologists defined that the anaerobic rod-shaped spore-forming bacteria were grouped in the genus *Clostridium* and the aerobic rod-shaped spore-forming bacteria were grouped in the genus *Bacillus* (Popoff, 2015). Therefore, the binomial name proposed by A. Prazmowski was accepted. Thus *C. butyricum* was the first isolated *Clostridium* strain becoming the type strain of the genus (Popoff, 2015).

Clostridium spp. are ubiquitous microorganisms that can be found in the soil, dust, plants, human skin, intestinal tract of birds, fish, and mammals. The medical importance of many species is well known. For instance, the presence of *C. butyricum* in the human gut is associated with attenuating inflammation and allergic diseases

(Guo et al., 2020). Due to the production of certain metabolites, such as butyrate, these bacteria help inhibit the growth of potential pathogens and promote an increase in the thickness of the intestinal mucosa (Stoeva et al., 2021). Other species, called solventogenic *Clostridium* spp., such as *C. acetobutylicum* and *C. beijerinckii* are known because of their biotechnological relevance due to the ability to produce solvents including acetone, butanol, and ethanol (Li et al., 2020). Although the majority of the species are harmless, several species, such as *C. difficile*, *C. perfringens*, *C. botulinum,* and *C. tetani* can cause human or animal disease (Uzal et al., 2018).

Clostridia species such as *C. thermocellum* (Viljoen et al., 1926; current valid name *Acetivibrio thermocellus,* Tindall et al., 2019), *C. cellulolyticum* (Petitdemange et al., 1984; or *Ruminiclostridium cellulolyticum*, Zhang et al., 2018), and *C. cellulovorans* can efficiently degrade cellulose from lignocellulosic biomass. To date, *C. thermocellum*, a thermophilic bacterium isolated in 1926 (Viljoen et al., 1926), is the most efficient cellulose solubilizer characterized (Paye et al., 2016). It possesses a multiprotein complex, called cellulosome (Lamed et al., 1983). This allows it to consume cellulose at a high rate of 2.5 g/L/h (Argyros et al., 2011), which is greater than that reported for other cellulolytic organisms, including *C. cellulovorans* that degrades at a rate of 0.053 g/L/h (Sleat et al., 1984). However, since it is a thermophilic organism, the expression of heterologous proteins for the production of solvents can be difficult. *C. cellulolyticum* is the model organism of mesophilic cellulolytic activity (Desvaux, 2005). Due to its ability to utilize pentoses from hemicellulose degradation, including xylose, arabinose, and ribose, in addition to the hexoses fructose, galactose and mannose, it has been considered a valuable candidate for CBP (Gowen & Fong, 2010). It has been already genetically engineered to produce ethanol and isobutanol directly from cellulose (Guedon et al., 2002; Higashide et al., 2011). In terms of the production of butanol from biomass in CPB, *C. cellulovorans* is considered a better candidate. Further details will be addressed in the following section.

1.3.2. *C. cellulovorans*

C. cellulovorans 743B (ATCC 35296) is a cellulolytic bacterium isolated from a wood chip pile (Sleat et al., 1984). Contrary to the other *Clostridium* spp., it stains Gram-negative. It is obligately anaerobic, catalase-negative, and does not reduce sulfate. It is a mesophilic bacterium with optimum growth at 37 °C and pH 7.0, even though it

can grow in a temperature range of 20–40 °C and a pH range of 6.4–7.8. Cells are non-motile rods with 2.5–3.5 µm length by 0.7–0.9 µm width. *C. cellulovorans* was described as a spore-forming bacterium, with oblong-shaped endospores, occurring either centrally or subterminally within the mature sporangium (Sleat et al., 1984). However, its ability to sporulate has not been shown ever since its description. Its colonies are creamy-white and opaque with 1–2 mm and it can assume a rhizoid shape (Sleat et al., 1984). *C. cellulovorans* genome sequencing was completed and resulted in a size of about 5.1 Mbp (megabase pairs), which is at least 1 Mbp larger than other cellulosome-forming clostridia, like *C. cellulolyticum* H10 (4.07 Mbp) and *Acetivibrio thermocellus* (*C. thermocellum*) ATCC 27405 (3.84 Mbp). About 4,220 open reading frames (ORFs) were predicted. Like other *Bacillota*, its genomic DNA has a low GC content of 31 % (Tamaru et al., 2010).

Besides cellulose, *C. cellulovorans* 743B can ferment cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, pectin, sucrose and xylan. The main products of the fermentation are short-chain fatty acids such as acetate, butyrate, lactate, and formate, and the gases H_2 and CO_2. Acetyl-CoA and butyryl-CoA are two important nodes in its theoretical metabolic network. They are related to the formation and reassimilation of acetate and butyrate (Wen et al., 2019). These two nodes can be a link between the carbon central metabolism and the butanol synthesis pathway with the CoA-dependent (carbon chain elongation) pathway (Wen et al., 2019). Briefly, carbon chain elongation is a process that converts volatile fatty acids (e.g. acetic acid, propionic acid, and butyric acid) and an electron donor (e.g. ethanol, lactate) into longer chain carboxylic acids (e.g. butanol) via reverse β-oxidation in an anaerobic culture (Bevilacqua et al., 2022; Roghair et al., 2018). Although *C. cellulovorans* wild-type cannot produce butanol, this has been attempted with several strategies. Wen and co-workers redirected the carbon flux from butyryl-CoA to butanol by overexpression of an alcohol aldehyde dehydrogenase (*adhE1*) from *C. acetobutylicum* ATCC 824 (Wen et al., 2019). In another publication, Wen and co-workers further changed the carbon flux by overexpressing a trans-enoyl-coenzyme A reductase (*ter*) from *Treponema denticola*, which can irreversibly catalyze the conversion of crotonyl-CoA to butyryl-CoA (Wen et al., 2020a). The authors also introduced an acid reassimilation pathway not coupled with acetone production to redirect the carbon flow from butyrate and acetate toward butyryl-CoA, by

overexpressing a butyryl-CoA−acetate CoA transferase (*cat1*) from *C. tyrobutyricum* DSM 2637 (Wen et al., 2020a). Nevertheless, the progress obtained to date is limited by the lack of efficient genetic tools. Tools such as the ClosTron system and CRISPR interference (CRISPRi) were employed for *C. cellulovorans* (Wen et al., 2017, 2019). However, it was not possible to obtain markerless gene knockouts. For instance, the deletion of the butyrate kinase (*buk*) would be essential to direct the butyrate flux to butanol, but even after long-term screening, a *buk* mutant was not obtained (Wen et al., 2019).

1.3.2.1. *C. cellulovorans* cellulases and non-cellulosomal enzymes

One of the most prominent characteristics of *C. cellulovorans* is the production of cellulosomal and non-cellulosomal enzymes able to efficiently degrade the cellulose and hemicellulose present in the lignocellulosic biomass (Inamori et al., 2016). After genome analysis, a total of 57 cellulosomal protein-encoding genes and 168 noncellulosomal (hemi)cellulolytic protein-encoding genes have been annotated (Doi et al., 1998; Matsui et al., 2013).

The noncellulosomal proteins are produced as precursors with N-terminal signal peptides but do not have dockerin domains, while the cellulosomal proteins included 4 scaffold proteins and 53 cellulosomal proteins with dockerin domains (Matsui et al., 2013). Interestingly, *C. cellulovorans* changes the production of secreted enzymes depending on the substrate available in the medium (Esaka et al., 2015; Matsui et al., 2013; Morisaka et al., 2012). Large protuberances are visible on the cell surface when it is incubated in cellulose (Fig. 1.2). This high cellulolytic capacity makes *C. cellulovorans* a promising candidate for producing industrial biofuels and biochemicals from lignocellulosic biomass.

1.4. Metabolic engineering tools for clostridia

Genetic improvement of microorganisms has contributed to better utilization of natural resources. Although progress has been made in clostridial genome engineering, synthetic biology tools are scarce or non-existent for many species.

The implementation of a genetic system for a non-model organism relies on the availability of a fully annotated genome sequence, identification of all appropriate

genetic parts, means of introducing DNA into the clostridial host, and understanding of restriction/modification (RM) barriers (Minton et al., 2016). In the upcoming paragraphs, these key aspects will be discussed.

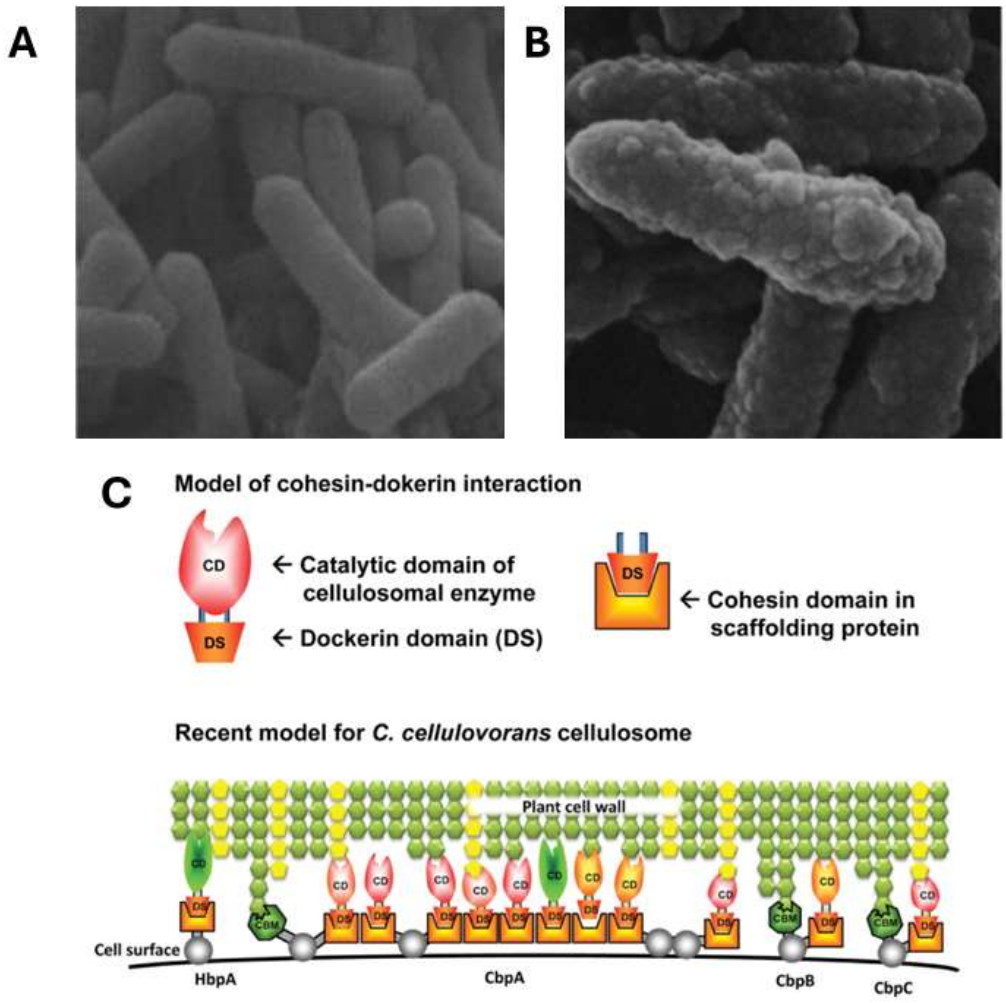

Figure 1.2 | Cell surface electron micrograph of *C. cellulovorans* incubated in a medium containing cellobiose (A) and cellulose (B). Schematic representation of *C. cellulovorans* cellulosomes (C). Adapted from Blair & Anderson (1998) and Tamaru et al. (2010).

1.4.1. Genome editing technologies for Clostridia

ClosTron, Allele-Coupled Exchange (ACE), and CRISPR/Cas9 are genome editing tools used for Clostridia. Shortly, the ClosTron system provides gene disruption by inserting the mobile intron from the *ltrB* gene of *Lactococcus lactis* (Ll.ltrB) into a target

locus in the chromosome (Heap et al., 2007). Although the ClosTron system has undergone refinements (Heap et al., 2010; Kuehne et al., 2011), two drawbacks remain. First, the curing of the plasmid is laborious (Wen et al., 2020b). Second, insertion mutations may lead to undesired polar effects (Dierick et al., 2023; Heap et al., 2012; Zhang et al., 2015). On the other hand, ACE relies on the homologous recombination process, that occurs naturally during DNA replication and repair (Li & Heyer, 2008). Briefly, this method relies on the selection of single-crossover clones (plasmid integration) followed by counterselection of double-crossover clones (plasmid excision) (Heap et al., 2012). A more detailed explanation of the ACE method will be discussed in the following paragraph. Recently, the CRISPR-Cas9 tool was adapted for use in clostridia to overcome constraints caused by the rare frequency of allelic exchange events. Clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) is part of the adaptive defense system found in bacteria (Hille & Charpentier, 2016; Makarova et al., 2006). Besides, it is the basis for a powerful genome editing tool (Doudna & Charpentier, 2014). Even though it is also a recombination-based method, the presence of the single guide RNA (sgRNA) associated with Cas endonuclease allows precise cleavage of the parent allele for further repair (Bruder et al., 2016; Cañadas et al., 2019; Pyne et al., 2016).

1.4.2. Genomic mutagenesis by Allele-Coupled Exchange (ACE)

One effective technique for modifying clostridial genomes is ACE (Heap et al., 2012). ACE is a two-step process based on the replacement of a specific region of DNA through homologous recombination (HR). HR is a natural process where two DNA molecules with identical or similar sequences are exchanged (Court et al., 2002). It is a mechanism to bring genetic diversification, such as antibiotic resistance, and preserve chromosome integrity (Smith, 1988; Thomas & Nielsen, 2005). According to the current knowledge about HR in bacteria, during the recombination process, RecA binds ssDNA forming a nucleoprotein filament present at a DNA break, and this complex searches for homologous dsDNA to use as a template for the repair (strand invasion) (Val et al., 2019). Thereafter, double-strand DNA breaks (which are a lethal condition) are repaired by the RecBCD pathway (Dillingham & Kowalczykowski, 2008), while single-strand DNA breaks are repaired by the RecF pathway (Morimatsu & Kowalczykowski, 2003). In both cases, the process is followed by branch migration,

strand exchange at the holiday junction, and resolution, resulting in the formation of a recombinant molecule (Handa et al., 2009; Hiom, 2009).

This mechanism has been used broadly to obtain markerless gene knock-out in clostridia. However, the application of the ACE method requires the construction of rationally designed shuttle vectors for *Escherichia coli* and *Clostridium* which carry a selective marker, a counterselection marker, and homologous flanks (sequences homologous to chromosomal DNA segments flanking the locus of interest, see Fig. 1.3).

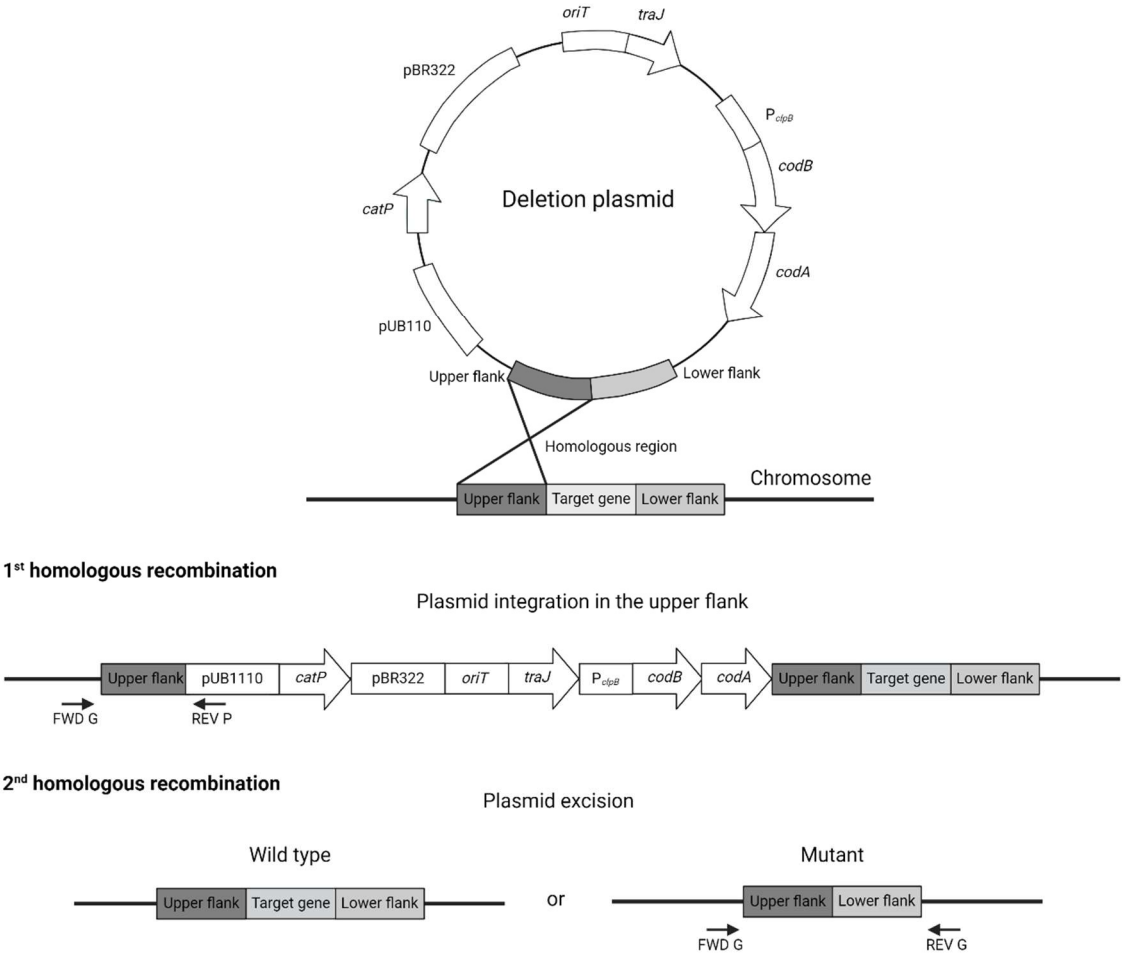

Figure 1.3 | Schematic representation of genomic mutagenesis by Allele-Coupled Exchange (ACE). The deletion vector containing flanking regions is transformed into the desired host. 1st homologous recombination (single-crossover integration) is stimulated by the cultivation of the organism on selective plates. Clones are then screened for the side of integration with a combination of chromosomal-specific and plasmid-specific primers (e.g., FWD G/REV P to verify the integration in the upper flank region). 2nd homologous recombination (plasmid excision) either recreates the wild-type or a mutated locus. FWD G stands for forward primer specific to the genome (or chromosomal region); REV P stands for reverse primer specific to the plasmid; REV G stands for reverse primer specific to the genome (or chromosomal region).

1.4.3. Synthetic biology genetic parts

Deletion and cloning vectors are plasmids containing certain features. Reliable genetic parts are essential for the construction of gene cassettes present in the plasmids used for genetic engineering. The synthetic biology basic parts are minimal sequence elements with biological functions in gene expression, including promoters, repressors, ribosome binding site, origin of replication, and transcriptional terminators (Gyulev et al., 2018).

1.4.3.1. Promoters and reporter systems

A promoter is a region of DNA upstream of a gene where relevant proteins, such as RNA polymerase and transcription factors, bind to initiate transcription of a gene. The knowledge about promoters is extremely valuable in synthetic biology. Constitutive promoters commonly used for Clostridia include P_{thlA} (thiolase) and P_{fdx} (ferredoxin), from *C. acetobutylicum* and *C. sporogenes*, respectively (Ehsaan et al., 2016; Girbal et al., 2003; Heap et al., 2010; Zhang et al., 2015). For instance, Bengelsdorf and co-workers constructed a recombinant *C. ljungdahlii* that harbors an artificial acetone synthesis operon under the control of P_{thlA} promoter from *C. acetobutylicum* (Bengelsdorf et al., 2016). The same promoter has also been used to construct FAST-tagged fusion proteins to monitor protein production during *Eubacterium limosum* growth (Flaiz et al., 2021). The P_{thl} from *C. perfringens* has also been used in metabolic engineering approaches. A recombinant strain of *C. acetobutylicum* with increased cellulolytic properties was constructed by introducing the genes *Cel9G*, *Cel48F*, and *Xyn10A* from *C. cellulolyticum* via ACE technology with expression driven by P_{thl} (Willson et al., 2016). The constitutive P_{fdx} from *C. sporogenes* has been employed in the ClosTron system for expression of the Ll.LtrB intron (Heap et al., 2010). It is also an important genetic part of expression plasmids used for the production of recombinant proteins in *E. coli* and *C. perfringens* (Takamizawa et al., 2004).

Inducible promoters allow controlled expression of a gene of interest. Constitutive promoters can be made inducible either by adding binding sites for transcriptional repressors or activators (Gyulev et al., 2018). A common challenge in the expression of heterologous proteins, such as enzymes or membrane proteins, is the metabolic burden imposed on the new host, resulting in poor growth. One broadly used system

to mitigate this problem is the lac operator-repressor system. Briefly, in the absence of lactose, the *lac* repressor (LacI) binds specifically to a lac operator sequence (*lacO*), inhibiting the *lac* promoter (Brown et al., 1967). For instance, a LacI-repressible version of P_{thlA} has allowed the cloning of the cellulolytic enzymes in *E. coli* (which is usually used for the construction of recombinant plasmids). Due to the success of this intermediate step, it was possible to obtain a *C. acetobutylicum* strain encoding various cellulosome genes (Kovács et al., 2013). Natural inducible promoters, such as the xylose promoter-repressor system (where the *xylA* promoter is repressed by XylR of *Staphylococcus xylosus*) have also been used for Clostridia (Girbal et al., 2003; Philipps et al., 2019).

Another type of promoter are fermentation phase-specific promoters. For instance, in *C. acetobutylicum* P_{ptb} (phosphotransbutyrylase), and P_{hydA} (hydrogenase A) are active during acidogenesis, while P_{bdhB} (NADH-dependent butanol dehydrogenase B), and P_{adhe2} are mainly active during solventogenesis (Feustel et al., 2004; Schulz et al., 2013). Unfortunately, the majority of the well-characterized promoters come from a few strains and their effectiveness is not always transferrable to other hosts (Joseph et al., 2018).

To generate a library of promoters with varying strengths, sensitivity to the inducer, and knowledge about “leaky” expression, reporter systems are required. Reporter systems based on enzymatic reactions, such as the chloramphenicol acetyltransferase (CAT) reporter system, were the first reporter systems used in the *Clostridium* genus (Gyulev et al., 2018). However, this method is not suitable for naturally chloramphenicol-resistant strains.

Fluorescent reporter systems have also been successfully used for clostridia. Of note, the oxygen sensitivity of *Clostridium* species limits the use of many reporter systems (Charubin et al., 2020). Contrary to green fluorescent proteins (GFPs), flavin-binding fluorescent proteins (FbFPs) are capable of maturation under anaerobic conditions. The FbFPs are small proteins (~11−15 kDa) with oxygen-independent fluorescent properties, using a flavin mononucleotide (FMN) cofactor as the chromophore (Gyulev et al., 2018). The reporter functionality has been already shown for many *Clostridium* species (Buckley et al., 2016; Molitor et al., 2016; Teng et al., 2015). Recently, a codon-optimized fluorescence-activating and absorption-shifting tag protein (FAST)

was described as a reporter system for *Clostridium* organisms (Streett et al., 2019). FAST is a small protein-tag (~14 kDa) engineered from the photoactive yellow protein (PYP) (Plamont et al., 2016). In the presence of a fluorogenic ligand (e.g. 4-hydroxy-3-methylbenzylidene-rhodanine, HMBR, commercialized as ^{TF}Lime) the fluorescence is activated. However, it is important to consider that some species, such as *C. acetobutylicum*, have a natural green auto-fluorescence increasing the background signal (Buckley et al., 2016). Therefore, it is necessary to have a control plasmid in gene expression experiments to accurately assess the results.

1.4.3.2. Origin of replication

The origin of replication (ori) is an essential part of the plasmids used to genetically manipulate bacteria. The ori is responsible for recruiting the proteins of the replication machinery and allowing the autonomous replication of the plasmid. Many oris have been described for *Clostridium* spp., such as those from pCB101 (from *C. butyricum* NCIB 7423) (Brehm et al., 1992), pBP1 (from *C. botulinum* NCTC 2916) (Davis, 1998), pCB102 (from *C. butyricum* NCIB 7423) (Minton & Morris, 1981), pCD6 (from *C. difficile* CD6) (Purdy et al., 2002), pIP404 (from *C. perfringen*) (Garnier & Cole, 1988), and p19 (from *C. carboxidivorans* P7T) (Bruant et al., 2010). Apart from *Clostridium* spp. oris, the oris of pIM13 (from *B. subtilis* BD1109), pUB110 (from *S. aureus*) (Lacey & Chopra, 1974), and pAMβ1 (from *Streptococcus lactis*) (Oultram & Young, 1985) are also functional in clostridia. The temperature-sensitive pWV01ts (from *L. lactis* subsp. *cremoris*) (Maguin et al., 1992) was found to be functional in *C. ljungdahlii* (Molitor et al., 2016). In 2009 Heap and co-workers published a series of *Clostridium–E. coli* shuttle plasmids (called pMTL80000 modular plasmids) containing various Gram-positive oris. This series is broadly used by the *Clostridium* research community (Heap et al., 2009).

Besides testing the compatibility of the ori with the host organism, it is important to know the segregational stability of the plasmid. It has been proposed that replication-defective plasmids are preferable when intended to use the ACE method for gene modification in clostridia (Minton et al., 2016). Therefore, the identification of replication-defective oris (also called “pseudo-suicide” replicon) is a crucial step in the development of a genetic system for a non-model organism.

1.4.3.3. Selection and counterselection markers

Selection markers are used to select cells that contain the plasmid of interest after transformation. A range of different selection markers that confer resistance against structurally dissimilar antibiotics are available for clostridia. Selection markers commonly used in clostridia are the antibiotic-resistance cassettes *catP* (encoding chloramphenicol acetyltransferase) and *ermB* (encoding rRNA adenine N-6-methyltransferase) (Heap et al., 2007; Jennert et al., 2000; Sloan et al., 1992). They confer resistance to chloramphenicol (or its analog thiamphenicol) and erythromycin, respectively. Alternatively, the antibiotic-resistance cassettes *tetA* (encoding tetracycline resistance protein) and *aad9* (encoding spectinomycin 9-adenylyltransferase) can also be used in clostridial hosts (Heap et al., 2009). They confer resistance to tetracycline and spectinomycin, respectively.

While selection markers confer a survival advantage, the counterselection markers result in cell death in the presence of the counterselective compound. Identifying a suitable counterselection marker and the appropriate conditions for its use can be a challenging aspect of developing genetic tools (Reyrat et al., 1998). The *mazF*-based, Δ*pyrE*-based, Δ*pyrF*-base, Δ*upp*-based, and *codBA*-based are examples of counterselection systems used to obtain gene knock-outs in clostridia.

A *mazF*-based counterselection method was established for clostridia by Al-Hinai and co-workers (2012) by combining a lactose-inducible promoter with a codon-optimized *mazF* toxin gene (Al-Hinai et al., 2012). Briefly, MazEF is a stress-induced toxin-antitoxin system found in *E. coli*. Under normal conditions, the antitoxin MazE binds to and inhibits the MazF (mRNA interferase responsible for inhibiting translation by cleaving mRNA), but in the presence of cellular stress, MazE is degraded and MazF is free, leading to cell death (Al-Hinai et al., 2012). The expression of *mazF* under the control of the lactose-inducible promoter allowed the use of lactose as the counterselective medium ingredient. The system was incorporated into the deletion plasmid allowing the generation of gene knock-outs and knock-ins in *C. acetobutylicum* (Al-Hinai et al., 2012).

The Δ*pyrE*-based, Δ*pyrF*-based, and Δ*upp*-based counterselection relies on the construction of an uracil auxotrophic strain. The *pyrE* and *pyrF* genes encode an

orotate phosphoribosyltransferase and an orotidine 5'-phosphate decarboxylase, respectiviely, which are part of the pyrimidine biosynthetic pathway. These enzymes also catalyze the conversion of 5-fluoroorotic acid (5-FOA) into 5-fluorouracil (5-FU), which is a highly toxic compound (Heap et al., 2012; Tripathi et al., 2010). Both Δ*pyrE* and Δ*pyrF* strains need uracil supplementation for growth and are 5-FOA resistant. However, the uracil auxotrophy can be restored to prototrophy via the expression of *pyrF* from a plasmid, providing a positive selection. The counterselection compound 5-FOA can be used to select against plasmid-expressed *pyrF*, providing a negative selection (Tripathi et al., 2010). Similarly, the *upp* gene encodes a uracil phosphoribosyltransferase, which also participates in the pyrimidine salvage pathway. This enzyme participates in the metabolism of the pyrimidine analog 5-FU (Dong & Zhang, 2014). Therefore, Δ*upp* mutants also require uracil supplementation and are 5-FU resistant. The combination of a uracil-deficient mutant with the mentioned counterselection markers and counterselective compounds (5-FOA or 5-FU) allowed the development of these counterselection systems. These systems were initially used for genetic modifications in *A. thermocellus* (*C. thermocellum*) (Tripathi et al., 2010) and *C. acetobutylicum* (Heap et al., 2012; Soucaille et al., 2008), but they are broadly applicable to other clostridia.

Contrary to the Δ*pyrE*-, Δ*pyrF*- or Δ*upp*-based systems, the *codBA*-based counterselection system does not require any previous deletion in the target host. The *codBA* system is based on the presence of a *codBA* cassette in the deletion plasmid and the use of 5-fluorocytosine (5-FC) as the counterselective compound (Kostner et al., 2013). The *codB* gene codes for cytosine permease and *codA* for cytosine deaminase, enzymes involved in cytosine uptake and utilization, respectively (Danielsen et al., 1992). CodA is responsible for the conversion of 5-FC to 5-FU (Tiraby et al., 1998). Therefore, in the presence of 5-FC wild-type cells that have lost the plasmid containing the counterselection cassette, as well as cells that have undergone the second homologous recombination leading to the desired mutation will grow. The *codBA* system has already been used by our group to generate markerless mutants of *C. saccharobutylicum* (Huang et al., 2018).

1.4.4. Introduction of DNA into cells

The introduction of foreign DNA into clostridial cells can be achieved via conjugation or electroporation (Foulquier et al., 2019; Huang et al., 2018; Lesiak et al., 2014; Pyne et al., 2013). In conjugation, genetic material is transferred directly from one bacterial cell to another after a bridge-like connection was established via a pilus (Gram-negative bacteria) or by the action of an adhesin (Gram-positive bacteria) (Liu et al., 2022). During conjugation the donor cell mobilizes a genetic element (plasmid or transposon) (Koraimann & Wagner, 2014). After nicking the mobilizable plasmid, the recipient cell receives a protein-ssDNA complex and synthesizes a complementary strand to produce a double-stranded circular plasmid (Liu et al., 2022). Although it is a relatively time-consuming method, conjugation allows the transfer of relatively large amounts of genetic material with minimal disruption of the cells. For instance, Lauer and co-workers were able to transfer a 17.9-kb gene cluster into *C. saccharobutylicum* using conjugation as the method (Lauer et al., 2022).

On the other hand, electroporation is a technique that uses an electric field at around 5–10 kV/cm for 5–10 μs presumably to generate pores in bacterial membranes, allowing the entry of the exogenous DNA molecule (Aune & Aachmann, 2010). This method is divided into two steps: the preparation of electro-competent cells (generally using sucrose treatment to provide osmoprotection) and the application of an electrical pulse (Pyne et al., 2013). After the electric pulse, cells are cultivated in a recovery medium, with further cultivation on a selective medium. *C. acetobutylicum* and *C. perfringens* were the first clostridial organisms transformed via electroporation (Allen & Blaschek, 1988; Oultram et al., 1988). Since then, other electroporation protocols have been published for other clostridia, such as for *C. pasteurianum* (Pyne et al., 2013) and *Clostridium* sp. AWRP (Kwon et al., 2024).

1.4.5. Restriction/modification (RM) systems

Bacteria developed multiple defense mechanisms to prevent phage infection, including modifications at their surface to avoid phage absorption, the CRISPR-Cas systems, and the restriction/modification systems (RM systems) (Van Houte et al., 2016).

In the early 1950s, the phenomenon of restriction and modification of DNA was observed for the first time (Bertani & Weigle, 1953; Luria & Human, 1952). Only 10 years later a molecular explanation for this was presented. While studying the growth of λ phages in *E. coli* strains, Arber and Linn observed that λ phages grown in one *E. coli* strain and then used to infect another *E. coli* strain produced fewer plaques. The *E. coli* strains that limited the growth of the phage were said to "restrict" the phage by a sequence-specific endonuclease that cuts the infecting λ DNA. The group also observed that λ phages taken from formed plaques could infect a new host strain and lead to the formation of a large number of plaques. Such phages were described as "modified" by the growth in the bacterial strain caused by sequence-specific methylation of the phage DNA (Arber & Linn, 1969).

A typical RM system is comprised of a restriction endonuclease (REase) and a methyltransferase (MTase) (Tock & Dryden, 2005). While the REase cleaves double-stranded DNA into fragments, the MTase adds methyl groups on the host's genome to ensure discrimination between self and non-self DNA, and therefore prevents the destruction of own DNA (Vasu & Nagaraja, 2013). Despite their importance in protecting the cells, RM is not essential for bacterial life (Thomas & Nielsen, 2005; Vasu & Nagaraja, 2013). RM systems have been classified into four groups according to enzyme composition, cofactor requirements, subunit organization, recognition sequence symmetry, and cleavage site of the enzyme (Roberts et al., 2003).

The enzymes from the type I RM systems are multisubunit proteins that function as a single protein complex both endonuclease and methyltransferase. They usually contain two restriction subunits, two modification subunits, and one specificity subunit. The cleavage occurs at variable distances from the recognition sequence, resulting in unspecific fragments (Roberts et al., 2003). The type II RM systems are the simplest and the most numerous RM systems. They are formed by individual REase and MTase enzymes encoded by separate genes that act independently. The type II REases recognize specific DNA sequences and cleave at constant positions (Roberts et al., 2003). The type III RM systems are composed of two genes encoding protein subunits that function either in DNA recognition and modification or restriction. Both subunits are required for restriction. The enzymes cleave at a specific distance away from one of the two copies of their recognition sequence (Roberts et al., 2003). The type IV RM

systems are composed of one or two genes encoding proteins that cleave only modified DNA, including methylated bases (Roberts et al., 2003).

1.4.5.1. *C. cellulovorans* RM system

RM systems are often responsible for low transformation efficiencies since the introduced plasmids are recognized as foreign genetic material, representing a strong barrier to metabolic engineering (Minton et al., 2016). An *in silico* analysis of the *C. cellulovorans* genome sequence using the online database REBASE (The Restriction Enzyme Database) predicted 12 theoretical restriction systems. These RM systems are subdivided into type I (n = 1), type II (n = 8), type III (n = 1), and type IV (n = 2) restriction systems (Yang et al., 2016). However, only 4 operons encode simultaneously a restriction endonuclease and methyltransferase, which according to the literature is the most abundant RM system found in nature (Roberts et al., 2015).

To date, the only characterization of *C. cellulovorans* RM system was performed by Yang and co-workers (Yang et al., 2016). The authors identified two RM systems, namely Cce743I and Cce743II, and established a system for plasmid *in vivo* methylation. These findings contributed to the construction of a *C. cellulovorans* strain able to produce butanol from crystalline cellulose using expression plasmids, but no chromosomal modification was achieved (Yang et al., 2016).

1.5 Thesis motivation

The increasing concern about global warming and climate change led to an increase in the interest in producing chemicals and fuels from renewable resources. In this context, biobutanol has been considered an interesting bulk chemical for the production of a large variety of chemicals. Additionally, due to its similarity to gasoline, it could be used as biofuel. Butanol can be produced by fermentation or petrochemically. The biotechnological production of butanol is achieved through ABE fermentation. However, one important aspect of ABE fermentation is the type of feedstock to be used. To avoid competition with edible feedstocks, studies regarding the production of biofuel have been focusing on the utilization the lignocellulosic material. Nevertheless, due to the complexity of lignocellulosic biomass, multiple treatment steps are required, which increases costs and the release CO_2 into the atmosphere. To overcome this, the co-culture of different cellulolytic and solventogenic

bacteria has been considered a promising strategy. In terms of cellulolytic microorganisms, *C. cellulovorans* is a strong candidate, due to the production of many cellulases able to degrade and utilize efficiently the cellulose present in the lignocellulosic biomass. However, synthetic co-cultures tend to be unstable and single members might get lost during cultivation. Engineering the strains to be metabolically dependent on each other can provide a solution. Unfortunately, although genetic tools to manipulate the solventogenic clostridia are now available, there is still a lack of tools for the genetic engineering of *C. cellulovorans*. In the present study, it was aimed to expand the spectrum of tools for the genetic engineering of *C. cellulovorans* and to obtain a strain of *C. cellulovorans* that will be part of a synthetic co-culture with a solventogenic *Clostridium* spp., which in combination are able to convert cellulose to butanol at high yield.

1.6 Aim of this work

This work aimed to construct *C. cellulovorans* strains suited as part of a synthetic consortium for the continuous production of n-butanol at high yield using engineered clostridia. Strains of *C. cellulovorans* should be contructed that are metabolically dependent on an acetate-producing clostridial strain. For that, the *C. cellulovorans* strains should only grow and produce butyrate from cellulose in the presence of acetate, which is to be provided by the solventogenic *Clostridium* spp., and on the other hand to supply the solventogenic clostridia with butyrate, which will be further converted to butanol. For that, the specific objectives were as follows:

- To improve the fundamental genetic tools needed to follow synthetic biology strategies with the host bacterium *C. cellulovorans*. This includes
  - the identification of suitable Gram-positive replicons,
  - the identification of an appropriate “pseudo-suicide” replicon,
  - the construction of a restriction-less mutant of *C. cellulovorans.*

- To engineer the metabolism of *C. cellulovovorans* by
  - replacement of *ptb-buk* genes by a *cat1* gene from *C. tyrobutyricum* to couple butyrate formation to acetate re-consumption to produce acetyl-CoA,
  - deletion of the *hydA* gene to obtain a strain unable to produce hydrogen and able to re-oxidize the NADH produced in the glycolytic pathway,
  - deletion of the *pta-ack* operon so that the strain can no longer produce acetate.

2. Material and methods

2.1. Microorganisms, media and culture conditions

2.1.1. Strains

The strains used in this study are listed in Table 2.1.

Table 2.1 | Bacterial strains used in this study.

Strain	Genotype and relevant characteristics	Source/reference
C. cellulovorans 743B	Wild type (WT), DSM 3052	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
C. cellulovorans 743B	WT, ATCC 35296	American Type Culture Collection (ATCC)
C. cellulovorans/82151	*C. cellulovorans* (DSMZ) with plasmid pMTL82151	This study
C. cellulovorans/83151	*C. cellulovorans* (DSMZ) with plasmid pMTL83151	This study
C. cellulovorans/87151	*C. cellulovorans* (DSMZ) with plasmid pMTL87151	This study
C. cellulovorans/88151	*C. cellulovorans* (DSMZ) with plasmid pMTL88151	This study
C. cellulovorans/89151	*C. cellulovorans* (DSMZ) with plasmid pMTL89151	This study
C. cellulovorans Δ1114	*C. cellulovorans* (DSMZ) with the locus Clocel_1114 deleted	This study
C. cellulovorans Δ2651	*C. cellulovorans* (DSMZ) with the loci Clocel_1114 and Clocel_2651 deleted	Zenz (2023)
C. cellulovorans Δ1114 Δ2651	*C. cellulovorans* (DSMZ) with the locus Clocel_2651 deleted	This study
C. cellulovorans Δ1114 Δptb-buk::cat1	*C. cellulovorans Δ1114* with the operon *ptb-buk* deleted and replaced by the *cat1* gene from *C. tyrobutyricum*	This study

Strain	Genotype and relevant characteristics	Source/reference
C. cellulovorans Δ1114 Δpta	*C. cellulovorans Δ1114* with the gene *pta* deleted	Kimadze, (2024)
C. tyrobutyricum G33R	WT	DSMZ
E. coli 10-beta NEB® Competent (High Efficiency)	*Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1 Δ(mrr-hsdRMS-mcrBC)*	New England BioLabs GmbH (NEB, Frankfurt am Main, Germany)
E. coli CA434	*E. coli* HB101 carrying the IncPβ conjugative plasmid, R702	Purdy et al. (2002)
Latilactobacillus curvatus TMW 1.2406	Prophage-free strain	In-house strain collection
Lat. curvatus TMW 1.591	Strain containing prophage	In-house strain collection

2.1.2. Media and supplements

2.1.2.1. 520: *Ruminoclostridium cellulolyticum* (cm3) medium

520 medium was prepared according to the DSMZ instructions (Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ, Braunschweig, Germany) with minor modifications. All components were dissolved in distilled water (dH_2O) to a final volume of 926 mL (Table 2.2). The medium was adjusted to pH 6.0 (the pH was set using 6 M HCl solution) and sterilized by autoclaving. Before use, the medium was supplemented with solutions filter-sterilized using a 0.2 µm pore size filter (Sartorius Minisart™ Plus, Sartorius, Göttingen, Germany). As carbon source, if not otherwise mentioned, 5 g/L D(+)-cellobiose was used.

The medium was sparged for 30–45 minutes with 100 % N_2 atmosphere for anaerobiosis and the final pH was 7.0–7.2. Solid media was prepared by adding 1 % agar (Carl Roth GmbH, Karlsruhe, Germany) to the liquid media before autoclaving. After solidification, the plates were transferred to an anaerobic tent for anaerobiosis. When required, liquid and solid media were supplemented with 15 µg/mL

thiamphenicol (if not mentioned otherwise). More details about the solutions used for the preparation of the medium can be found in 2.1.2.9.

Table 2.2 | 520: *Ruminoclostridium cellulolyticum* (cm3) medium composition.

Component	Amount	Source
$FeSO_4 \cdot 7H_2O$ (0.1 % w/v in 0.1 N H_2SO_4) solution	1.25 mL	Merck KGaA, Darmstadt, Germany
Na-resazurin (0.1 % w/v)	0.50 mL	Sigma Aldrich Chemie GmbH, Steinheim, Germany
$MgCl_2 \cdot 6H_2O$ (1 M) solution	0.98 mL	Merck KgaA
$CaCl_2 \cdot 2H_2O$ (1M) solution	0.51 mL	Alfa Aesar, Massachusetts, USA
10 X NKK solution	100 mL	Self-made
SL-10 trace elements solution	1 mL	Self-made
Yeast extract	2 g	Carl Roth GmbH
dH_2O	926 mL	
Solutions added after autoclaving:		
L-cysteine-$HCl \cdot H_2O$ (5 %, w/v) solution	10 mL	Carl Roth GmbH
D(+)-cellobiose (10 %, w/v) solution	50 mL	Carl Roth GmbH
Na_2CO_3 (1 M) solution	14 mL	Merck KgaA

2.1.2.2. 104c: PY + X medium

104 c PY + X medium was prepared according to the DSMZ instructions. The medium components can be found in Table 2.3. Before autoclaving the pH was adjusted to 7. The medium was sparged with 100 % N_2 for 30–45 minutes. More details about the salt solution used for the preparation of the medium can be found in 2.1.2.9.

Table 2.3 | 104c: PY + X medium composition.

Component	Amount	Source
Trypticase peptone	5 g	Carl Roth GmbH
Meat peptone	5 g	Carl Roth GmbH
Yeast extract	10 g	Carl Roth GmbH
Salt solution	40 mL	Self-made
Na-resazurin (0.1 % w/v)	0.50 mL	Sigma Aldrich Chemie
Na_2CO_3	1 g	Merck KgaA
dH_2O	910 mL	
L-cysteine-HCl·H_2O	0.5 g	Carl Roth GmbH
Solution added after autoclaving:		
D(+)-glucose monohydrate (10 %, w/v) solution	50 mL	Carl Roth GmbH

2.1.2.3. Modified AEA sporulation medium

Modified AEA sporulation medium (17170 AEA sporulation broth, base, modified) was prepared according to the Sigma recipe. The medium components can be found in Table 2.4. Solid media was prepared by adding 1 % agar to the liquid media before autoclaving.

Table 2.4 | Modified AEA sporulation medium composition.

Component	Amount	Source
Biopeptone	10 g	Carl Roth GmbH
Yeast extract	10 g	Carl Roth GmbH
Na_2HPO_4	4.36 g	PanReac AppliChem
$KHSO_4·7H_2O$	0.25 g	Sigma Aldrich Chemie

Component	Amount	Source
$NH_4CH_3CO_2$	1.5 g	Carl Roth GmbH
$MgSO_4 \cdot 7H_2O$	0.2 g	Merck KgaA
dH_2O	936.32 mL	
Solution added after autoclaving:		
Na_2CO_3 (1 M) solution	35.08 mL	Merck KgaA
$Cl_2Co \cdot 6H_2O$ (0.32 %, w/v) solution	13.28 mL	Sigma Aldrich Chemie
$C_6H_7NaO_6$ solution (1.5 %, w/v)	5.32 mL	Sigma Aldrich Chemie
D(+)-cellobiose (10 %, w/v) solution	10 mL	Carl Roth GmbH

2.1.2.4. Defined minimal media

Defined minimal media (DMM) was prepared as described before (Mearls, 2013) with minor modifications. The medium components can be found in Table 2.5. The pH was adjusted to 7.2. Solid media was prepared by adding 1 % agar to the liquid media before autoclaving.

Table 2.5 | Defined minimal media.

Component	Amount	Source
KH_2PO_4	1.04 g	Merck KgaA
K_2HPO_4	1.11 g	PanReac AppliChem
$NaHCO_3$	2.5 g	J.T. Baker Chemical, Deventer, Holland
$MgCl_2 \cdot 6H_2O$	0.2 g	Merck KgaA
NH_4Cl	0.4 g	PanReac AppliChem
$CaCl_2 \cdot 2H_2O$	0.05 g	Alfa Aesar
Na-resazurin (0.1 % w/v) solution	0.5 mL	Sigma Aldrich Chemie GmbH

Component	Amount	Source
dH_2O	988 mL	
Solution added after autoclaving:		
L-cysteine-HCl·H_2O (5 %, w/v) solution	2.5 mL	Carl Roth GmbH
D(+)-cellobiose (10 %, w/v) solution	9 mL	Carl Roth GmbH

2.1.2.5. Luria-Bertani medium

Luria-Bertani (LB) medium was prepared according to Sambrook and co-workers (1989). The medium components can be found in Table 2.6. The pH was measured and adjusted to 7.0–7.4 using 1 M NaOH if necessary. LB agar plates were prepared by adding 1 % agar into the liquid medium before autoclaving. When required, the medium was supplemented with 25 µg/mL chloramphenicol and/or 12 µg/mL tetracycline.

Table 2.6 | Luria-Bertani medium composition.

Component	Amount	Source
Tryptone	10 g	Carl Roth GmbH
Yeast extract	5 g	Carl Roth GmbH
NaCl	10 g	Carl Roth GmbH
dH_2O	1000 mL	

2.1.2.6. Super optimal broth with catabolite repression medium

Super optimal broth with catabolite repression (SOC) medium was prepared according to Cold Spring Harbor Protocols (2018). The medium components can be found in Table 2.7. The pH was adjusted to 7.0 using 1 M NaOH if necessary. After autoclaving, the medium was kept at 4 ºC until use. 1 M $MgCl_2 \cdot 6H_2O$ solution and 2M D(+)-glucose monohydrate solution were added right before use. More details about the solutions used for the preparation of the medium can be found in 2.1.2.9.

Table 2.7 | Super optimal broth with catabolite repression medium composition.

Component	Amount	Source
Tryptone	20 g	Carl Roth GmbH
Yeast extract	5 g	Carl Roth GmbH
NaCl	0.5 g	Carl Roth GmbH
KCl	0.18 g	Baker Hughes Chemicals GmbH, Celle, Germany
dH_2O	980 mL	
Solution added after autoclaving:		
D(+)-glucose monohydrate (2M) solution	10 mL	Carl Roth GmbH
$MgCl_2 \cdot 6H_2O$ (1 M) solution	10 mL	Merck KgaA

2.1.2.7 Modified De Man, Rogosa and Sharpe Medium (mMRS)

Modified De Man, Rogosa and Sharpe Medium (mMRS) was prepared as described previously (Ambros & Ehrmann, 2022). The medium components can be found in Table 2.8. The pH was adjusted to 6.2–6.5 with 6 M HCl before autoclaving. Solid media was prepared by adding 1.5 % agar-agar to the liquid media before autoclaving.

Table 2.8 | Modified De Man, Rogosa and Sharpe medium composition.

Component	Amount	Source
Trypton/pepton from casein	10 g	Carl Roth GmbH
Meat extract	10 g	Carl Roth GmbH
Yeast extract	5 g	Carl Roth GmbH
$K_2HPO_4 \cdot 3H_2O$	6 g	Carl Roth GmbH
$C_2H_3NaO_2 \cdot 3H_2O$	28.3 g	Merck KGaA
$C_6H_{14}N_2O_7$	2 g	Merck KGaA

Component	Amount	Source
MgSO4·7H2O solution (10 %, w/v)	2 mL	Merck KGaA
MnSO4·H2O solution (10 %, w/v)	0.5 mL	Merck KGaA
Tween 80®	1 mL	PanReac AppliChem
dH_2O	946.5 mL	
Solution added after autoclaving:		
D(+)-glucose monohydrate solution (10 %, w/v)	50 mL	Carl Roth GmbH

2.1.2.8. Antibiotics and additives

Thermosensitive solutions, such as vitamins and antibiotics, were added in the autoclaved medium after colling to approximately 50 ºC. Antibiotics and additives used in this study are listed in Table 2.9. Antibiotic solutions prepared in 70 % or above ethanol (EtOH, AppliChem GmbH) were used without prior filtration. EtOH solutions with different concentrations were prepared using ethanol absolute for molecular biology (AppliChem). Antibiotic solutions were aliquoted to avoid repetitive freeze/thaw cycles and stored at –20 °C. 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU) were freshly prepared for each for each usage.

Table 2.9 | Antibiotics stock solutions and additives.

Antibiotics and additives	Stock solution concentration	Working concentration	Solvent	Source
Chloramphenicol	25 mg/mL	25 µL/mL	95 % EtOH (v/v)	SERVA Electrophoresis GmbH, Heidelberg, Germany
Thiamphenicol	10 mg/mL	15 µL/mL**	50 % EtOH (v/v)	Sigma Aldrich Chemie GmbH
Tetracycline*	12 mg/mL	12 µL/mL	70 % EtOH (v/v)	Merck KGaA
5-fluorocytosine (5-FC)*,***	10 mg/mL	250 µL/mL	dH_2O	TCI, Tokyo, Japan

Antibiotics and additives	Stock solution concentration	Working concentration	Solvent	Source
5-fluorouracil (5-FU)***	10 mg/mL	50 µL/mL	dH_2O	J&K Scientific, Beijing, China
Adenosine	75 mM	0.1 mM	DMSO	PanReac AppliChem, ITW Reagents, Darmstadt, Germany
Guanosine	75 mM	0.1 mM	DMSO	PanReac AppliChem
Mitomycin C	2 mg/mL	20 µg/mL **	dH_2O	Sigma Aldrich

*Light sensitive, ** different concentrations were required depending on the experiment, ***short incubation at 37 ºC to facilitate solubilization.

2.1.2.9. Sugar solutions

10 % stock solution (w/v) of D(+)-cellobiose (Carl Roth GmbH) was prepared using dH_2O. The solution was stirred with heating until completely solubilized. Although the heating, no change in the color was observed, indicating no sugar degradation. A 10 % stock solution (w/v) of D(+)-glucose monohydrate (Carl Roth GmbH) was prepared using dH_2O and stirred at room temperature. 2 M D(+)-glucose was prepared by dissolving 36.03 g D(+)-glucose monohydrate in 100 mL dH_2O. A 44 % stock solution (w/v) of D-(+)-glucose monohydrate was prepared using dH_2O and stirred at room temperature. 8 % (w/v) stock solution of L(+)-arabinose was prepared using dH_2O. 50 % stock solution (w/v) of sucrose was prepared using dH_2O. All sugar solutions were filter-sterilized before use.

2.1.2.10. Phosphoric acid swollen cellulose (PASC)

Phosphoric acid swollen cellulose (PASC) was prepared as described by Zhang and co-workers (Zhang et al., 2006). Briefly, 0.2 g of microcrystalline cellulose (Avicel®) was added to a 50 mL centrifuge tube, and 600 µL dH_2O was added to obtain a cellulose-suspended slurry. 10 mL ice-cold 86.2 % H_3PO_4 (v/v) was slowly added to the slurry with vigorous stirring. Before the addition of the last 2 mL of phosphoric acid (H_3PO_4), the cellulose solution was mixed evenly. After turning transparent, the cellulose solution was maintained on ice for 1 h with occasional stirring. Then, 40 mL ice-cold dH_2O was added, in 10 mL portions with vigorous stirring between additions, generating a white precipitate. The precipitate was centrifuged at 5.000 g, 4 ºC for 20

min. The pellet was resuspended in 40 mL ice-cold water and centrifuged at 5.000 g, 4 ºC for 20 min. This step was repeated four times for the complete removal of H_3PO_4. After, 500 µL of 2 M Na_2CO_3 and 45 mL of ice-cold dH_2O were added to the pellet. After centrifugation, the pH of the supernatant was measured (pH 5–7). The pellet was resuspended in 1 mL dH_2O. All the preparation was performed in a 4 ºC room. 1 mL of the solution was dried using the SpeedVac and the PASC concentration (%) was calculated using the following equation:

$$\text{Concentration (\%)} = \frac{\text{sample weight dry} - \text{tube weight}}{\text{sample weight wet} - \text{tube weight}} \text{ x } 100$$

2.1.2.11. General solutions

10 X NKK solution

10 X NKK solution was prepared by adding 13 g of $(NH_4)_2SO_4$ (PanReac AppliChem), 15 g of KH_2PO_4 (Merck KGaA), 29 g of K_2HPO_4 (PanReac AppliChem) in 1000 mL dH_2O.

Trace elements solution

Trace elements solution was obtained by adding 10 mL of HCl (25 %, v/v, PanReac AppliChem) solution, 1.5 g of $FeCl_2 \cdot 4H_2O$ (Alfa Aesar), 70 mg of $ZnCl_2$ (Sigma Aldrich Chemie GmbH), 100 mg of $MnCl_2 \cdot 4H_2O$ (Alfa Aesar), 6 mg of H_3BO_3 (PanReac AppliChem), 190 mg of $CoCl_2 \cdot 6H_2O$ (Merck KGaA), 2 mg of $CuCl_2 \cdot 2H_2O$ (Alfa Aesar), 24 mg of $NiCl_2 \cdot 6H_2O$ (Sigma Aldrich Chemie GmbH), and 36 mg of $Na_2MoO_4 \cdot 2H_2O$ (Merck KgaA) in 990 mL dH_2O 0.1 % (w/v).

Na-resazurin solution

Na-resazurin solution was prepared by adding 10 mg of Na-resazurin (Sigma Aldrich Chemie GmbH) in 10 mL of dH_2O. The solution was mixed, filter-sterilized, aliquoted in 2 mL portions, and stored at –20 ºC.

L-cysteine-HCl·H_2O solution

5 % (w/v) L-cysteine-HCl·H_2O solution was freshly prepared by adding 0.75 g of L-cysteine-HCl·H_2O (Carl Roth GmbH) in 15 mL dH_2O. 0.5 %, w/v was prepared by adding 0.075 g of L-cysteine-HCl·H_2O in 15 mL dH_2O. The solutions were filter-sterilized before use.

$MgCl_2 \cdot 6H_2O$ solutions

1M $MgCl_2 \cdot 6H_2O$ solution was prepared by adding 20.33 g of $MgCl_2 \cdot 6H_2O$ (Merck KgaA) in 100 mL of dH_2O. 2 M $MgCl_2 \cdot 6H_2O$ solution was prepared by 40.66 g of $MgCl_2 \cdot 6H_2O$ in 100 mL of dH_2O. The solutions were sterilized by autoclaving.

$CaCl_2 \cdot 2H_2O$ solution

1M $CaCl_2 \cdot 2H_2O$ solution was prepared by adding 14.70 g $CaCl_2 \cdot 2H_2O$ (Alfa Aesar) in 100 mL of dH_2O. The solution was sterilized by autoclaving.

$FeSO_4 \cdot 7H_2O$ solution

0.1 % (w/v) $FeSO_4 \cdot 7H_2O$ solution was prepared by adding 0.1 g $FeSO_4 \cdot 7H_2O$ to 100 mL 0.1 N H_2SO_4. 1 N H_2SO_4 solution was prepared by adding 1.37 mL 97 % H_2SO_4 (Merck KGaA) in 48.63 mL dH_2O. A 1:10 was performed to obtain 0.1 N H_2SO_4.

Na_2CO_3 solution

1 M Na_2CO_3 solution was prepared by adding 52.99 g of Na_2CO_3 (Merck KgaA) in 500 mL of dH_2O. The solution was aliquoted in 50 mL portions and transferred to anaerobic serum bottles. The solution was purged 100 % CO_2 atmosphere and sealed with butyl rubber stoppers (Glasgerätebau Ochs, Bovenden, Germany) and metal caps (Glasgerätebau Ochs, Bovenden, Germany). The solution was sterilized by autoclaving. 2 M Na_2CO_3 solution was prepared by adding 10.59 g of Na_2CO_3 in 100 mL of dH_2O.

$MgSO_4 \cdot 7H_2O$ solution

10 % (w/v) $MgSO_4 \cdot 7H_2O$ solution was prepared by adding 1 g of MgSO4·7H2O in 10 mL dH_2O.

$MnSO_4 \cdot H_2O$ solution

10 % (w/v) $MnSO_4 \cdot H_2O$ solution was prepared by adding 1 g of $MnSO_4 \cdot H_2O$ in 10 mL dH_2O.

Salt solution for 104c medium

Salt solution for 104c medium was prepared by dissolving 0.25 g $CaCl_2 \cdot 2H_2O$, 0.5 g MgSO4·7H2O, 1 g K_2HPO_4, 1 g KH_2PO_4, 10 g $NaHCO_3$, 2 g NaCl in 1000 mL dH_2O.

0.9 % saline solution

0.9 % saline solution (w/v) was prepared by dissolving 9 g NaCl in 1000 mL dH_2O. The solution was autoclaved.

7 vitamins solution

7 vitamins solution was prepared by dissolving 10 mg Vitamin B12, 80 mg p-aminobenzoic acid, 20 mg D-(+) biotin, 200 mg nicotinic acid, 100 mg Ca pantothenic acid (vitamin B5), 300 mg pyridoxine hydrochloride and 200 mg thiamine hydrochloride in 1000 mL ddH_2O. The solution was filter-sterilized and stored at 4 ºC.

L-tryptophan solution

L-tryptophan solution was prepared by dissolving 0.1064 g L-tryptophan (Sigma Aldrich Chemie GmbH) in 100 mL dH_2O. The solution was incubated at 40°C and sporadically shaken until completely homogeneous. After filter sterilization, the solution was stored at 4 ºC.

2.1.2.12. Buffers

10 X Phosphate-Buffered Saline (PBS) solution

10 X PBS stock solution was prepared by combining 17.8 g $Na_2HPO_4 \cdot 2H_2O$ (PanReac AppliChem), 2.4 g of KH_2PO_4, 80 g of NaCl, 2 g of KCl for a final volume of 1000 mL ddH_2O (the pH was 6.8, but when diluted to 1x PBS the pH was 7.4). The solution was sterilized by autoclaving.

Tris Buffer (pH 8.0)

1 M Tris-HCl solution was prepared by dissolving 121.1 g Tris base in 800 mL dH_2O. The pH was adjusted to 8 using 6 M HCL. The final volume was adjusted to 1000 mL. The solution was sterilized by autoclaving.

0.5 M EDTA

0.5 M ethylenediaminetetraacetic acid (EDTA) solution was prepared by dissolving 186.12 g EDTA in 800 mL dH_2O. The pH was adjusted to 8 using 1 M NaOH. The final volume was adjusted to 1000 mL. The solution was sterilized by autoclaving.

TE Buffer (pH 8.0)

TE buffer (T10E1 buffer) was prepared by mixing 10 mL 1 M Tris-HCl solution and 2 mL 0.5 M EDTA in 988 mL dH_2O.

2.1.3. Cultivation conditions

2.1.3.1. Conditions for *Clostridium* spp.

C. cellulovorans 743B was cultured under anaerobic conditions. Anaerobic cultivation was done in anaerobic culture tubes (Hungate types, Avantor®, VWR International GmbH, Darmstadt Germany) or anaerobic serum bottles sealed with butyl rubber stoppers under 100 % N_2 atmosphere. Hungates and serum bottles were filled with liquid medium up to 50 % of their capacity at least 1 hour before use. For cultivation on solid media, the plates were maintained in the anaerobic tent (anaerobic vinyl tent, type B, COY Products, Inc., Grass Lake, USA) for at least 2 days before use to ensure complete anaerobic condition. For incubation, agar plates were maintained in a 2.5 L anaerobic jar (AnaeroJar™, Thermo Scientific™, Oxoid™, United Kingdom) containing a 2.5 L sachet (AnaeroGen™, Thermo Scientific™, Oxoid™). If not mentioned otherwise, *C. cellulovorans* was cultivated in 520 medium, containing 2 g/L yeast extract and 5 g/L cellobiose. Cultivation of *C. tyrobutyricum* G33R was performed in the same manner as for *C. cellulovorans*, except that the media used for cultivation was 104c medium.

For growth curve experiments, pre-cultures were prepared by picking one single colony and inoculating in 5 mL 520 medium with incubation at 37 °C, without shaking, overnight (16 h ± 1). The OD_{600nm} was measured and the main culture was inoculated to an initial OD_{600nm} of 0.1. The growth was determined by measuring the optical density (OD_{600nm}, Spectrophotometer Jenway 7310, Staffordshire, United Kingdom), the turbidity using a densitometer (McFarland Densitometer DEN-1B, Grant Instruments™ Grant Bio™, Beaver Falls, USA), pH changes in the medium, and protein concentration. A negative control containing only sterile medium was also included. All experiments were performed with at least three biological replicates and two technical replicates.

To ensure healthy growth, bacterial cultures were checked microscopically. For that, 10 µL culture was used. The samples were analyzed using 100 X immersion objective

under phase contrast (AXIO Scope.A1, Carl Zeiss, Oberköchen, Germany). Images were captured using a camera (AxioCam HRc, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) coupled to the microscope, and analyzed using the software AxioVision Release 4.7.2 (Carl Zeiss Vision, Imaging Systems, Oberköchen, Germany).

2.1.3.2. Conditions for aerobic bacteria

E. coli and *Latilactobacillus curvatus* strains were cultured aerobically in culture tubes (VWR International GmbH). Colonies were kept on solid media for up to four weeks. After that, new plates were prepared from fresh overnight cultures.

For *E. coli* growth curve experiments, 96-well culture plates (sterile, F-bottom, with lid, Greiner Bio-One International GmbH, Frickenhausen, Germany) were used. Pre-cultures were prepared by picking one single colony and inoculating in 5 mL LB medium (with the proper antibiotic), with incubation at 37 °C, at 180 rpm, overnight (16 h ± 1). The OD_{600nm} was measured and 1 mL bacterial suspension for a final OD_{600nm} 1 was prepared in a sterile microcentrifuge tube. Then, the wells were filled with 180 µL medium. The medium was inoculated with 20 µL of bacterial suspension. The plate was covered with a breathseal sealer (Greiner Bio-One International GmbH) and incubated in the microplate reader at 37 ºC, 300 rpm, for 24 h ± 1, with measurement every 30 min. A negative control containing only sterile medium was also included. All experiments were performed with at least three biological replicates and six technical replicates.

For prophage induction using mitomycin C (MMC), pre-cultures of *Lat. curvatus* were prepared by picking one single colony and inoculating in 5 mL mMRS, with incubation at 30 °C, static, overnight (16 h ± 1). After incubation, the OD_{600nm} was measured and the main culture was inoculated to an initial OD_{600nm} of 0.05. After reaching an OD of 0.1, MMC was added for a final concentration of 20 µg/mL.

2.1.4. Glycerol stocks for aerobic bacteria

50 % glycerol solution (v/v) was prepared by diluting 86 % glycerol (PanReac AppliChem) in dH_2O. The solution was sterilized by filtration. To prepare the stock, 500 µL glycerol solution and 500 µL overnight culture were transferred into a 2 mL screw

top tube. Before freezing at –80 °C, the sample was gently mixed 5-6 times to obtain a homogeneous suspension.

For the stock re-activation, a sterile inoculation loop was used to scrape off bacteria from the frozen top of the stock, with further inoculation on agar plate or 5 mL in liquid medium. When liquid medium was used, the culture was subsequently streaked on agar plate to obtain a single colony.

2.1.5. Glycerol stocks for anaerobic bacteria

The preservation of *Clostridium* spp. was performed as described by Schneider (2024). First, 12.5 mg L-cysteine-HCl·H_2O was dissolved in 25 mL ddH_2O. The pH was adjusted to 7 using 1 M NaOH. After, 86 % glycerol was diluted in the cysteine solution to obtain a final concentration of 50 % (v/v). 0.5 mg Na-resazurin was dissolved in 50 mL 50 % (v/v) glycerol solution. Then, the solution was distributed in 2 mL portions in Hungate tubes. The tubes were kept under an oxygen-free vacuum environment in the airlock of the anaerobic tent for 30 min to remove oxygen. After that, the tubes are closed inside the anaerobic tent and then autoclaved.

To prepare the anaerobic glycerol stock, 2 mL of overnight culture were transferred to the Hungate tubes containing 50 % glycerol solution (v/v). The mixture was gently mixed to ensure homogeneity and the tubes were immediately transferred to –80 °C. For the stock re-activation, the samples were thawed on ice. After, 2 mL stock was inoculated in 5 mL medium (2 duplicates were prepared per sample). The suspension was incubated at 37 ºC overnight (passage 1 = P1). A second culture using 0.5 mL of the previous culture was used to inoculate a new culture (P2) and ensure that the strain was fully re-activated. Finally, the culture was streaked on solid plates for further use.

2.1.6. Sporulation

2.1.6.1. Cultivation

C. cellulovorans was cultivated on agar plates with several compositions. 520 medium (containing yeast extract, here called 520 2Y) was used as a control. The yeast extract was replaced by casamino acids (520 2Cas), tryptone (520 2T), or tryptone carbohydrates free (520 2TCF). All media were prepared with different cellobiose

concentrations (0.05 %, 0.5 % and 1 %). Additionally, the DMM and modified AEA sporulation media were used. One single colony was streaked on the agar plates and the plates were incubated for 5–15 days at 37 ºC before harvesting.

2.1.6.2. Endospores purification and pasteurization

After incubation, the biomass present on the agar plate was collected and purified as described before (Weldy et al., 2020), with minor modifications. Using an inoculation loop the biomass was collected and resuspended in a microcentrifuge tube containing 2 mL sterile ddH_2O. After, the sample was centrifuged at 11.000 rpm for 2 min. The pellet was washed twice with 2 mL ddH_2O. Then, the pellet was resuspended in 1.5 mL ddH_2O. Samples were incubated at 4 ºC for 24 h up to one week to facilitate endospore release from the mother cell. Then, endospores were purified using a sucrose gradient. Briefly, 1.5 mL of spore suspension was carefully layered onto the top of 10 mL 50 % sucrose solution (w/v). The sample was centrifuged at 3.600 rpm for 20 min at 4 °C to separate the cell debris from the spores. After centrifugation, the upper cell debris layer on the top and the sucrose solution were carefully removed. The pellet was resuspended in 500 µL ddH_2O and transferred to a microcentrifuge tube. The suspension was vortexed and centrifuged at 14.000 g for 5 min at room temperature. After, the pellet was washed 5 X with 1 mL ddH_2O. After the final wash, the pellet was resuspended in 1 mL ddH_2O. Then, 250 µL of the spore suspension was transferred to 5 mL 520 medium. Per condition, 4 cultures were prepared since 4 different heat treatments were applied (80 ºC, 1 min; 80 ºC, 2 min; 80 ºC, 11 min). After, the samples were cooled at room temperature, with further incubation at 37 ºC. All procedures were performed in the anaerobic condition, except the centrifugation steps.

2.1.6.3. Spores staining

The smear was prepared by placing 10 µL of spore suspension in the center of a slide for microscopy. The smear was air-dried and heat-fixed. After, the slide was placed on a staining rack over a bath of steaming water. The sample was saturated with 5 % malachite green solution (w/v) and steam-heated for 5 minutes (when required, more dye was added). The excess dye was carefully removed and the sample was cooled for 2 minutes. After, the sample was vigorously washed with dH_2O on both sides (avoiding placing the flush of water directly on the smear). Then, the sample was

counterstained with safranin (Gram's safranine solution ready-to-use, Merck) for 5 minutes. The sample was washed and air-dried. Later, 10 µL dH_2O was added to the stained smear, and a cover slip was placed. The sample was examined under the oil immersion lens using the bight-field microscope.

2.1.7. Prophage induction in *C. cellulovorans*

For prophage induction, *C. cellulovorans* overnight cultures were used to prepare main cultures with a starting OD_{600nm} of 0.1. The cultures were incubated at 37 ºC until OD_{600nm} of 0.2, 0.5, and 1 was reached. After, the MMC to a final concentration of 0, 0.3,3, and 20 µL/mL was added. At 1-hour intervals, the cultures were monitored to determine cell lysis. *L. curvatus* TMW 1.2406 and *L. curvatus* TMW 1.5912.1.3.2 were used as negative and positive controls, respectively, for prophage induction MMC.

2.2. Molecular biology

2.2.1. Plasmids

The plasmids used in this study can be found in Table 2.10.

Table 2.10 | Plasmids used in this study. MCS stands for multiple cloning site.

Plasmid	Relevant characteristics	Reference
pMTL81151	pCB101, Cm^R, ColE1, *traJ*, MSC	Nigel Minton (unpublished)
pMTL82151	pBP1, Cm^R, ColE1, *traJ*, MSC	Heap et al. (2009)
pMTL83151	pCB102, Cm^R, ColE1, *traJ*, MSC	Heap et al. (2009)
pMTL84151	pCD6, Cm^R, ColE1, *traJ*, MSC	Heap et al. (2009)
pMTL85151	pIM13, Cm^R, ColE1, *traJ*, MSC	Heap et al. (2009)
pMTL87151	pUB110, Cm^R, ColE1, *traJ*, MSC	Nigel Minton (unpublished)
pMTL88151	p19, Cm^R, ColE1, *traJ*, MSC	Nigel Minton (unpublished)

Plasmid	Relevant characteristics	Reference
pMTL89151	pAMβ1, Cm^R, ColE1, *traJ*, MSC	Nigel Minton (unpublished)
pKVM4	pE194(ts), $ErmC^R$, *bla*^{R}, pBR322, *traJ*, MSC	Kostner et al. (2017)
pMTL8x151_pE194(ts)	Derivate from pMTL83151 with the temperature sensitive Gram-replicon pE194(ts)	This study
placORM	Derivate from pACYC184 plasmid, p15A1, Tc^R, Clocel_4007 and Clocel_4008	Aline Schöllkopf (unpublished)
pBAD_myc_his	*araC*, P_{BAD}, Amp^R, pBR322, MCS	Guzman et al. (1995)
pHE1-C2	pIM13, Cm^R, pBR322, *oriT*, *traJ*, P_{fdx} from *C. sporogenes*, *codBA* operon from *C. ljungdahlii*	Holger Edelmann (unpublished)
pMTL83151_codBA	Derivate from pMTL83151 with the P_{fdx} from *C. sporogenes*, *codBA* operon from *C. ljungdahlii* present in the plasmid pHE1-C2	This study
pMTL83151CodBA_araBAD	Derivate from pMTL83151_codBA with the P_{BAD} and the repressor *araC* from the plasmid pBAD_myc_his	This study
pMTL83151codBA_ARAi	Derivate from pMTL83151_codBA with the P_{ptk} and the repressor *araR* from *C. acetobutylicum*	This study
pHE1-B	pIM13, Cm^R, pBR322, *oriT*, *traJ*, P_{clpB} from *S. aureus*, *codBA* operon from *C. ljungdahlii*	Holger Edelmann (unpublished)
pLA_del_ori3	Derivate from pHE1-B, pCB102	This study
pLA_ori7	Derivate from pHE1-B, pUB110	This study
pLA_del_ori8	Derivate from pHE1-B, p19	This study

Plasmid	Relevant characteristics	Reference
pLA_del_4005_ori3	Derivate from pLA_del_ori3 containing homologous flanks to the ORF Clocel_4005	Baumgartner (2023)
pLA_del_4006_ori3	Derivate from pLA_del_ori3 containing homologous flanks to the ORF Clocel_4006	Baumgartner (2023)
pLA_del_2651_ori3	Derivate from pLA_del_ori3 containing homologous flanks to the ORF Clocel_2651	Baumgartner (2023)
pLA_del_1114_ori3	Derivate from pLA_del_ori3 containing homologous flanks to the ORF Clocel_1114	Baumgartner (2023)
pLA_del_4005_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the ORF Clocel_4005	This study
pLA_del_4006_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the ORF Clocel_4006	Zenz (2023)
pLA_del_2651_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the ORF Clocel_2651	Zenz (2023)
pLA_del_1114_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the ORF Clocel_1114	This study
pLA_del_4005_ori8	Derivate from pLA_del_ori8 containing homologous flanks to the ORF Clocel_4005	Baumgartner (2023)
pLA_del_4006_ori8	Derivate from pLA_del_ori8 containing homologous flanks to the ORF Clocel_4006	Baumgartner (2023)
pLA_del_2651_ori8	Derivate from pLA_del_ori8 containing homologous flanks to the ORF Clocel_2651	Baumgartner (2023)
pLA_del_1114_ori8	Derivate from pLA_del_ori8 containing homologous flanks to the ORF Clocel_1114	Baumgartner (2023)
pLA_del_ptbbuk_cat1_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the *ptb-buk* operon in *C. cellulovorans* with the *cat1* gene	This study

Plasmid	Relevant characteristics	Reference
	from *C. tyrobutyricum* in between the flanking region	
pAS11_sgRNA1	pCB102, Cm^R, ColE1, *traJ*, CRISPR-Cas9 nickase	Aline Schöllkopf (unpublished)
pNickase_ptbbuk_cat1_sgRNA1	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_ptbbuk_cat1_ori7	Zenz (2023)
pNickase_ptbbuk_cat1_sgRNA3	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_ptbbuk_cat1_ori7	Zenz (2023)
pLA_del_pta_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the *pta* gene in *C. cellulovorans*	This study
pNickase_pta_sgRNA1	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_pta_ori7	Kimadze (2024)
pNickase_pta_sgRNA3	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_pta_ori7	Kimadze (2024)
pLA_del_hyd_4097_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the ORF Clocel_4097	This study
pNickase_ hyd_4097_sgRNA1	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_hyd_4097_ori7	Kimadze (2024)
pNickase_ hyd_4097_sgRNA2	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_hyd_4097_ori7	Kimadze (2024)
pLA_del_hyd_2243_ori7	Derivate from pLA_del_ori7 containing homologous flanks to the ORF Clocel_2243	This study
pNickase_ hyd_2243_sgRNA1	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_hyd_2243_ori7	Kimadze (2024)

Plasmid	Relevant characteristics	Reference
pNickase_ hyd_2243_sgRNA2	Derivate from pAS11_sgRNA1 containing the repairing sequence present in the pLA_del_hyd_2243_ori7	Kimadze (2024)

2.2.2. Chromosomal DNA isolation from *Clostridium* spp.

Chromosomal DNA of *Clostridium* spp. was isolated using a Master Pure™ Complete DNA and RNA Purification Kit (Epicentre, Biozym Scientific GmbH, Hessisch Oldendorf, Germany) following the manufacturer's instructions with modifications. Briefly, 2 mL of an overnight culture were centrifuged at 11.000 rpm for 1 min and the pellet was resuspended in 1 mL 1 x PBS and centrifuged at 11.000 rpm for 1 min. 950 µL of the supernatant was removed and the bacterial biomass was resuspended in the remaining 50 µL. 10 µL of lysozyme (50 mg/mL, Sigma Aldrich, St. Louis, Missouri, USA) were added to the suspension and the solution was incubated for 30 min at 37 °C. After, 300 µL of the tissue and cell lysis solution (T&C solution) and 1 µL of protein kinase K (50 mg/mL, Carl Roth GmbH) was added. The suspension was incubated at 65 °C, 800 rpm for 15 min. The suspension was cooled down on ice for about 5 min, followed by the addition of 10 µL of RNAse A (10 mg/mL, AppliChem GmbH) and an additional incubation at 37 °C for 30 min. Subsequently, it was incubated on ice for 5 min before the addition of 175 µL of the MPC protein precipitation reagent. The suspension was mixed for 10 s using a vortex. It was centrifuged at 11.000 rpm, 4 °C, for 10 min. After, 500 µL of the supernatant was transferred to a new tube and 500 µL of ice-cold isopropanol (AppliChem GmbH) was added. The solution was mixed by inverting the tube 30–40 times. The sample was again centrifuged at 11000 rpm, 4 °C for 10 min. The resulting pellet was washed with 900 µL 70 % ice-cold ethanol and then centrifuged 11.000 rpm, 4 °C for 2 min. The supernatant was removed, and the DNA pellet was incubated at 37 °C for about 10 min (or until the ethanol had been completely evaporated). The pellet was resuspended in 50 µL of sterile ddH_2O and incubated at room temperature for 30 min. Samples were stored either at 4°C or at –20 ºC for long-term storage.

2.2.3. Chromosomal DNA isolation from *Clostridium* spp. colonies

Chromosomal DNA isolation from *Clostridium* spp. colonies were performed using the Master Pure™ Complete DNA and RNA Purification Kit following the manufacturer's instructions with modifications. The biomass present on an agar plate (approximately the size of a colony) was resuspended in 500 µL 1 x PBS and centrifuged at 11.000 rpm for 1 min. The supernatant was removed and 100 µL T&C lysis solution premixed with 10 µL lysozyme solution (50 mg/L) was added. The suspension was incubated at 37 ºC, 800 rpm for 30 min. After, 100 µL T&C lysis solution premixed with 0.4 µL proteinase K solution (50 mg/L, Carl Roth GmbH) was added and the sample was incubated at 65 ºC, 800 rpm for 15 min. The suspension was cooled down on ice for about 5 min. Then, 120 µL MPC protein precipitation reagent was added, and the sample was mixed by vertexing. The sample was centrifuged at 11.00 rpm, 4ºC, for 10 min. 250 µL of the supernatant was transferred to a new tube and 250 µL of ice-cold isopropanol was added. The solution was mixed by inverting the tube 20–30 times, followed by centrifugation at 11.00 rpm, 4ºC, for 10 min. The supernatant was discarded, and the pellet was washed twice with 500 µL 70 % ice-cold ethanol. The ethanol was removed, and the DNA pellet was incubated at 37 °C for about 10 min (or until the ethanol had been completely evaporated). After, the DNA pellet was resuspended in 30 µL of sterile ddH_2O and incubated at room temperature for 30 min. Samples were stored either at 4 °C or at –20 ºC for long-term storage.

2.2.4. Plasmid isolation from *E. coli*

For isolation of plasmids from *E. coli* the the NucleoSpin® Plasmid DNA purification kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) was used following the manufacturer's instructions with minor modifications. Briefly, 4 mL of the *E. coli* overnight culture was centrifuged at 11.000 rpm for 30 s. The supernatant was removed, and the pellet was resuspended in 500 µL A1 buffer. Then, 500 µL A2 buffer was added, and the suspension was mixed by inverting carefully the tube 8 times. After 5 min incubation at room temperature, 600 µL A3 buffer was added. The tube was inverted thoroughly until the liquid was colorless. To purify the lysate, the suspension was spun down at 11.000 rpm for 10 min. 700 µL of the supernatant were transferred to the column containing a collection tube, and centrifuged at 11.000 rpm g for 1 min. The flow-through liquid was discarded and the step was repeated a second

time. Then the silica membrane was washed by adding 500 µL AW Buffer followed by centrifugation at 11.000 rpm for 1 min and the flow through liquid was discarded. Then, 600 µL A4 buffer was added followed by centrifugation at 11.000 rpm for 1 min and the flow through liquid was discarded. After, an additional centrifugation step at 11.000 rpm for 2 min was performed to completely dry the silica membrane. The column was placed in a 1.5 mL reaction tube and 25 µL ddH_2O was loaded for the elution of the plasmid DNA. This was followed by incubation for 1 min at room temperature with subsequent centrifugation at 11.000 rpm for 1 min. An additional 25 µL ddH_2O was loaded on the membrane and the last step was repeated. Samples were stored either at 4°C or at –20 ºC for long-term storage.

2.2.5. Polymerase Chain Reaction (PCR)

2.2.5.1. Standard PCR

Q5® High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt, Deutschland) was used for reactions requiring proofreading, such as for the amplification of PCR fragments used for molecular cloning. For a 50 µL reaction, 32.5 µL ddH_2O, 10 µL 5 X Q5® reaction buffer, 2.5 µL forward primer, 2.5 µL reverse primer, 1 µL 10 mM dNTPs (Biozym), 1 µL template DNA and 0.5 µL Q5® DNA polymerase was used. For the template DNA either for genomic DNA (10 ng/µL) or plasmid (1 ng/µL) was used. All reactions were prepared on ice. The reaction tube was then placed in the thermal cycler (Biometra TRIO - Triple Powered PCR thermal cycler, Analytik Jena). The following setup was used: Initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 50–72 °C (depending on the primers) for 30 s, extension at 72 °C for 30 s/kb and a final extension at 72 °C for 2 min. The denaturation, annealing, and extension steps were generally repeated 25–30 times. The extension time was set according to the length of the PCR product and the annealing temperature was determined using the NEB Tm Calculator (version 1.16.5).

2.2.5.2. Colony PCR for Gram-negative bacteria

The biomass of one colony was removed from the selection plate with a pipette tip and transferred to a new selection plate. The remaining biomass on the pipette tip was resuspended in 10–50 µL ddH_2O by pipetting up and down. 1 µL of the suspension was used as template for a PCR reaction. The PCR was carried out using Phire Hot Start II DNA Polymerase (Thermofisher Scientific). For a 20 µL reaction, 12.2 µL

ddH_2O, 4 µL 5 X Phire Green reaction buffer, 1 µL forward primer, 1 µL reverse primer, 0.4 µL 10 mM dNTPs, 1 µL template, and 0.4 µL Phire Hot Start II DNA Polymerase was used. All reactions were prepared on ice. The reaction tube was then placed in the thermocycler. The following setup was used: Initial denaturation at 98 °C for 3 min, denaturation at 98 °C for 10 s, annealing at 50–72 °C (depending on the primers) for 30 s, extension at 72 °C for 15 s/kb and a final extension at 72 °C for 1 min. The denaturation, annealing, and extension steps were generally repeated 25–30 times. The extension time was set according to the length of PCR product and the annealing temperature was determined using Thermo Fisher Scientific Tm Calculator.

2.2.5.3. Colony PCR for Gram-positive bacteria

The colony PCR for Gram-positive bacteria was prepared as described before (Andreas Abstreiter, unpublished). The biomass of one colony was removed from the selection plate with a pipette tip and transferred to a new plate with replica on a selection plate. The remaining biomass on the pipette tip was resuspended in 50 µL 1 X TE buffer. 1 µL Proteinase K (12.5 mg/mL) was added and mixed. The suspension was incubated in the thermocycler for 10 min at 65 ºC, followed by heat-inactivation for 5 min at 99 ºC. 1 µL of the suspension was used as template for a PCR reaction. The PCR was carried out using Phire Hot Start II DNA Polymerase. For a 10 µL reaction, 9.05 µL ddH_2O, 3 µL 5X Phire Green reaction buffer, 0.75 µL forward primer, 0.75 µL reverse primer, 0.3 µL 10 mM dNTPs, 1 µL template and 0.15 µL Phire Hot Start II DNA Polymerase was used. All reactions were prepared on ice. The reaction tube was then placed in the thermocycler. The following setup was used: Initial denaturation at 98 °C for 1 min, denaturation at 98 °C for 10 s, annealing at 50–72 °C (depending on the primers) for 15 s, extension at 72 °C for 15 s/kb and a final extension at 72 °C for 1 min. The denaturation, annealing, and extension steps were generally repeated 25–30 times. The extension time was set according to the length of PCR product and the annealing temperature was determined using Thermo Fisher Scientific Tm Calculator.

2.2.6. Nucleic acid quantification

The Nanodrop® spectrophotometer (ND-1000, V3.8.1, Thermo Scientific) was used to quantify the DNA concentration and assess the purity of samples. The DNA concentration was measured at a wavelength of 260 nm. The ratio of absorbance at

260 nm and 280 nm was used as an indicator of proteins in the sample. A ratio of 1.8 was accepted as pure for DNA. The ratio of absorbance at 260 nm and 230 nm was used as an indicator of unwanted organic compounds in the sample. A ratio of > 1.8 was accepted as pure for DNA.

2.2.7. Gel electrophoresis

DNA fragments were separated via gel electrophoresis. Considering that DNA fragments are negatively charged, they will migrate in the gel toward the positively charged electrodes. Fragments with different sizes lead to different throughput times, allowing the estimation of the size. Fragment separation was performed using the power supply Phero-stab 500 (Biotec-Fischer, GmbH, Reiskirchen, Germany) at 120 V, constant current, for 35 minutes. 0.8 % (w/v) agarose gels (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) were prepared using 1 X TAE buffer solution. Horizontal electrophoresis chambers were filled with approximately 25 mL agarose solution. 5 µL sample was mixed with 1 µL 6 X Loading Dye (Thermo Scientific) to increase the density of the samples. 3 µL GeneRuler 1 kB DNA ladder (Thermo Scientific) was used for fragment size determination. After, the gel was stained in aqueous ethidium bromide solution (2.5 µM) for 10–20 min. Then it was briefly washed in water for discoloration. Fragments were visualized under UV light using the AlphaImager® MINI gel documentation system (Biozym Scientific GmbH, Oldendorf, Germany).

2.2.8. DNA fragments purification from the agarose gel

When required, DNA fragments needed for cloning were isolated from agarose gel. The gel electrophoresis was performed as described in 2.2.7, except for the gel loading. For this purpose, 50 µL sample was loaded in the agarose gel (45 µL PCR product mixed with 9 µL loading dye) and 5 µL DNA ladder. The agarose gel purification was performed using Zymoclean™ Gel DNA Recovery kit (Zymo Research, Freiburg, Germany) according to the protocol provided by the manufacturer. Briefly, DNA fragment was excised from the agarose gel using X-tracta generation II (Biozym) and transferred into a 1.5 mL microcentrifuge tube. Then, 3 volumes of ADB buffer were added per volume of agarose excised from the gel. The sample was incubated at 52 °C for up to 10 minutes (until completely melted). For DNA fragments larger than 8

kb, one additional volume of water was added to the mixture for better DNA recovery. The melted agarose was transferred to the column coupled to the collection tube. The sample was centrifuged at 11.000 g for 1 min. After, the flow-through was discarded and 200 µL wash buffer was added. The sample was centrifuged at 11.000 g for 30 s. This procedure was repeated and the column was transferred into a new 1.5 mL microcentrifuge tube. 6 µL ddH_2O was added on the top of the silica membrane and the sample was centrifuged at 11.000 g for 1 min. The same procedure was repeated and samples were kept at 4 ºC for short-term use.

2.2.9. PCR clean-up

PCR clean-up was performed using NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Shortly, the biding conditions were adjusted by mixing 45 µL PCR product with 90 µL NTI buffer. The solution was loaded in the clean-up column and centrifuged at 11.000 g for 30. The flow-through was discarded and the silica membrane was washed twice with 700 µL NT3 buffer. The flow-through was discarded. After the last centrifugation step, the flow-through was discarded and an additional 1 min centrifugation step was used to completely dry the silica membrane. The elution of the DNA was performed in 2 steps to increase the recovery yield. For that, the clean-up column was placed into a new 1.5 mL microcentrifuge tube and 15 µL ddH_2O was added on the top of the silica membrane. After 1 min incubation at room temperature, the sample was centrifuged at 11.000 g for 1 min. The same procedure was repeated and samples were kept at 4 ºC for short-term use.

2.2.10. Molecular cloning

2.2.10.1. NEBuilder® assembly

NEBuilder® assembly was used to clone multiple DNA fragments into a vector. Inserts and vectors were obtained via PCR. After verification of PCR products by analytic gel electrophoresis, the vector was digested with FD-*Dpn*I (Thermo Scientific) to remove plasmid DNA used as template in the PCR reaction. The reactions were prepared as in 2.2.5.1. The PCR products were purified as described in 2.2.8 and 2.2.9. The assembly of the fragments was performed using NEBuilder® HiFi DNA Assembly Master Mix (NEB). 100 ng of vector with a 10-fold molar excess of each insert was

used. The reaction mix was incubated at 50 ºC for 1 h. After, 4 µL reaction mix was transformed into chemically competent *E. coli* 10-beta NEB® as described in 2.2.10.3. For screening of the recombinant DNA, colony PCR was performed. Overnight cultures of the positive candidates were prepared for further plasmid isolation as described in 2.2.4. Isolated plasmids were digested with appropriate restriction enzymes as described in 2.2.10.1. Positive candidates were sent for sequencing as described in 2.2.10.2.

2.2.10.2. Restriction-based cloning

Restriction-based cloning was used to clone a DNA fragment into a vector. Inserts were generated via PCR and vectors were obtained after plasmid isolation. Inserts and vectors were restricted digest with complementary restriction enzymes. For this purpose, 5 µL 10 X CutSmart buffer (NEB), substrate (up to 1 µg), 1 µL restriction enzyme "X", 1 µL restriction enzyme "Y" and ddH_2O (up to 50 µL) were mixed. The reaction was incubated at 37 ºC for 1 h, followed by heat-inactivation at 80 ºC for 20 min. After digestion, vectors were dephosphorylated to avoid re-circularization during ligation. For that, 45 µL digested plasmid reaction, 5 µL 10 X Antarctic Phosphatase buffer (NEB), and 1 µL Antarctic Phosphatase (NEB) were mixed. The reaction was incubated at 37 ºC for 1 h, followed by heat-inactivation at 80 ºC for 2 min. Inserts and vectors were purified from agarose gel as in 2.2.8. The ligation was performed using T4 ligase (NEB), using 25–50 ng of vector with 3-fold molar excess of each insert. The reaction was incubated at 16 ºC, overnight (16 h ± 1). After, 5 µL reaction mix was transformed into chemically competent *E. coli* 10-beta NEB® as described in 2.2.10.3. For screening of the recombinant DNA, colony PCR was performed. Overnight cultures of the positive candidates were prepared for further plasmid isolation as described in 2.2.4. Isolated plasmids were digested with appropriate restriction enzymes as described in 2.2.10.1. Positive candidates were sent for sequencing as described in 2.2.10.2.

2.2.10.3. DNA transfer in *E. coli*

Preparation of competent cells

Competent cells were prepared by inoculation 100 mL LB medium with *E. coli* overnight culture to obtain an OD_{600nm} of 0.1. The culture was grown until an OD_{600nm} of 0.5–0.6. Then, 2 X 50 mL culture was transferred to a centrifuge tube and

centrifuged at 4500 rpm, 0 °C for 5 min. The supernatant was removed, and the pellet resuspended in 25 mL 100 mM $CaCl_2$ (pre-cool at 0 °C). The suspension was centrifuged, and the pellet was resuspended in 5 mL 100 mM $CaCl_2$ (pre-cool at 0 °C). 2 mL glycerol (87 %) was added, and the suspension was aliquoted in 100 µL portions. The competent cells were stored at –80 °C.

Heat shock bacterial transformation

The transformation of *E. coli* strains with recombinant plasmid DNA was performed via heat shock transformation, a simple procedure to induce the uptake of exogenous DNA by the formation of pores in the chemically competent cells. For that, 100 µL of chemically competent *E. coli* cells were thawed on ice for about 5 min (or until no ice crystal was observed) followed by the addition of about 100 ng DNA solution and incubation on ice for 30 min. The suspension was heat shocked for 30 s at 42 °C and incubated on ice for 5 min. After, 900 µL of SOC medium was added and cells were incubated for 60 min at 37 °C and 800 rpm. After, the suspension was spun down at 14.000 rpm for 2 min. 800 µL of the supernatant was removed and the biomass was resuspended in the remaining liquid. Then, the cell suspension was plated on solid agar LB plates with the appropriate selective marker and incubated overnight (± 16 h) at 37 °C.

2.2.10.4. Restriction digest

Restriction digest was used to analyze plasmids by comparing the resulting restriction pattern with the *in silico* restriction pattern. For this purpose, 6 µL ddH_2O, 1 µL 10 X Fast digest Green Buffer (Thermo Fisher Scientific), 1 µL plasmid DNA (up to <1 µg), 1 µL Fast-digest restriction enzyme “X” (Thermo Fisher Scientific), and 1 µL Fast-digest restriction enzyme “Y” was used. The reaction mix was incubated at 37 ºC for 15 min, followed by heat-inactivation at 80 ºC for 5 min. To digest the plasmid used as template for PCR reactions, 42.5 µL PCR product, 2.5 µL FD-*Dpn*I (ThermoFisher Scientific) and 5 µL 10 X Fast Digest buffer (without loading dye) were used. The reaction was incubated at 37 °C for 60 min, followed by heat-inactivation at 80 ºC for 5 min. After restriction digest, samples were analyzed by gel electrophoresis.

2.2.10.5. Sequencing

The reactions for Sanger sequencing were prepared from either purified plasmid or PCR-product samples. Analytic Sanger DNA sequencing was performed by Azenta

Life Sciences (Griesheim, Germany). The reactions were prepared according to the manufacturer's instructions. In brief, 5 µL template and 5 µL primer was mixed in a 1.5 mL reaction tube. For plasmid, a final concentration of 30–100 ng/µL was used, while for PCR products 10–50 ng/µL was used. Primer solutions at 10 µM were used, to obtain a final concentration of 5 µM in the reaction.

2.3. Screening of restriction endonucleases

2.3.1. Preparation of the crude free extract

Cell free extract (CFE) was prepared according to Yang and co-workers (X. Yang et al., 2016), with modifications. In the anaerobic tend, centrifuge tubes were filled with 50 mL *C. cellulovorans* culture in the exponential phase (OD_{600nm} = 0.5–0.6). The culture was centrifuged at 4500 g for 10 min at 4 °C. The supernatant was removed, and the same tube was filled with an additional 50 mL *C. cellulovorans* culture. The culture was centrifuged, and the supernatant was removed. The pellet was washed with 50 mL 1 X PBS. After centrifugation, the supernatant was removed, and 1 mL lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10 mM β-mercaptoethanol) was added and carefully mixed with the cells avoiding bubbles. Exactly 1 mL of the suspension was transferred to a 2 mL screw cap tube with the silica beads (0.1 mm, Lysing Matrix B provided in the FastRNA® Pro Blue Kit, MP Biomedicals GmbH, Eschwege, Germany). The cells were disrupted using the homogenizator FastPrep-24™ Classic bead beating grinder and lysis system (MP Biomedicals GmbH) for 30 s at a setting of 6.0 m/s with a 5 min pause on ice. This step was repeated 10 times. The lysate was centrifuged at 16000 g for 30 min at 4 °C. Approximately 750 µL of the supernatant was carefully transferred to a new 2 mL tube. Then, 50 µL of 1 M NaCl was added and the sample was incubated on ice for 10 min, with further centrifugation. The supernatant was transferred to a new tube, mixed with the same volume of glycerol (96 %), and stored at –20 °C for short-term use. The analysis of protein concentration was performed using Bradford assay. The bovine serum albumin (BSA, NEB) was diluted in glycerol to obtain the standard curve.

2.3.2. Restriction assay

The restriction assay was prepared similarly to Karl (Karl, 2018). Initially, 20 µL BSA (10 mg/mL) was mixed with 200 µL CFE. Then, 8 µL CFE, 10 µL plasmid solution (100

ng/µL) and 2 µL NEBuffer 2 (NEB) were mixed. The reaction was incubated at 37 °C in the thermocycler for 1, 2, and 4 h. After, samples were analyzed by gel electrophoresis. Samples with no CFE, as well as no plasmid, were used as control.

2.4. Genetic manipulation of *C. cellulovorans*

2.4.1. *C. cellulovorans* transformation

2.4.1.1. *In vivo* DNA methylation with native methyltransferases

Prior to transformation, plasmids underwent *in vivo* DNA methylation using *C. cellulovorans* native methyltransferases. For that, the methylation plasmid placORM and the interest plasmid were co-transformed into the *E. coli* 10-beta NEB®. An overnight liquid culture was used to inoculate 5 mL of LB medium containing the proper antibiotic and 10 mM isopropylthio-β-galactoside (IPTG, for the induction of the methylases) to an OD_{600nm} of 0.1. The cultures were incubated at 37 °C, overnight, 180 rpm, with further plasmid isolation as described in 2.2.4. When the *in vivo* DNA methylation was carried out to proceed with the conjugation protocol, the cultures were incubated at 37 °C, 180 rpm, until reaching OD_{600nm} of 0.5–0.6.

2.4.1.2. Electroporation

Electroporation was performed as previously described (Bao et al., 2019; X. Yang et al., 2016), with minor modifications. In the anaerobic tend, ice-cold centrifuge tubes were filled with 50 mL *C. cellulovorans* culture in the exponential phase (OD_{600nm} = 0.5–0.6). The culture was centrifuged at 4500 g for 10 min at 4 °C. The supernatant was removed, and the pellet was washed with 50 mL ice-cold anaerobic electroporation buffer (270 mM sucrose; 1 mM $MgCl_2$; 5 mM sodium phosphate buffer, pH 7.4). After centrifugation, the pellet was resuspended in 400 µL electroporation buffer. 5−8 µg *in vivo* methylated plasmid (less than 10 µL) was added to the 400 µL cell suspension. Then, the mixture was transferred to a prechilled electroporation cuvette (0.2 cm inter-electrode distance), and an electrical pulse at 1.8 kV, 25 µF, and 800 Ω (Bio-Rad Gene Pulser) was applied (time pulse 4−6 ms). The cell suspension was transferred to 5 mL pre-warmed medium and incubated at 37 °C for recovery. After overnight incubation, the 2.5 mL suspension was inoculated in 10 mL medium supplemented with 10 µg/mL thiamphenicol incubated at 37 ºC for 3−7 days. If case obvious growth was observed, 0.5 mL of culture was inoculated into 5 mL growth

medium supplemented with 15 µg/mL thiamphenicol and incubated at 37 ºC for 1−2 days. Transformation efficiency (TE) was defined as the number of colony forming units (CFU) produced per microgram of plasmid DNA used. TE was determined using the following equation Liu et al., 2018):

$$\mathrm{TE\ (CFU/\mu g)} = \frac{\mathrm{number\ of\ transformants}}{\mathrm{\mu g\ of\ DNA}} \times \frac{\mathrm{final\ volume\ at\ recovery\ (mL)}}{\mathrm{volume\ plated\ (mL)}}$$

2.4.1.3. Triparental conjugation

Triparental conjugation was performed as previously described (Aline Schöllkopf, unpublished), with modifications. Pre-cultures of *E. coli* 10-beta NEB® with interest plasmid and methylation plasmid (donor), *E. coli* CA434 (helper), and *C. cellulovorans* (acceptor) were prepared as described in 2.1.3. and incubated overnight. After overnight incubation, the main cultures of donor, helper, and acceptor were prepared. For the donor strain, 5 mL of medium containing the proper antibiotic and IPTG was inoculated to an OD_{600nm} of 0.1. The culture was incubated at 37 ºC, 180 rpm, until reaching an OD_{600nm} of 0.5. For the helper strain, 5 mL of medium containing the proper antibiotic was inoculated to an OD_{600nm} of 0.05. The culture was incubated at 37 ºC, 180 rpm until reaching an OD_{600nm} of 0.5. For the acceptor strain, hungates containing 5 mL of medium were inoculated with 0.1 and 0.5 mL pre-culture. The culture was incubated at 37 ºC until reaching an OD_{600nm} of 0.5–0.6. When all cultures reached the proper density, 1 mL of the donor and 1 mL helper cultures were transferred to a microcentrifuge tube and centrifuged at 11000 rpm at room temperature for 1 min. The cell pellet was washed twice with 1 X PBS. Then, cell pellet and *C. cellulovorans* culture were transferred into the anaerobic tent. The cell pellet was resuspended in 250 µL of recipient culture. After, the suspension was distributed in six drops (about 40 µL per drop) onto agar plates of 520 medium supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), containing glucose and lacking any antibiotic. After drying, the mating plate was inverted and transferred to an anaerobic GasPak jar and incubated for 20–24 h at 37 °C. Afterward, the plate was transferred to the anaerobic tent. The cell biomass was collected using an inoculation loop and resuspended in 500 µL 1 X PBS. The homogeneous suspension was spread into 4 selection plates (520 2T medium-containing 10 µg/mL thiamphenicol). When using restriction-less strains of *C. cellulovorans*, the biomass was resuspended in 1 mL 1 X PBS, with further 10-fold serial dilutions (up to 10^{-5}). 10

µL of each dilution was spotted on the agar plate using the track-dilution method. The plates were inverted and transferred to an anaerobic GasPak jar and incubated for 48–72 hours at 37 °C.

To determine the donor:helper:acceptor ratio, the main cultures were diluted. A 10-fold serial dilution (up to 10^{-7}) in 0.9 % NaCl was performed for *E. coli* strains; a 10-fold serial dilution (up to 10^{-5}) in 1 X PBS was performed for *C. cellulovorans*. After, 10 µL of each dilution was spotted on the suitable agar plate using the track-dilution method. *E. coli* strains were incubated overnight and *C. cellulovorans* strains were incubated for 48 hours, at 37 ºC, before CFU counting. Transconjugation efficiency (TCE) was determined using the following formula:

$$\text{TCE (CFU/mL)} = \frac{\text{number of transconjugants (CFU/mL)}}{\text{number of acceptor (CFU/mL)}}$$

2.4.1.4. Confirmation of the transformants and transconjugants

Transformants and transconjugates phenotypic characteristics, such as colony shape, size, and morphology were observed. 20 colonies were analyzed via colony PCR to confirm *C. cellulovorans* identity, as well as the presence or absence of the target loci. After confirmation, part of the biomass of the colonies was collected to perform chromosomal DNA isolation, as described in 2.2.3. The identity was confirmed through the amplification of the 16S rDNA or specific locus via PCR, followed by sequencing.

2.4.1.5. Plasmid integrity confirmation

To confirm the integrity of the plasmids transformed into *C. cellulovorans*, 2 mL of an overnight culture was used for total DNA isolation (genomic DNA [gDNA] and plasmids) as described in 2.2.2. The DNA concentrations were measured. Afterward, 25 µL (>1000 ng) of DNA solution was transformed into *E. coli* 10-beta NEB®, as described in 2.2.10.3. The extracted plasmid was verified by restriction digestion with *Acs*I (NEB) and *Fse*I (NEB), followed by gy gel electrophoresis. The obtained bands were compared with the original plasmids (positive control).

2.4.2. Plasmid segregational stability assay

To investigate plasmid segregational stability, 100 µL of overnight culture, obtained by cultivating the transconjugants in 520 medium with 15 µg/mL thiamphenicol at 37 ºC,

was transferred to 5 mL thiamphenicol-free medium and incubated for 24 h. The culture was subcultured 3 times at 24 h intervals in thiamphenicol-free medium. Every new culture was obtained by inoculation 100 µL of the previous culture. The plasmid retention was measured every 24 h as follows. First, a 10-fold serial dilution was performed by mixing 100 µL culture with 900 µL 1 X PBS (up to 10^{-5}). The dilution was distributed in 10 µL portions onto plates without thiamphenicol using the track-dilution method. The plates were incubated at 37 ºC for 48 h. After, 50 colonies selected at random were further replicated onto selective plates containing 15 µg/mL thiamphenicol and incubated at 37 ºC for 48 h. The colonies that had grown in these plates were counted as thiamphenicol-resistant. Once only cells retaining the plasmid can grow, the number of thiamphenicol-resistant colonies was taken as an indicator of stability. A schematic representation of this experiment is given in Figure 2.1.

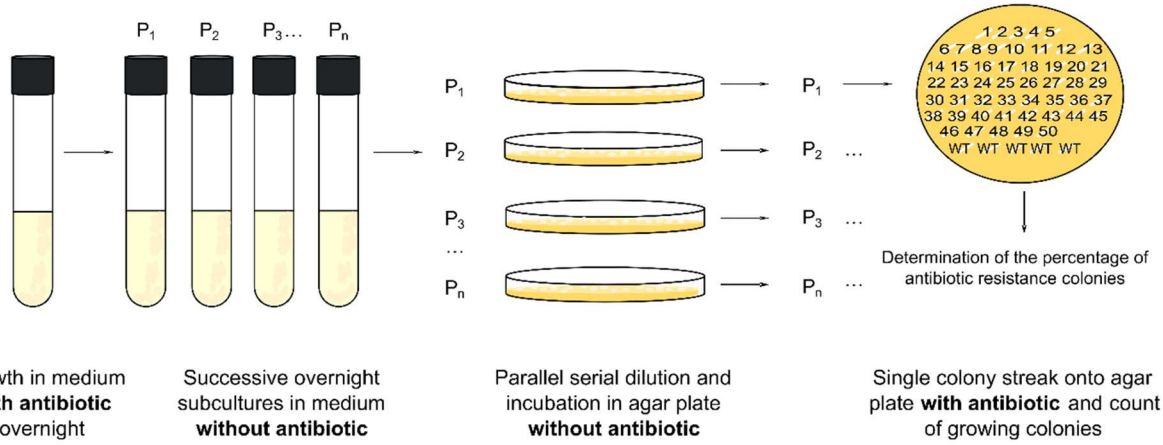

Figure 2.1 | Schematic representation of plasmid segregational stability assay (P = passage).

2.4.3. Plasmid copy number

Plasmid copy number was determined by Real-time quantitative PCR (qPCR) as previously described (DeLorenzo et al., 2018; Lee et al., 1993). Primer design and optimization were performed as recommended in the literature (Bustin et al., 2009; Taylor et al., 2010). The target gene (*catP*) and reference gene (ORF Clocel_3734) were designed using the online tool PrimerQuest™ (IDT, Coralville, USA). Primers were defined by a length of approximately 20 nt and a fragment size of ~100 bp. A gradient PCR was used to identify the optimal annealing temperature of the used

primer sets. The used annealing temperature (Ta) for *catP* was 64.5 °C, while the Ta for Clocel_3734 was 59 °C. The PCR efficiency was determined by amplification of a dilution series of DNA (50 pg–50 ng). The plasmid pMTL83151 and gDNA of *C. cellulovorans* were used to determine the dynamic range. qPCR was performed in triplicates with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, USA). The final primer concentration was 250 nM and the amount of DNA used as template was 50 ng per reaction. The reaction was run on a Bio-Rad® CFX96™ using the following thermo cycling protocol: 98 °C for 3 min, followed by 40 cycles of 98 °C for 10 s, and 64.5 or 59.0 °C for 10 sec, 60.0 ºC for 15 sec and fluorescence measurement. qPCR reaction was prepared by mixing 10 µL SsoAdvanced™ Universal SYBR® Green Supermix 2 X, 0.5 µL forward primer (5 mM), 0.5 µL reverse primer (5 mM), 1 µL DNA sample (50 ng/µL), and 8 µL nuclease-free H_2O. Three biological replicates and three technical replicates were prepared. To determine the copy number ratio, an external standard dilution curve of cycle threshold (CT) versus log (copy number) was performed and a linear regression was performed to confirm the linearity. The following equation was used to determine the DNA amplicon copy number (Whelan et al., 2003):

$$DNA\ (copy) = \frac{N_A \times DNA\ target\ amount\ (g\ \mu l^{-1})}{M_W}$$

Where N_A = 6.02 x 10^{23} mol^{-1} (Avogadro constant) and M_W = DNA total length (bp) x 660 (g/mol/dp) x 10^9. The standard curves were used to determine the number of copies of each plasmid in the sample on the basis of the measured sample CT values. The following equation was used to determine the quantity of each amplicon (Real-Time PCR Applications Guide, Bio-rad):

$$Quantity = 10^{\frac{Ct-b}{m}}$$

Where m is the slope and b is the y-intercept. The copy number ratio was then determined by dividing the number of copies of each plasmid by the number of copies of the chromosome.

The PCR efficiency for the used annealing temperatures was determined by amplification of a dilution serie of plasmid or gDNA (50 pg – 50 ng). A linear regression

was performed to confirm the linearity by plotting the cycle CT values versus the log of the DNA input. The qPCR efficiency was calculated using the following equation:

$$PCR\ efficiency = 100\ x\ (10^{-1/m}\ -1)$$

An efficiency of 1 means a doubling of product in each cycle. A melt curve analysis was performed at the end of each PCR program to confirm the absence of unspecific products.

2.4.4. 5-fluorouracil (5-FC) and 5-fluorocytosine (5-FU) minimum inhibitory concentrations (MIC)

Initially, to evaluate the usability of the *codBA* system for counter-selection, the 5-fluorouracil (5-FU) and 5-fluorocytosine (5-FC) minimum inhibitory concentrations (MIC) were determined. For that, hungates containing 5 mL 520 medium supplemented with 5-FU or 5-FC at final concentrations ranging from 0–200 µL/mL were prepared. After, the medium was inoculated with 0.5 mL *C. cellulovorans* WT overnight culture. The cultures were incubated at 37 ºC for 48 h. The concentration where no growth was observed was determined as the MIC. Secondly, to find the optimal condition for counter-selection, 520 medium agar plates containing either 2 g/L tryptone or 2 g/L casamino acids (technical) were prepared. After autoclaving, besides the addition of cellobiose, L-cysteine-HCl·H_2O, and Na_2CO_3, the media was supplemented with 1.5 mL thiamphenicol (10 mg/mL), 1.33 mL adenosine (75 mM), 1.33 mL guanosine (75 mM), 1 mL 7 vitamins solution, 1 mL L-tryptophan solution, and 5-FU or 5-FC (for a final concentration ranging from 0–200 µL/mL). When the plates were anoxic, approximately 20 µL cultures of *C. cellulovorans* strains containing codBA deletion plasmids were spread on the agar plates. The concentration where no growth was observed was determined as the MIC. Three biological replicates, with two technical replicates, were prepared.

2.4.5. Generation of gene knock-in and knock-out

The generation of marker-less gene knock-in and knock-out in *C. cellulovorans* was based on the Allele-Coupled Exchange (ACE) method using the *codBA*-based counterselection. For that, deletion plasmids containing two homologous flanks located up and downstream of the gene of interest (GOI) were constructed. The flank

fragment had a length of 0.75–1 kb and was designed to obtain an in-frame deletion (Fig. 2.2). The upstream and downstream flanking regions were amplified using chromosomal DNA from *C. cellulovorans* WT as a template, and the backbone was amplified using the plasmid either pLA_del_ori3, pLA_del_ori7 or pLA_del_ori8 as template. The fragments were assembled using NEBuilder® assembly.

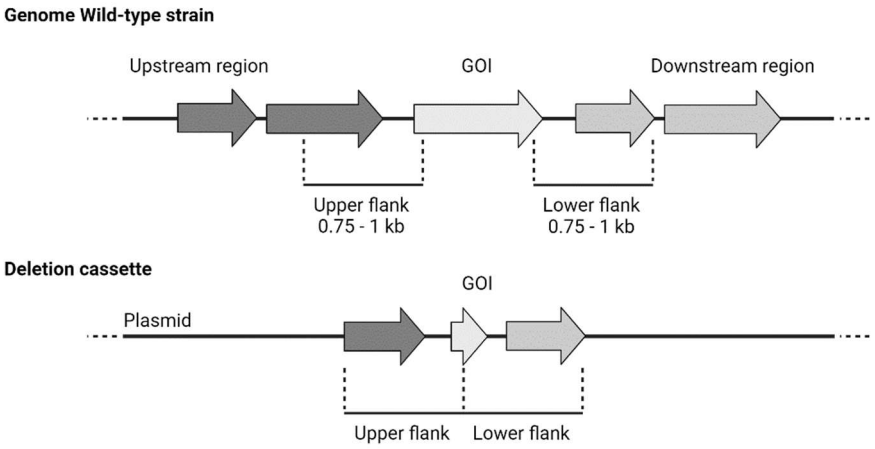

Figure 2.2 | Schematic representation of the in-frame deletion cassette creation. The homology arms (upper and lower flanks) are specific to the gene of interest (GOI).

In vivo methylated plasmids were transferred into *C. cellulovorans* via triparental conjugation. 3 colonies of *C. cellulovorans* containing the *codBA* deletion vector were successively streaked on 520 medium containing 2 g/L tryptone and 2.5 µL/mL thiamphenicol for the removal of *E. coli* and selection of plasmid integration by homologous recombination. After the 3–5 passages, when only *C. cellulovorans* was present on the agar plate, 24–100 colonies underwent colony PCR to verify the presence of the insertion/deletion. After, positive candidates were streaked on the counter-selection plate. 50 colonies selected at random were streaked on 520 medium agar plate without antibiotic and replica on 520 medium agar plate containing 15 µL/mL thiamphenicol. Clones that lost the plasmid were selected for chromosomal DNA isolation, for further analysis via PCR. The correct clones were identified using PCR using the high-fidelity Q5® polymerase and sequencing.

2.5. Analytical methods

2.5.1. Analysis of fermentation products using Gas chromatography

The quantity of the fermentation products was analyzed using gas chromatography (GC) as described before (Lesiak, 2014). To ensure adequate measurements two different standards were used during every GC run. The external standard consists of an acid solvent mixture containing the most common acids/solvents in the ABE fermentation.

Initially, an acid/solvent mix (0.5 %, w/w) was prepared by combining 200 µL 5 % acetone (w/w), 200 µL 5 % methanol (w/w), 200 µL 5 % ethanol (w/w), 200 µL 5 % isopropanol (w/w), 200 µL 5 % isobutanol (w/w), 200 µL 5 % butanol (w/w), 200 µL 5 % acetic acid (w/w), 200 µL 5 % propionic acid (w/w), 200 µL 5 % butyric acid (w/w) and 200 µL of GC-water in a 2 mL tube. All 5 % solvent solution was prepared in GC-water. GC-water was prepared by adjusting the pH of ddH_2O to 2.0 using HCl. Then, the acid/solvent mix was serially diluted in GC-water to obtain the final concentrations of: 0.4 %, 0.2 %, 0.1 %, 0.05 %, 0.025 %, 0.005 %. 500 µL of each dilution was transferred into a GC vial for further analysis. The internal standard stock (0.5 %, v/v) was prepared by mixing 9 mL of GC-water with 1 mL 5 % 1-Propanol (w/w). 500 µL of the internal standard stock was transferred into a GC vial for further analysis. For the sample preparation, 1 mL culture was centrifuged at 15.000 g for 20 min at 4 °C. 750 µL of the supernatant was transferred into a new tube and kept at –20 °C for at least 8 h. The remaining supernatant was discarded and the pellet underwent protein concentration analysis using the Bradforad assay. After the incubation at 20 °C, the sample was centrifuged at 15.000 g for 20 min at 4 °C. Then, 100 µL of the supernatant was mixed with 50 µL of 0.5 % internal standard stock and 350 µL of GC-water in a GC-vial. The solution was well mixed by vortexing. The sample was stored at 4 °C until use or at –20 °C for long-term storage. 400 µL of the remaining supernatant was transferred into a new tube for further analysis.

GC analysis was performed using GC – 2010 AOC-20i auto-injector (Shimadzu Corporation, Kyoto, Japan) using nitrogen as carrier gas. Two technical replicates were prepared and the GC Solution Software (Shimadzu) was used for data analysis.

2.5.2. Determination of substrate consumption (determination of polysaccharide and disaccharide utilization)

To estimate the substrate consumption during growth in cellulose, cellulose extraction was performed as described before (Bimmer, 2023). For that, 1 mL culture was centrifuged at 4.500 rpm at 10 ºC for 10 min. The pellet was resuspended in 1 M NaOH for removal of the cells and the medium. The solution was transferred into a 2 mL screw cap tube and incubated in the water bath at 100 °C for 30 min. After, the sample was cooled for 3 min on ice and centrifuged at 10.000 rpm at 10 ºC for 10 min. The pellet was neutralized using 1 mL 5 M ammonium acetate, and the sample was centrifuged again. After, the pellet was washed in 1.5 mL ddH_2O. The washing step was repeated three times or until the pH was neutral. The pellet was dried in the SpeedVac for approximately 2.5 h. After, the Saeman hydrolysis was carried out by adding 175 µL 72 % sulfuric acid (v/v) to the sample and incubation at room temperature for 1 h (the sample was repeatedly mixed). Then, 825 µL ddH_2O was added to adjust the acid concentration to approximately 12.6 %. The sample was mixed by vortexing and incubated in the water bath at 100 °C for 30 min. Then, the sample was centrifuged at 10.000 rpm at 10 ºC for 5 min. The supernatant was used to estimate the glucose content using the Anthrone assay. For that, 90 µL ddH_2O and 10 µL of the supernatant were transferred into a 96-well plate in triplicates. Then, 200 µL of freshly prepared pre-cooled Anthrone reagent (0.2 % anthrone (w/v) in 96 % sulfuric acid, Carl Roth GmbH) was added to each well.

The microplate was incubated on ice for 5 min and covered with a foil. The plate was heated at 80 ºC for 30 min. After cooling at room temperature, the absorbance was measured at 620 nm. A glucose standard curve ranging from 0 to 20 µg/mL was included in each experiment. The amount of cellulose was determined from the released glucose according to the equation (Haffner et al., 2013):

$$[mcellulose] = 0.9\ x\ [mglucose]$$

2.5.3. Determination of protein concentration

Bradford assay was used to determine the total protein concentration as previously described (Bradford, 1976), with modifications. To investigate growth performance in insoluble substrates, such as cellulose, the protein concentration was used as an

indicator of growth. For that, 1 mL bacterial culture was collected and centrifuged at 10750 rpm, room temperature, for 1 min. The pellet was washed twice in 1 mL 0.9 % NaCl solution (w/v). The pellet was resuspended in 1 mL TE buffer and incubated for 30 min at 95 °C in a thermoblock. The sample was incubated at 26 °C for approximately 30 min or until it was at room temperature. The sample was mixed by vortexing and centrifuged. Then, 150 µL of the supernatant was transferred into a 96-well plate with 3 to 6 technical replicates. 150 µL room temperature Bradford reagent (Coomassie Plus (Bradford) Assay Kit, Thermo Fisher) was added into each well and mixed with the sample. Controls containing 150 µL of TE buffer and 150 µL Bradford reagent were prepared. The microplate was incubated for 10 min in the dark. After, the OD_{595nm} was measured. A calibration curve with BSA was prepared in the 1–25 µg/mL working range. The standard curve was used to determine the protein concentration for each unknown sample. Alternatively, 10 µL of the supernatant was transferred into the wells and mixed with 300 µL room temperature Bradford reagent. A calibration curve with BSA was prepared in the 100–1500 µg/mL working range. For the determination of the protein concentration in the cell free extract, 150 µL of CFE was mixed with 150 µL room temperature Bradford reagent. Alternatively, 10 µL of CFE was mixed with 300 µL room temperature Bradford reagent. All the other procedures were performed as described above. BSA was prepared in 10 % glycerol to have the same diluent as the samples.

2.5.4. *In silico* DNA manipulations

Benchling (https://www.benchling.com/, San Francisco, USA) and Clone Manager Professional 9 Software (Sci-Ed Software, Cary, NC) were used for *in silico* DNA manipulations, such as molecular cloning, restriction digest analysis, primer designing, PCR reactions, and analysis of the sequencing results. Primers for qPCR were designed using PrimerQuest™ (https://eu.idtdna.com/PrimerQuest/, Integrated DNA Technologies, Inc., Coralville, USA). Prophage genomic analysis was performed using PHASTER (https://phaster.ca/) (Arndt et al., 2016). RM genomic analysis was performed using REBASE (http://rebase.neb.com) (Roberts et al., 2023).

2.5.5. Whole genome Illumina sequencing

For whole genome sequencing, genomic DNA was extracted as described in 2.2.2. Genome assembly and assembly statistics were performed using UniCycler (the sequencing and assembly were kindly prepared by Dr. Klaus Neuhaus's team). Assembly visualization was achieved using Integrative Genomics Viewer (IGV, https://igv.org/) (Robinson et al., 2023). Single nucleotide polymorphisms (SNP) analysis was achieved using the tool snippy in the Galaxy (https://usegalaxy.org/, Galaxy version 23.2.2.dev0).

2.6. Statistical analysis

Experiments are in general based on at least three independent biological replicates. If applicable, several technical replicates of a sample were measured. The measured values are given as mean (MV) ± standard deviation (SD). All analyses were performed in GraphPad Prism 8 (trial version; San Diego, CA, USA).

3. Results

3.1. Insights into *C. cellulovorans* bacteriology

3.1.1. Growth behavior

C. cellulovorans is a mesophilic bacterium, the temperature and pH for optimum growth are 37 °C and 7.0, respectively, even though it can grow in a temperature range of 20–40 °C and a pH range of 6.4–7.8. Cells are non-motile rods with 2.5–3.5 µm length by 0.7–0.9 µm width (Sleat et al., 1984). The establishment of the optimum growth conditions, which is a crucial step in the development of a genetic system, was accomplished previously in our group. The 520 medium (*Ruminiclostridium cellulolyticum* (CM3) medium), was selected for cultivation. Cellobiose and glucose were found to be the best carbon sources (Aline Schöllkopf, unpublished). Additionally, it was observed that yeast extract can be interchangeably replaced by tryptone, casamino acids, or even a synthetic mix of amino acids.

However, when insoluble components such as cellulose derivatives are present in the medium, growth cannot be determined optically. On the other hand, growth can also be evaluated, for instance, by protein determination using a Bradford assay. Yet, further optimizations of the standard procedure for *C. cellulovorans* were still necessary. To determine the growth on cellulose, two kinds of cellulose were used, Avicel® (microcrystalline cellulose) and PASC (phosphoric acid swollen cellulose, a regenerated amorphous cellulose, which is made from Avicel through phosphoric acid dissolution followed by regeneration). As can be seen in Figure 3.1 A, *C. cellulovorans* WT 743B is able to efficiently grow either on cellobiose or cellulose, although a faster growth in cellobiose was observed. However, it was observed that the growth on 0.5 % cellulose is delayed compared to 0.3 % cellulose, but a higher protein concentration was observed in the medium with 0.5 % cellulose, indicating a better growth on microcrystalline cellulose.

After cultivation with PASC and Avicel®, it was possible to observe visually that the amount of cellulose became less than half of the negative control (media without bacteria). The cellulose remaining in the medium after 14 days of incubation was hydrolyzed into glucose using sulfuric acid (Saeman hydrolysis). Then, the

concentration of reducing sugar was measured by the Anthrone assay. As can be seen in Figure 3.1 B, *C. cellulovorans* could utilize almost all cellulose in the medium, but more than 35 % of cellobiose (1.74 g/L) in the medium was not utilized.

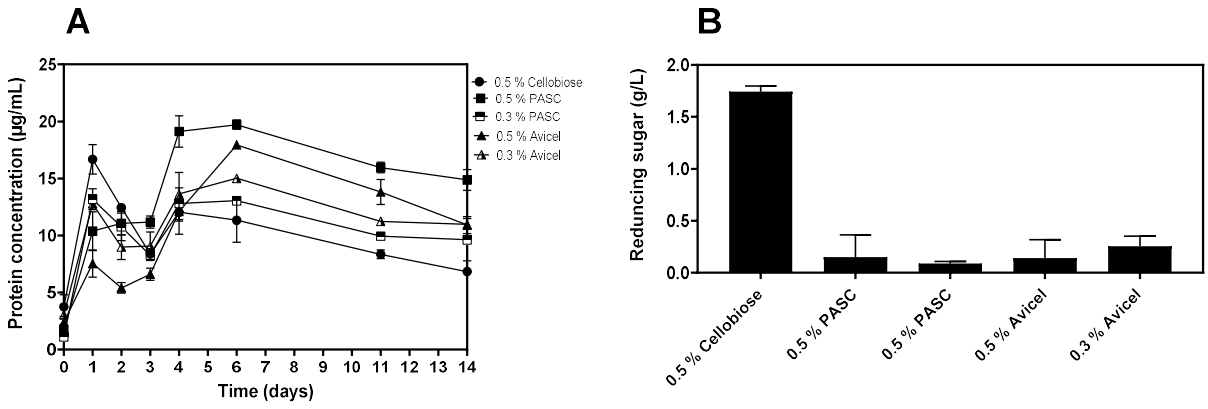

Figure 3.1 | Growth of *C. cellulovorans* on Avicel® (microcrystalline cellulose) and PASC (amorphous cellulose). (A) The growth was evaluated by determining the protein concentration using a Bradford assay. (B) Estimation of cellulose utilization by the determination of residual substrate (as reducing sugar after acid hydrolysis) left in the media after 14 days of incubation. Cellobiose was used as positive control.

Although metagenomic analysis from IMNGS (www.imngs.org) and Microbeatlas (www.microbeatlas.org) based on the global distribution of *C. cellulovorans* 16S rDNA sequence data has found this sequence worldwide in soil (n = 3150), aquatic (n = 1721), animal (n = 1021) and plant samples (n = 543), only one strain of *C. cellulovorans* has been isolated to date. To investigate potential genetic differences that could arise from the cultivation in different culture collections, the growth behavior and genome sequence of *C. cellulovorans* 743B strains obtained from DSMZ (DSM 3052) and American Type Culture Collection (ATCC 35296) were analyzed. As can be seen in Figure 3.2, *C. cellulovorans* from DSMZ reached a slightly higher cell density. Independent of the origin of the type-strain, *C. cellulovorans* growth behavior was frequently unpredictable. Whole genome sequencing of both strains revealed only a few differences. *C. cellulovorans* from ATCC possessed in its genome 15 alterations compared to the genome sequence available in NCBI (GenBank: CP002160.1), while *C. cellulovorans* from DSMZ possessed 10 alterations. To notice, all alterations

present in the strain from ATCC were also present in the DSMZ strain. Only in the ATCC strain, the ORFs Clocel_0871 (putative transport system permease protein), Clocel_2482 (putative amino acid adenylation domain protein), and Clocel_3117 (putative hemolysin-type calcium-binding region) had alterations (Supplementary data, Table 1).

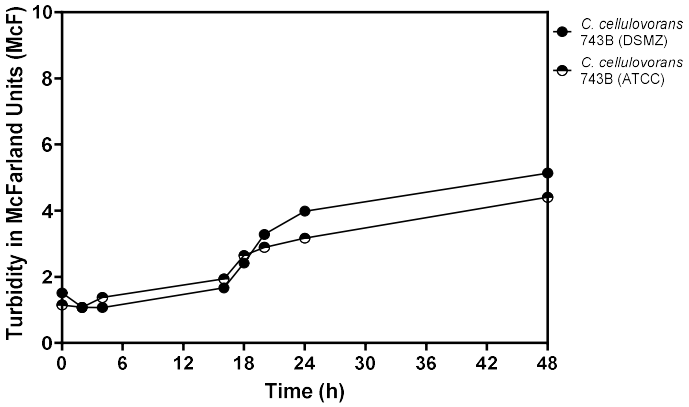

Figure 3.2 | Growth of *C. cellulovorans* 743B from the German Collection of Microorganisms and Cell Cultures GmbH (DSM 3052) and American Type Culture Collection (ATCC 35296) in 520 medium with 2 g/L yeast extract and 5 g/L cellobiose. The growth was evaluated by determining the turbidity using a densitometer.

3.1.2. Evaluation of fermentation products and pH changes in the medium during growth

Although *C. cellulovorans* cannot produce the 4-carbon primary alcohol butanol, the main product of ABE fermentation, it does produce the corresponding fatty acid butyrate. It has been published that butyrate supplementation leads to improvement in final butanol yield for butanol-producing bacteria (Wang, Li & Blaschek, 2013). In the present project, one aim was to construct a *C. cellulovorans* strain that is able to convert cellulose to butyrate at a higher yield, which in turn should serve as a substrate for butanol production by a solventogenic clostridia. Therefore, it was important to know the *C. cellulovorans* fermentation products as well as the yield of each product.

For that, the fermentation products in the media were quantified using gas chromatography (GC). In accordance with the literature, the most abundant

fermentation products found were butyrate and acetate. Trace amounts (< 1 g/L) of ethanol, isopropanol, and butanol were found, but these compounds were under the reliable detection limit of the GC device. The final yield was similar under all conditions tested (≅ 2.5 g/L butyrate and ≅ 0.5 g/L acetate). After 1 day no significant further increase in fatty acid production was observed when the growth was performed on cellobiose. However, a gradual and less abrupt increase in fatty acid production over time was observed when the organism was grown on cellulose, except in the medium with 0.5 % Avicel (Fig. 3.3).

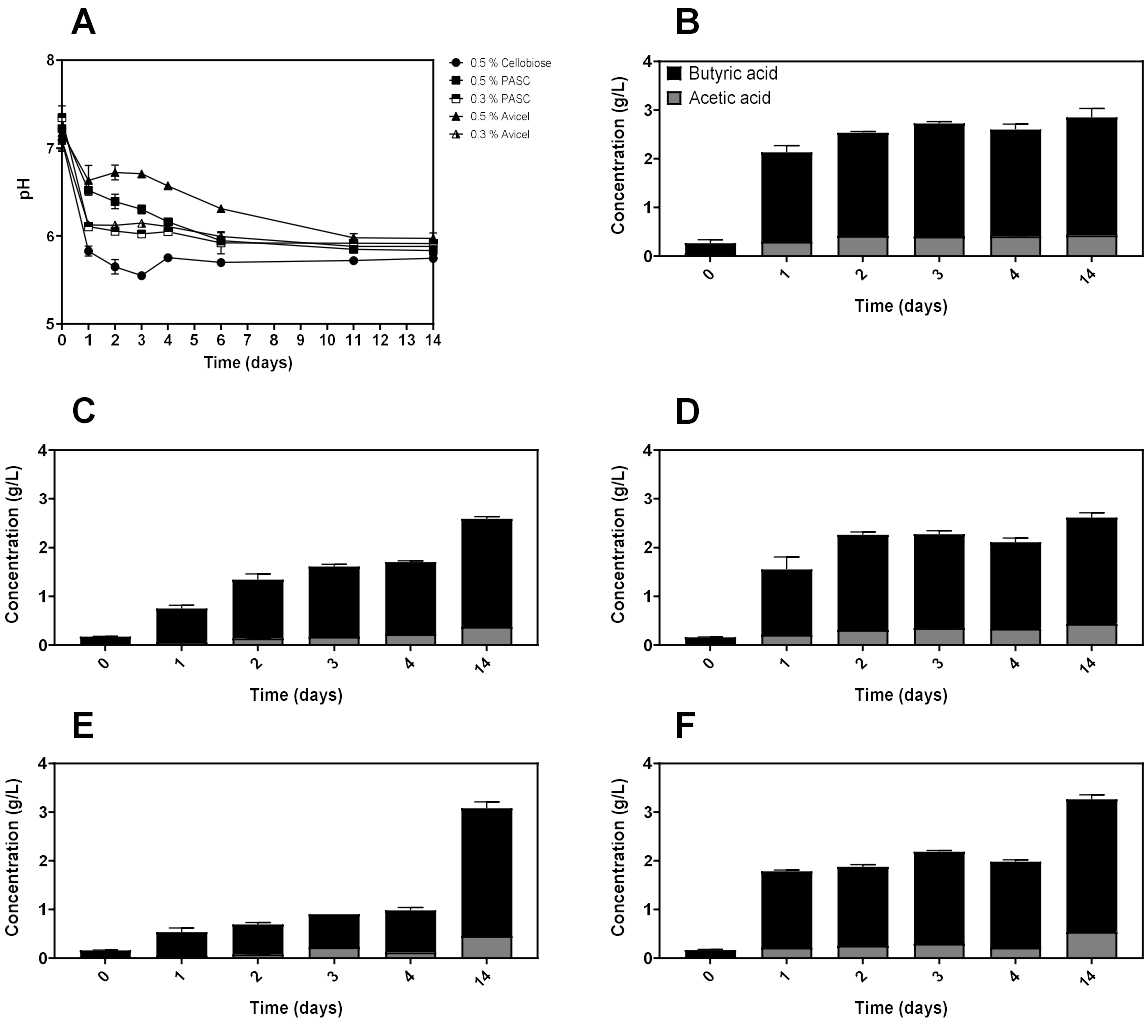

Figure 3.3 | (A) Changes in the medium pH value during *C. cellulovorans* growth and quantification of the main fermentable products. Cells were grown in 10 mL of 520 medium with 2 g/L yeast extract and either 5 g/L cellobiose (B), 5 g/L Avicel (C), 3 g/L Avicel (D), 5 g/L PASC (E) or 3 g/L PASC (F).

Additionally, the pH changes during growth of *C. cellulovorans* on cellulose were investigated. This is of particular relevance in the context of co-cultures including this organism. Synthetic microbial co-cultures have been receiving special attention recently. However, the different requirements for the growth of different species are a barrier to overcome. The co-culture of *C. cellulovorans* and *C. beijerinckii* is a paradigmatic example. *C. cellulovorans* is able to decompose lignocellulosic material but cannot produce butanol. On the other hand, *C. beijerinckii*, just like *C. acetobutylicum*, is unable to decompose lignocellulosic material efficiently, but is a good butanol producer. Importantly, the pH required for butanol fermentation (pH 4.5–5.5) is much lower than the optimal pH for *C. cellulovorans* (6.4–7.8), leading to insufficient saccharification and consequently affecting the growth of both strains (Wen et al., 2017).

The sensitivity of *C. cellulovorans* to low pH values is an obstacle to the implementation of CBP, thus it is important to evaluate the changes in the pH during growth of this bacterium on different substrates. To this end, the pH of *C. cellulovorans* cultures growing in media with cellobiose or cellulose was measured using a photometric pH assay as previously described (Takao et al., 2020). As can be seen in Figure 3.3 A, faster acidification was observed with cellobiose as the substrate, possibly caused by its faster assimilation in comparison when cellulose is used as C-source. Interestingly, the pH value during growth on cellulose was never much lower than the pH described to be optimal for growth. Perhaps, this observation is due to the fact that slower growth leads to slower production of fatty acids (e.g. butyrate), at a rate that the bacterium can adjust its metabolism to re-equilibrate the pH.

3.1.3. Sporulation

C. cellulovorans was described as an endospore-forming bacterium (Sleat et al., 1984). Obtaining spores would be a great advantage for the long-term storage and easy activation from stock suspension (compared to cells cryopreserved in glycerol stocks at –80 ºC). On the other hand, sporulation is not a desirable trait from the biotechnological point of view since improved cellulose degradation has been reported for cellulolytic bacteria with abolished sporulation (Li et al., 2014).

In order to investigate *C. cellulovorans* sporulation, different conditions were tested. Cells were cultivated in different media for different incubation times. Medium 520 agar plates with yeast extract, tryptone, carbohydrate-free tryptone, casamino acids, and no vitamin were used. 520 medium with yeast extract and low cysteine concentration (0.05 g/L) was also prepared. Cells were incubated for 5, 7, or 15 days at 37 ºC. After the incubation time, cells were harvested and heat-shocked at 70 or 80 ºC for 0, 1, 2, 11, and 30 min. Then, cells were incubated in 520 liquid media, on 520 agar plates, and both medium types supplemented with L-alanine, L-asparagine, D-glucose, D-fructose, and K (Ala-AGFK), since it was reported that this mixture can trigger germination receptors (Yi & Setlow, 2010; Cruz-Mora et al., 2015; Bressuire-Isoard, Broussolle & Carlin, 2018). All cultures were monitored microscopically. As can be seen in Figure 3.4 A, cells with atypical morphology were observed after prolonged incubation. However, no growth was observed in the pasteurized and non-pasteurized samples (even after one month incubation at 37 °C). Surprisingly, *C. cellulovorans* "endospore" suspensions, stored aerobically (in Eppendorf tubes) for 2 months at 4 °C, when inoculated in fresh medium grew within 48 h (without heat activation). Microscope observation confirmed *C. cellulovorans* identity (Figure 3.4 B).

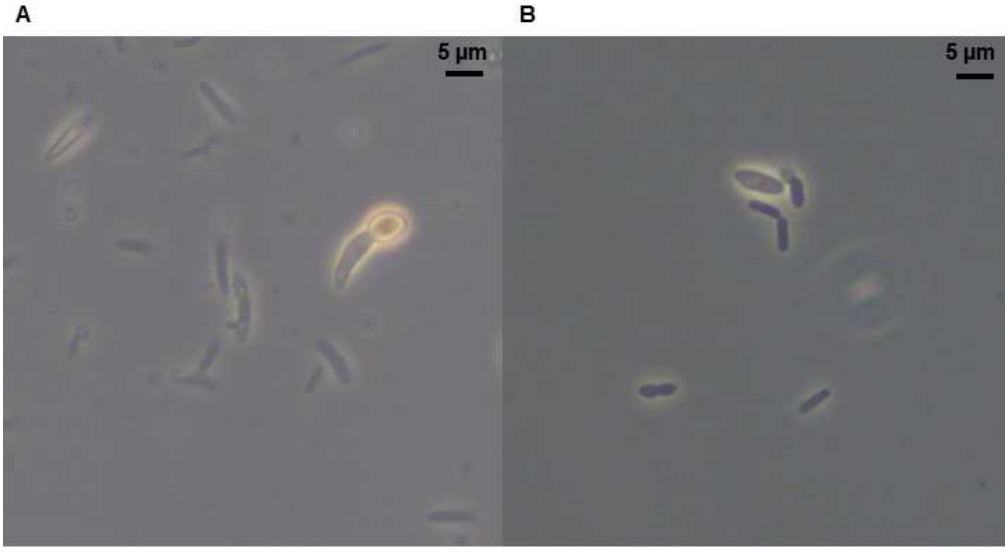

Figure 3.4 | *C. cellulovorans* potential endospores. (A) *C. cellulovorans* incubated for 15 days at 37 ºC on 520 agar plate with yeast extract and (B) *C. cellulovorans* culture incubated at 37 °C for 48 h inoculated with "endospore" suspensions maintained at 4 ºC for 2 months.1000 X magnification.

Additionally, the Schaeffer-Fulton method, a widely used endospore staining procedure, was employed to demonstrate endospores in *C. cellulovorans* cultures and, as a positive control, a spore suspension of *C. acetobutylicum* was used. Although a single spore was observed once, the swollen cells of *C. cellulovorans* do not have endospores (Figure 3.5). However, further experiments are required to show if the strain is still able to form endospores and to find the proper conditions for *C. cellulovorans* sporulation.

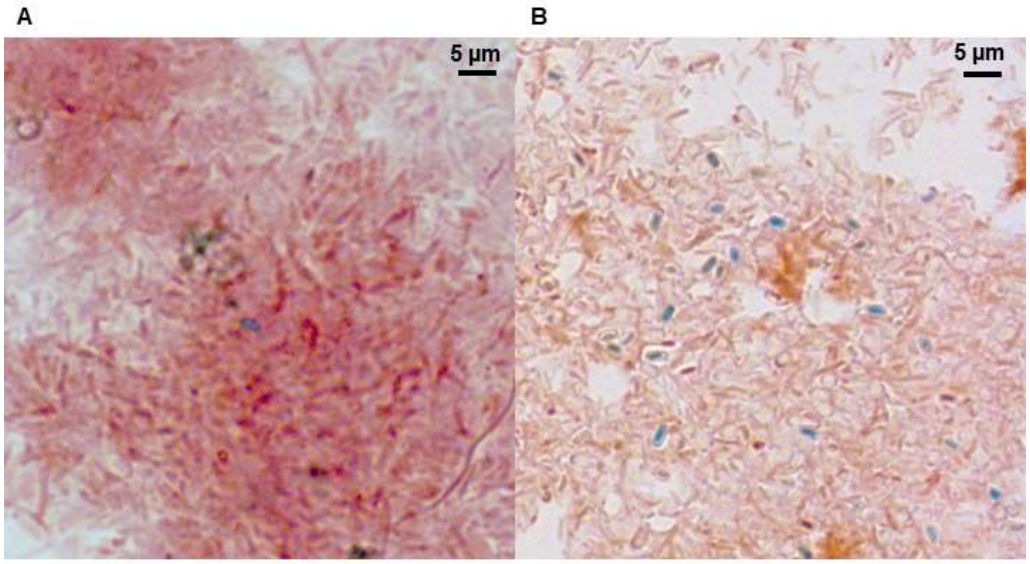

Figure 3.5 | *C. cellulovorans* vegetative cells and potential endospore. (A) *C. cellulovorans* cells after Schaeffer-Fulton staining and (B) *C. acetobutylicum* spore suspension used as a positive control. Vegetative cells stain red-pinkish and spores stain green. 1000 X magnification.

3.1.4. Phage induction

Due to recurring problems in the cultivation of *C. cellulovorans*, it was hypothesized that potential prophage induction in the host cells had led to growth problems. To identify potential prophage sequences within the *C. cellulovorans* genome, the online tool PHASTER (www.phaster.ca) was used. A total of 4 intact prophage regions were identified. The first prophage region, with a size of 64.6 kb, encodes 58 putative proteins (ORFs Clocel_0740 to Clocel_0807). The second prophage region has a size of 39.8 kb with 36 putative coding sequences (ORFs Clocel_2413 to Clocel_2458). The third prophage region is 25.2 kb in size and putatively encodes 30 proteins (ORFs

Clocel_3220 to Clocel_3550). The fourth prophage region finally, with a size of 22.5 kb, contains 23 ORFs (Clocel_3956 to Clocel_3979). Attempts to obtain a cured strain were performed. For that, UV light and mitomycin C (MMC) were used as inducers. Strains of *Latilactobacillus sakei* with and without intact prophages were used as the positive and negative control, respectively. UV exposure for 10 min did not induce cell lysis in *C. cellulovorans* or the *Lat. sakei* positive control. To test the usability of MMC, *C. cellulovorans* cultures were grown to an OD_{600nm} of 0.2 and 0.5. After, 0, 0.03, 0.3, 3, and 30 µL/mL of MMC were added to the cultures. The density of the cultures was measured periodically in intervals of 1 h, but no cell lysis was observed. However, *C. cellulovorans* stopped growth in the cultures with 3 and 30 µL/mL of MMC. As expected, MMC induced prophages resulted in significant cell lysis in the *Lat. sakei* cells with intact prophages. In a further experiments, 3 µL/mL of MMC was added to a *C. cellulovorans* culture in the late exponential phase (overnight culture), but still no cell lysis was observed. Thus, further experiments are still required to identify the proper conditions for prophage induction in *C. cellulovorans*.

3.1.5. Screening for restriction endonucleases

The restriction/modification (RM) systems are defense mechanisms against foreign DNA widely found in prokaryotic organisms. The RM systems are comprised of restriction endonucleases and methyltransferases. While the restriction endonucleases (REase) cleave double-stranded DNA, in general after recognition of specific sequences, the methyltransferase (MTase) adds methyl groups on bases within the recognition sequences in the host's genome to ensure discrimination between self and non-self DNA, thereby preventing the destruction of own DNA.

The RM systems are often responsible for low transformation efficiencies since the DNA of internalized plasmids are recognized as foreign genetic material. Therefore, they represent a strong barrier to metabolic engineering. The RM systems are grouped into four types based on their subunit composition, sequence recognition, cleavage position, cofactor requirements, and substrate specificity. An *in silico* analysis of the *C. cellulovorans* genome sequence using the online database REBASE (The Restriction Enzyme Database) predicted 12 theoretical restriction systems. These RM systems are subdivided into type I (n = 1), type II (n = 8), type III (n = 1) and type IV (n = 2) restriction systems (Fig. 3.6). However, only 4 operons encode simultaneously a

restriction endonuclease and methyltransferase, which according to the literature is a requirement for a functional RM system (Roberts et al., 2003).

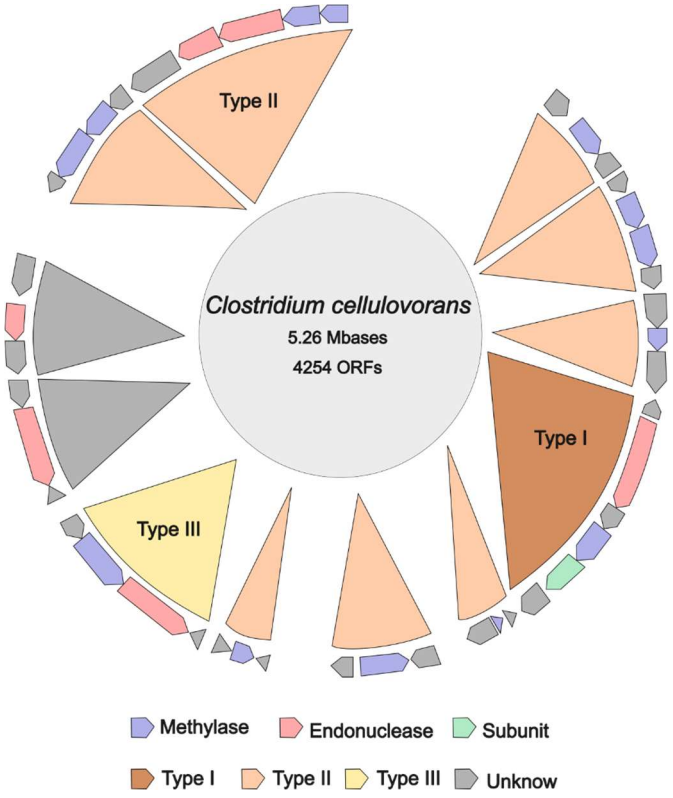

Figure 3.6 | Schematic representation of *C. cellulovorans* putative RM systems. Adapted from REBASE.

To evaluate the endonucleases activity a restriction assay was performed using cell free extract (CFE). The treatment of a plasmid DNA with the CFE should show a similar restriction patterns as predicted in the REBASE database. The recognition sequences for the endonucleases encoded by ORFs Clocel_4005 and Clocel_4006 is GACGC and CCWGG, respectively. These recognition sequences correspond to the commercially available restriction enzymes *Hga*I and *Eco*RII, respectively. Using both enzymes simultaneously 22 fragments are expected for restriction digestion of plasmid pLA_del_4005_ori8, which are: 10, 13, 17, 30, 34, 36, 59, 62, 73, 85, 98, 115, 150, 157, 191, 298, 331, 370, 529, 572, 1510 and 5926 bp. Although it is not possible to observe clear specific bands after the digestion with the CFE, the plasmid pLA_del_4005_ori8 was digested when incubated with the CFE (Figure 3.7). To test

if the plasmid was protected from restriction by the CFE if propagated in the presence of the methylation plasmid, *E. coli* cells harboring both pLA_del_4005_ori8 and placORM (methylation plasmid) were grown in liquid medium and the plasmids were isolated. As can be seen in Figure 3.7, there was no difference whether pLA_del_4005_ori8 was isolated from cells containing the methylation plasmid or not.

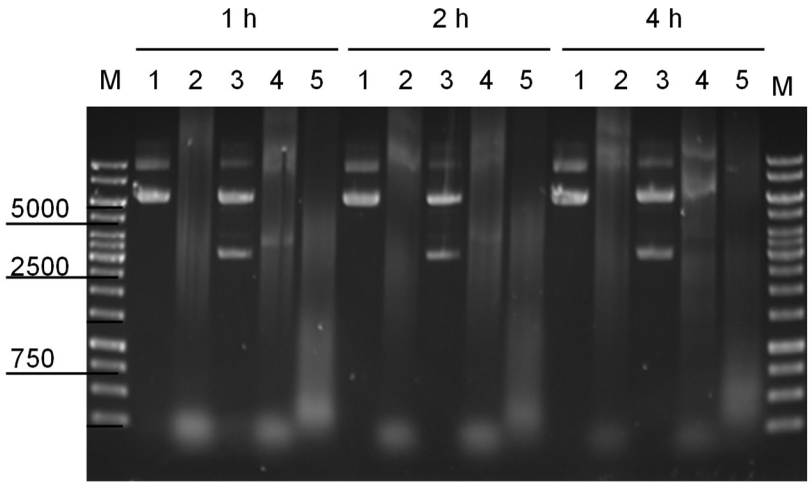

Figure 3.7 | Restriction digest of pLA_del_4005_ori8 or pLA_del_4005_ori8 together with the methylation plasmid (placORM) using *C. cellulovorans* cell free extract (CFE). The incubation was performed at 37 °C for 1, 2 or 4 h. M: GeneRuler 1 kb DNA Ladder, 1: plasmid pLA_del_4005_ori8, 2: plasmid pLA_del_4005_ori8 + CFE, 3: plasmid pLA_del_4005_ori8 + methylation plasmid, 4: plasmid pLA_del_4005_ori8 + methylation plasmid + CFE, 5: ddH_2O + CFE. M = GeneRuler 1 kb DNA Ladder was used as marker (M) and bands are expressed in base pairs (bp). Adapted from Baumgartner (2023).

To additionally test other digestion conditions, several dilutions of the CFE were prepared (1:2, 1:4 and 1:6), but when the CFE was diluted, no digestion was observed (data not shown). Also, the reaction buffer was supplemented with 1 mM ATP, since the cleavage activity for endonucleases from the type I group is ATP-dependent. However, also under these conditions, plasmid incubation with CFE still did not yield specific bands (data not shown). These experiments were performed within the Bachelor's thesis of Jana Baumgartner (Baumgartner, 2023) planned in support of this research project and co-supervised by me.

Nevertheless, it is important to consider that for type I restriction endonucleases the recognition site but not the cleavage site shows sequence specificity. Besides, unpredictable results can be observed for enzymes with star activity. The results observed here indicate that it is possible that in *C. cellulovorans*, the type I RM system plays an important role. It is important to note that the methylation plasmid used in these experiments expressed a putative type II RM system (ORFs Clocel_4007 and Clocel_4008). Therefore, future experiments should consider using a methylation plasmid that expresses a type I RM system.

3.2. Development of a markerless genome modification system and generation of a 'domesticated' strain of *C. cellulovorans*

3.2.1 Electroporation

Electroporation has been described for *C. cellulovorans* using *in vivo* methylation to protect plasmids against the restriction enzyme machinery (Yang et al., 2016). Different pMTL80000 shuttle plasmids (pMTL82151, pMTL83151, pMTL84151, and pMTL85151), with and without *in vivo* methylation were used. Electroporation was performed as described before, but no transformants were obtained after incubation in selective media. Thus, various improvement attempts have been tested, such as harvesting the cells at different stages of their growth (OD of 0.2–0.3, early exponential phase; OD of 0.5–0.6, middle exponential phase; and OD of 0.8–1.0, late exponential phase), using different amount of DNA (2 and 6 µg), and using a cell wall weakening agent (1 % glycine). After electroporation treatment, growth was always observed after overnight incubation in the recover medium, except when cells had been treated with glycine before electroporation, but still, no transformants were obtained. It is important to notice that although glycine has been used to enhance electrotransformation in Gram-positive bacteria, like *C. pasteurianum* (Pyne et al., 2013), it led to delayed growth in *C. cellulovorans* (OD of 0.3 after 24 h incubation) and it also had an impact on the size of the cells (Fig. 3.8). It is also worth noting that the pulse time observed was 10–30 ms, contrary to the 4–6 ms obtained before (Yang et al., 2016).

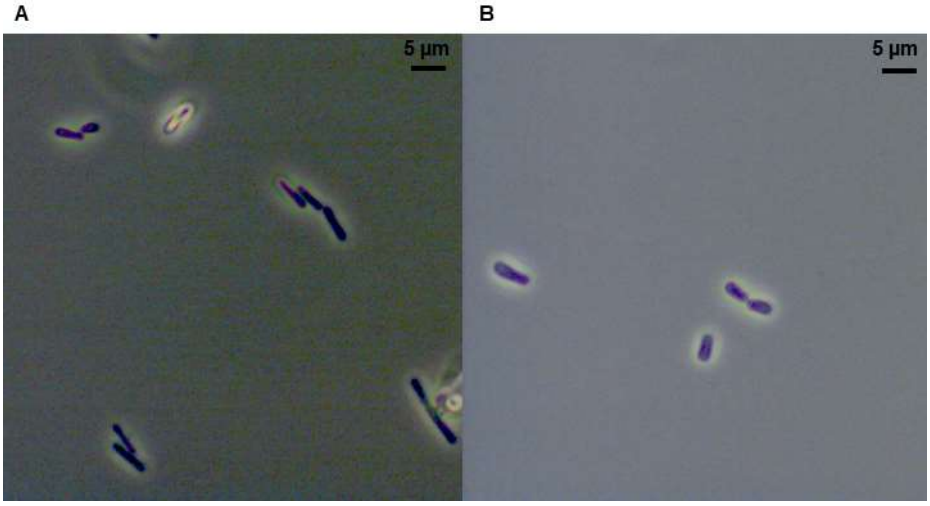

Figure 3.8 | (A) *C. cellulovorans* culture before and (B) after glycine treatment. After 24 h incubation in medium containing glycine an OD_{600nm} of 0.3 was measured, indicating that this treatment led to growth delay.

Since electroporation was not found to be applicable for transformation of *C. cellulovorans* in our hands, further attempts with this technique were dismissed and the use of triparental conjugation from *E. coli* was established in our group for the introduction of recombinant plasmids into *C. cellulovorans*.

3.2.2. The *codBA*-based counterselection technique

The Allele-Coupled Exchange (ACE) method allows the insertion and excision of a heterologous DNA sequence with a broad range of size or complexity (Heap et al., 2012; Minton et al., 2016). This strategy relies on the homologous recombination process, which occurs naturally in all species during DNA replication and repair of strand breaks. In the first step of ACE, a deletion vector containing homologous flanks to the gene of interest (GOI) is transferred into the host. The presence of a selection marker in a suicide deletion vector allows the selection of clones that have integrated the vector into the genome by incubation on a selective medium. In the second step of ACE, the vector must be excised again from the genome. For that, a counterselection marker must be present in the selection vector. Thus, after integration, clones should be incubated in the presence of the counterselective compound to select the clones that have lost the vector after the second recombination event.

To find a counterselection marker that can be used in the host, it is essential to (i) analyze the genome of the host organism to verify the absence of the marker and (ii) to ensure the availability of a counterselective compound that can be easily added to the medium. The *codBA* counterselection is based on the presence of the *codBA* cassette in the deletion plasmid and the use of the 5-fluorocytosine (5-FC) as the counterselective compound. While *codB* codes for a transporter for the uptake of 5-FC, *codA* encodes a cytosine deaminase converting non-toxic 5-FC into the highly toxic 5-fluorouracil (5-FU). Therefore, in the presence of 5-FC, only strains without *codBA*, which after successful application of the two-step ACE procedure described above represent either wild-type clones or mutants with the desired chromosomal deletion, can grow. The *codBA* operon genes (CLJU_c19220 and CLJU_c19230, respectively, Fig. 3.9) from *C. ljungdahlii* were previously used for genomic modification in *C. saccharobutylicum* (Huang et al., 2018). *In silico* analysis of the *C. cellulovorans* genome using The Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) was performed to identify sequences similar to the *codB* and *codA* genes from *C. ljungdahlii*. No significant similarities were identified, indicating the absence of the *codB* and *codA* genes in *C. cellulovorans* genome. Subsequently, the determination of minimal inhibitory concentrations (MIC) of 5-FC and 5-FC was performed to confirm this result.

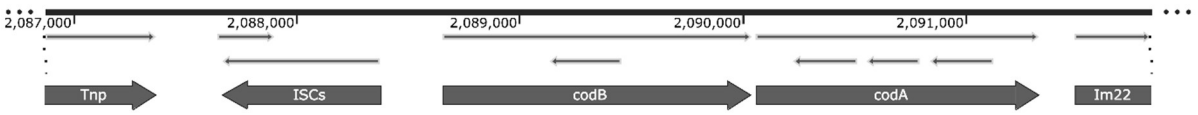

Figure 3.9 | Genomic region of the *codBA* operon in *C. ljungdahlii*. Tnp, transposase; ISCs, putative Iron–sulfur (Fe/S) cluster binding protein; *codB*, cytosine permease (predicted nucleoside transporter); *codA*, cytosine deaminase; Im22, putative immunity protein 22 of polymorphic toxin system.

Usually, yeast extract is added in the medium as a source of amino acids, nucleic acid bases and vitamins. However, according to the literature, it might interfere with the uptake of the 5-FC (Huang et al., 2018). Therefore, different concentrations of yeast extract were tested. Additionally, the MIC was performed with medium containing tryptone (instead of yeast extract) or medium with neither yeast extract nor tryptone.

In medium containing 0.2 % yeast extract, *C. cellulovorans* wild-type was able to grow on all tested concentrations of 5-FC in a range of up to 1500 µg/mL (Table 3.1). Confirming the absence of a functional *codA* gene. Interestingly, at reduced concentrations of yeast extract, 5-FC showed a negative effect on the growth. This might be due to the metabolization of 5-FC in unexpected pathways. Additionally, the MIC of 5-FU was also determined to ensure its toxic effect, which is necessary for the deletion system. Even a small concentration of 5-FU such as 6.25 µg/mL was sufficient to inhibit the growth under all tested conditions (Table 3.2).

Table 3.1 | Growth of *C. cellulovorans* 743B in 520 medium containing 5-fluorocytosine (5-FC) and different concentrations of yeast extract and tryptone.

Condition	5-FC concentration (µg/mL)					
	0	93.75	187.5	375	750	1500
0.2 % yeast extract	+	+	+	+	+	+
0.02 % yeast extract	+	+	+	+/-	-	-
0.002 % yeast extract	+	+	-	-	-	-
0.2 % tryptone	+	+	+	+	+/-	-
No yeast extract and no tryptone	+	+	+	-	-	-

+ = growth +/- = mild growth - = no growth

Table 3.2 | Growth of *C. cellulovorans* 743B in 520 medium containing 5-fluorouracil (5-FU) and different concentrations of yeast extract and tryptone.

Condition	5-FU concentration (µg/mL)								
	0	0.39	0.78	1.58	3.12	6.25	12.5	25	50
0.2 % yeast extract	+	+	+	+	+/-	-	-	-	-
0.02 % yeast extract	+	+	+/-	-	-	-	-	-	-
0.002 % yeast extract	+	+	+	-	-	-	-	-	-
0.2 % tryptone	+	+	+	+/-	-	-	-	-	-
No yeast extract and no tryptone	+	+	-	-	-	-	-	-	-

+ = growth +/- = mild growth - = no growth

3.2.3. Promoter optimization for efficient *codBA* expression

The *codBA* cassette from *C. ljungdahlii*, used for genome modification in *C. saccharobutylicum* (Huang et al., 2018), was expressed under the functional control of the constitutive P_{clpB} promoter from *Staphylococcus aureus* (Kostner et al., 2017). However, sequencing analysis revealed mutations in the promoter sequence (Holger Edelmann, unpublished). With this in mind, constitutive and inducible promoters were cloned in the upstream region of the pMTL83151 plasmid containing the *codBA* cassette to evaluate the usability of different promoters. The plasmid pMTL83151_codBA was constructed using the primers pMTL83151_backbone_fw and pMTL83151_backbone_rv for the amplification of the backbone (pMTL83151), and the primers CodBA_S_pMTL83151_fw and CodBA_S_pMTL83151_rev for the amplification of the insert (*codBA* region present in the plasmid pHE1-C2). The primers seq_pMTL83151_codBA_fw, seq_pMTL83151_codBA_rv, CodBA_Seq2, CodBA_Seq3 and CodBA_Seq4 were used for sequencing (Table 7.1). All primer sequences are listed in the appendixes. The molecular cloning of the plasmid pMTL83151_codBA was successful. However, it was not possible to clone this plasmid together with the methylation plasmid in the *E. coli* donor strain to be used for conjugation. Possibly, this effect was observed because the P_{fdx} is a strong promoter (Gyulev et al., 2018) and the expression of a membrane protein, such as the cytosine permease encoded by the *codB* gene, may pose a problem for *E. coli*, resulting in poor growth/inefficient recovery after transformation.

To overcome this problem, the *codBA* operon was placed under the control of regulated promoters. To this end, two different regulable arabinose-inducible gene expression systems were used. The *araBAD* system, the most well-known arabinose inducible system, uses AraC as a repressor to control the expression of downstream genes under the control of the promoter P_{BAD}. This system is derived from *E. coli* and has been used in several Gram-negative and Gram-positive bacteria (Carroll et al., 2007; Sukchawalit et al., 1999). The ARAi system derived from *C. acetobutylicum* consists of the promoter P_{ptk} and the repressor AraR (Zhang et al., 2015). This system was successfully used for genetic modifications in *C. cellulolyticum*. In both cases, transcription is blocked when arabinose is absent. Both systems were cloned into pMTL83151_codBA, resulting in the plasmids pMTL83151codBA_araBAD and pMTL83151codBA_ARAi.

The plasmid pMTL83151codBA_araBAD was constructed using the primers pMTL83151CodBA_backbone_S_fw and pMTL83151CodBA_backbone_S_rv for the amplification of the backbone (pMTL83151_CodBA), and the primers araBAD_S_fw and araBAD_S_rv were used for the amplification of the insert (*araC* repressor and P_{araBAD} present in the plasmid pBAD_myc_his). The primers check_pMTL83151codBA_araBAD_fw, check_pMTL83151_codBA_araBAD_rv and Seq_araBADP_codB were used for sequencing (Table 7.2).

The plasmid pMTL83151codBA_ARAi was constructed using the primers pMTLcodBA_fw and pMTLcodBA_rv for amplification of the backbone (pMTL83151_CodBA), and the primers araR_fw, araR_rv, ptk_fw and ptk_rv were used for amplification of the insert (genomic DNA of *C. acetobutylicum* was used as template for the amplification of the arabinose repressor (*araR*) and P_{ptk} promoter). The primers check_pMTL83151codBA_ARAi_fw, check_pMTL83151codBA_ARAi_rv and Seq_ptk_codB were used for sequencing (Table 7.3).

Next, growth curves of *E. coli* in LB medium supplemented with 0, 0.1, 1, and 10 g/L L-arabinose were performed. As can be seen in Figure 3.10, the addition of L-arabinose leads to weaker growth of *E. coli* when either araBAD or ARAi plasmids were present, although a similar growth in LB and LB with 10 g/L L-arabinose was observed for *E. coli* harboring the pMTL83151codBA_ARAi plasmid. This result indicates that the expression of an additional permease may have affected *E. coli* metabolism. The plasmids pMTL83151 and the pMTL83151_codBA (containing the P_{fdx} promoter - not inducible) were used as controls. The growth of *E. coli* with the pMTL83151_codBA was similar under all tested conditions.

Then, the plasmids pMTL83151codBA_araBAD, and pMTL83151codBA_ARAi were *in vivo* methylated in *E. coli* carrying the methylation plasmid placORM and transferred into *C. cellulovorans* via triparental mating. After conjugation, it was possible to obtain 1 colony with the pMTL83151codBA_araBAD and 3 colonies with the pMTL83151codBA_ARAi plasmid. The presence of the *codBA* operon was confirmed by PCR (Fig. 3.11). A faint band was observed for one of the colonies with the plasmid pMTL83151codBA_ARAi. To confirm the identity of *C. cellulovorons*, the ORFs Clocel_3986 and Clocel_3987 were amplified from the gDNA (using the primers AS11

and AS12, Table 7.1) and sent for sequencing. An identity greater than 97 % was observed for all colonies.

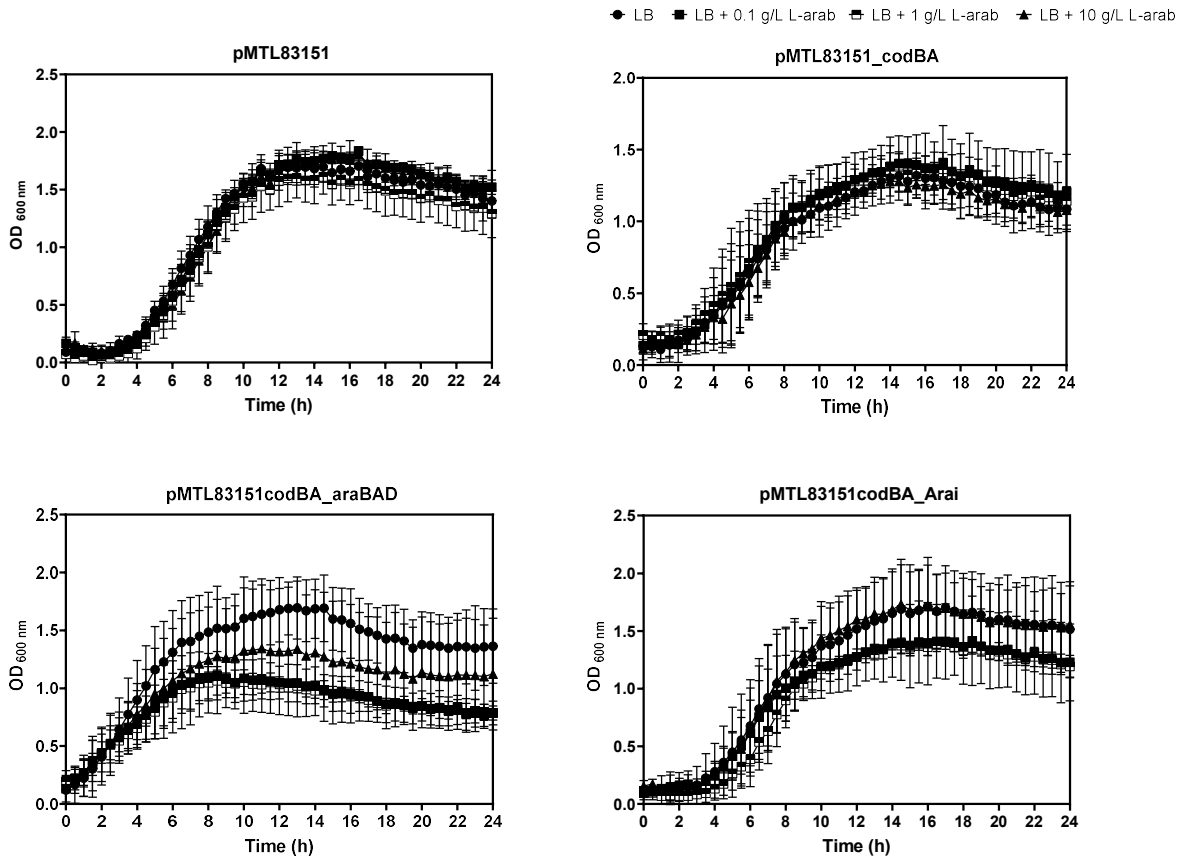

Figure 3.10 | Growth curves of *E. coli* strains harboring different recombinant plasmids with *codBA* and arabinose-inducible promoters (pMTL83151codBA_araBAD and pMTL83151codBA_Arai). *E. coli* strains harboring recombinant plasmids without the *codBA* cassette (pMTL83151) and with the *codBA* cassette under the control of the P_{fdx} promoter (pMTL83151codBA) were used as controls. The growth was performed in LB media supplemented with 0, 0.1, 1, and 10 g/L L-arabinose (L-ara) for induction.

Further, the determination of the 5-FC MIC of the transconjugants was performed. To this end, 520 agar plates containing 0, 25, and 200 µg/mL of 5-FC were prepared. Besides, several concentrations of cellobiose and L-arabinose were also tested. As can be seen in Table 3.3, *C. cellulovorans* colonies with codBA plasmid contructs were not sensitive to 5-FC as expected. The negative control (cells carrying pMTL83151) was also resistant to 5-FC.

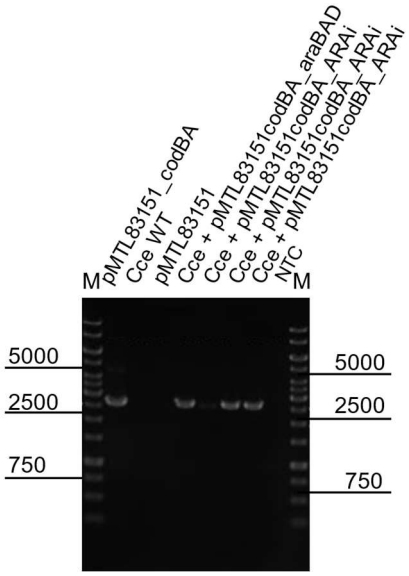

Figure 3.11 | PCR amplification of the *codBA* operon (size: 2.675 kb) in *C. cellulovorans* transconjugant clones obtained after triparental conjugation. The pMTL83151_codBA plasmid was used as a positive control for PCR. *C. cellulovorons* (Cce) wild type gDNA was used as a negative control. The pMTL83151 plasmid was used as a negative control as well. The single clone of *C. cellulovorons* + pMTL83151codBA_araBAD and all three clones of *C. cellulovorons* + pMTL83151codBA_ARAi were analyzed. NTC = non-template control, M = GeneRuler 1 kb DNA Ladder was used as marker (M) and bands are expressed in base-pairs (bp).

Table 3.3 | 5-Fluorocytosine (5-FC) minimum inhibitory concentration (MIC) for *C. cellulovorans* harboring different recombinant *codBA* plasmids.

Condition		**5-FC MIC (µg/mL)**			
Cellobiose (g/L)	L-Arabinose (g/L)	pMTL83151 codBA_araBAD (colony 1)	pMTL83151 codBA_ARAi (colony 1)	pMTL83151 codBA_ARAi (colony 2)	pMTL83151 codBA_ARAi (colony 3)
5	0	> 200	> 200	> 200	> 200
5	0.1	> 200	> 200	> 200	> 200
5	1	> 200	> 200	> 200	> 200
5	10	> 200	> 200	200	200
1	10	> 200	> 200	> 200	> 200
0	10	no growth	> 200	no growth	no growth

Inhibition of growth was observed just for the cells with pMTL83151codBA_ARAi (colonies 2 and 3) on plates with 5 g/L cellobiose and 10 g/L L-arabinose. Subsequently, determination of the MIC was performed just for colonies 2 and 3 with the pMTL83151codBA_ARAi plasmid using 0, 25, 50, 100, and 200 µg/mL of 5-FC in

plates with 5 g/L cellobiose and 10 g/L L-arabinose. It was observed that 200 µg/mL of 5-FC inhibited the growth. This result indicates that the *codBA* operon under the control of the ARAi system was expressed. However, the MIC was higher than the 30 µg/mL observed for *C. saccharobutylicum* (Huang et al., 2018), suggesting that the operon was not well expressed, either due to insufficient functioning of the selected promoters or insufficient induction. It is noteworthy that *C. cellulovorans* can grow on liquid medium with L-arabinose as the sole C-source. However, the growth is delayed when compared to liquid medium containing cellobiose. Only after 48 h incubation growth was observed. This indicates that the use of an arabinose-inducible promoter for the expression of the *codB* and *codA* gene might not be sufficient. At the time the present study was being conducted, the use of the mutated P_{clpB} resulted in successful genome modification in *C. acetobutylicum* (Holger Edelmann, unpublished). Therefore, for the upcoming experiments, the usability of the P_{clpB} to regulate the *codBA* operon expression was also investigated.

3.2.4. Identification of pseudo-suicide replicon

ACE vectors typically contain a suitable replication defective Gram-positive replicon, also called “pseudo-suicide” replicon. The presence of a “pseudo-suicide” replicon in the ACE vectors is crucial for two reasons. First, it helps to introduce the vector into the host, since the transfer of suicide vectors is frequently difficult in clostridia (Cartman & Minton, 2010; Minton et al., 2016). Second, it allows the differentiation between integrant clones (cells in which the marker becomes associated with the chromosome after the plasmid’s integration during the first homologous recombination event) and clones with the plasmid replicating autonomously (Kuehne et al., 2011). While the use of a suicide vector allows the selection of the integrant clones under antibiotic selection, the use of a “pseudo-suicide” replicon allows the enrichment of the integrant clones from a population of cells. The growth rate of integrant clones is not limited by the rate at which the pseudo-suicide vector can replicate and segregate into daughter cells (Kuehne et al., 2011). Therefore, integrants grow faster and produce visibly larger colonies since all of the progenies carry a copy of the resistance cassette present in the ACE vector (Ehsaan et al., 2016).

First, the compatibility of different Gram-positive replicons in *C. cellulovorans* was investigated. For that, shuttle plasmids from the pMTL80000 modular plasmid series

were transferred into *C. cellulovorans* via transconjugation. All tested plasmids contained the *catP* gene as the selective marker (conferring chloramphenicol/thiamphenicol resistance), the Gram-negative replicon ColE1 (for plasmid replication in Gram-negative bacteria, such as *E. coli*), the *traJ* gene (for the activation of conjugation genes) and a multiple cloning site (MCS); they only differ in the Gram-positive replicon. In addition, the Gram-positive replicon in the pMTL83151 plasmid was exchanged by the temperature-sensitive (ts) replicon from pE194, resulting in pMTL8x151_pE194(ts). The pKVM4 plasmid (Kostner et al., 2017) was used as source of the insert and the replicon pE194(ts) was obtained using the primers pE194ts_AscI_fw and pE194ts_FseI_rv (Table 7.4). The pMTL83151 plasmid was used as the source of the vector. The restriction enzymes *Asc*I and *Fse*I were used to digest the backbone and insert. After digestion, the products were ligated using T4 DNA ligase. The resulting product was transformed into chemically competent *E. coli* and isolated using standard procedures. All tested plasmids and their respective Gram-positive replicon are listed in Table 3.4.

Table 3.4 | Gram-positive origins of replication present in the shuttle vectors used in this study and strains where the replicon was originally isolated from.

Plasmids	Gram-positive replicon	Origin host of the replicon	Reference
pMTL81151	pCB101	*C. butyricum* NCIB 7423	Brehm et al. (1992)
pMTL82151	pBP1	*C. botulinum* NCTC 2916	Davis (1998)
pMTL83151	pCB102	*C. butyricum* NCIB 7423	Minton, N. & Morris, G. (1981)
pMTL84151	pCD6	*C. difficile* CD6	Purdy et al. (2002)
pMTL85151	pIM13	*Bacillus subtilis* BD1109	Monod et al., (1986)
pMTL87151	pUB110	*Staphylococcus aureus*	Lacey, R. & Chopra, I. (1974)
pMTL88151	p19	*C. carboxidivorans* P7T	Bruant et al. (2010)
pMTL89151	pAMβ1	*Streptococcus lactis*	Oultram, J. & Young, M. (1985)
pMTL8x151_pE194(ts)	pE194(ts)	*S. aureus*	Iordănescu (1976)

Among all tested plasmids, the pMTL82151, pMTL83151, pMTL87151, pMTL88151 and pMTL89151, containing the replicons pBP1, pCB102, pUB110, p19 and pAMβ1, respectively, were successfully transconjugated into *C. cellulovorans*. Thus, five suitable Gram-positive origins of replication (ori) were identified (Fig. 3.12).

Subsequently, the integrity of the plasmids after conjugation was analyzed. All plasmids were isolated from *C. cellulovorans* using the standard procedure for DNA extraction. Thus, after DNA extraction, the total DNA (genomic DNA [gDNA] and plasmids) was transferred back into *E. coli* (re-transformation).

In the following, the plasmids were isolated from *E. coli* using the standard procedure and digested with the enzymes *Asc*I and *Fse*I. For the plasmid pMTL82151 (5.251 kb) the expected fragment lengths were 2.851 kb and 2.403 kb. For pMTL83151 (4.476 kb) the expected fragment lengths were 2.851 kb and 1.625 kb. For pMTL87151 (3.868 kb) the expected fragment lengths were 2.851 kb and 1.017 kb. For pMTL88151 (4.798 kb) the expected fragment lengths were 2.851 kb and 1.947 kb. For pMTL89151 (5.771 kb) the expected fragment lengths were 2.920 kb and 2.851 kb.

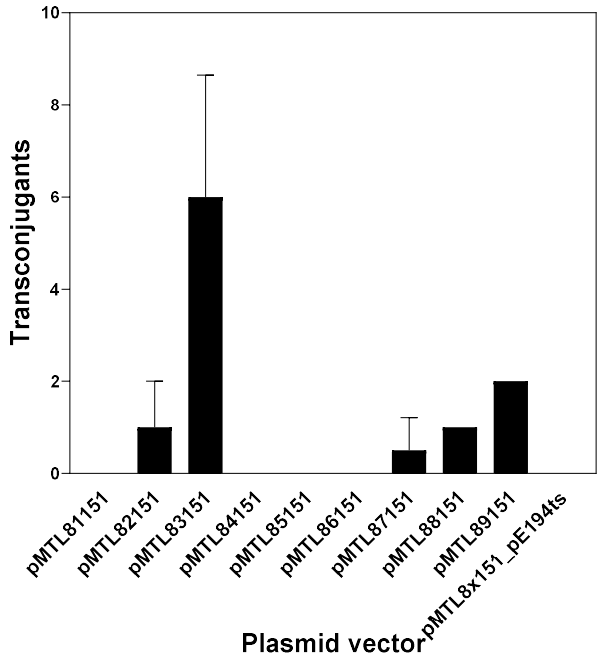

Figure 3.12 | Number of *C. cellulovorans* transconjugants obtained using different shuttle plasmids from the pMTL80000 series and the plasmid containing the thermosensitive (ts) replicon pMTL8x151_pE194ts. This figure is adapted from Almeida et al. (2024, submitted for publication).

As can be seen in Figure 3.13, unexpected bands were observed, probably due to incomplete digestion of the plasmids and star activity of the restriction endonucleases. However, all tested plasmids showed similar band patterns before and after transconjugation in *C. cellulovorans*. The only exception is plasmid pMTL89151, indicating that the plasmid underwent genetic modification in *C. cellulovorans*.

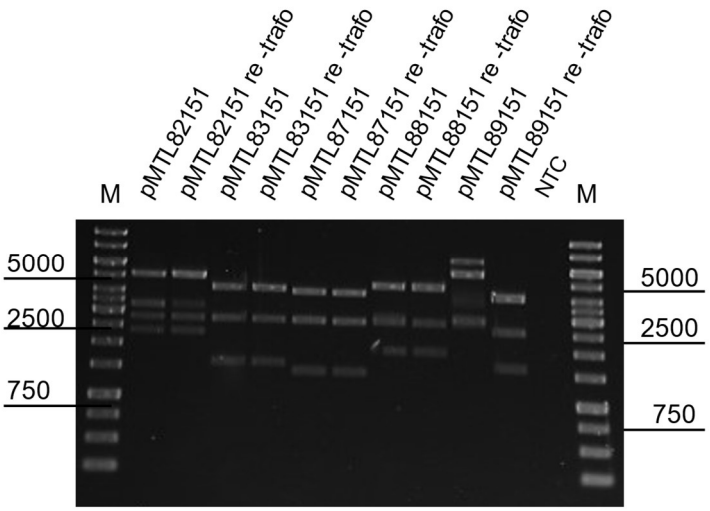

Figure 3.13 | Confirmation of the plasmid integrity after transconjugation in *C. cellulovorans*. Plasmids were isolated from *E. coli* and from *C. cellulovorans* (re-trafo) were digested with *Asc*I and *Fse*I. NTC = non-template control, M = GeneRuler 1 kb DNA Ladder was used as marker (M) and bands are expressed in base-pairs (bp).

Thereafter, the plasmid stability was evaluated to find the most unstable replicon. For that, *C. cellulovorans* strain containing the plasmids pMTL82151, pMTL83151, pMTL87151, pMTL88151 and pMTL89151 were inoculated in liquid medium containing 15 µg/mL thiamphenicol to ensure the presence of the plasmid. Then, the cultures were subcultured in medium lacking antibiotic to promote plasmid loss during segregation. After each subculture, the cells were spread on plates (also lacking antibiotic) to isolate single colonies. A total of 50 single colonies were streaked on a plate containing 15 µg/mL to determine the percentage of thiamphenicol-resistant colonies. Since only bacteria retaining the plasmid can grow on the selective plate, the number of thiamphenicol-resistant colonies was taken as an indicator of stability.

Plasmid pMTL87151, containing the replicon pUB110, was found to be the plasmid with the most unstable replication in *C. cellulovorans*. As can be seen in Figure 3.14, already after the first subculture in medium without antibiotic, all selected colonies had lost the plasmid. Followed by the pMTL82151 and pMTL8815, containing respectively the replicons pBP1 and p19. For these plasmids approximately 25 % of the clones still kept the plasmid even after four subculturing steps in medium without antibiotic. For the plasmid pMTL89151, approximately 70 % of the cells kept the plasmid after the fourth subculturing step. The most stable plasmid was pMTL83151, containing the replicon pCB102, where 100 % of the cells kept the plasmid even without selective pressure. With this in mind, pUB110 was selected to be part of the deletion vectors used in this work. However, since the number of transconjugants of the plasmid pMTL87151 was extremely low, the replicon p19 (middle-stable) and pCB102 were also cloned in the deletion vectors in an attempt to obtain more clones with the deletion vectors after conjugation. The plasmid stability assay was performed together with Maria Marquez and Selina Zenz during their research internship in the framework of this thesis under my co-supervision.

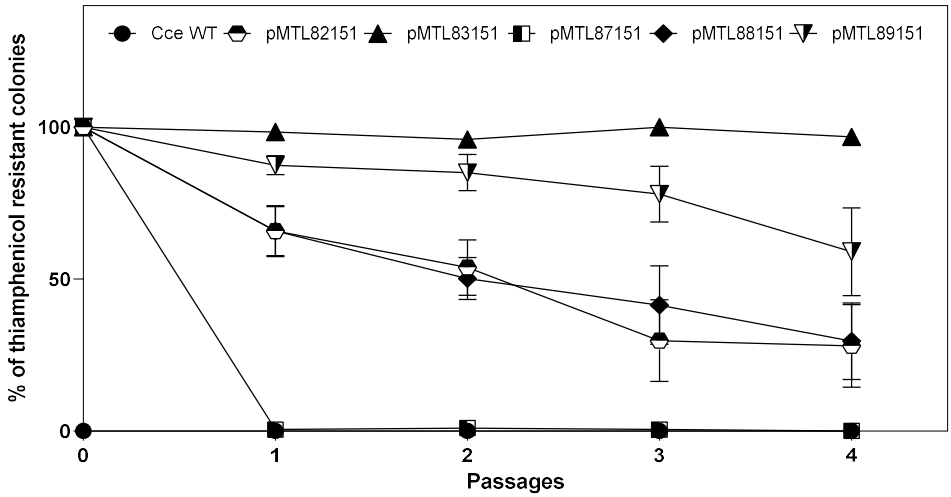

Figure 3.14 | Plasmid segregational stability in *C. cellulovorans* after four successive subcultures in medium without antibiotic. A total of 50 colonies were spread on selective plates after each subculture (passage). *C. cellulovorans* (Cce) WT was used as negative control. Values are averages of at least three biological replicates and error bars represent the standard error of mean. This figure is adapted from Almeida et al. (2024, submitted for publication).

Additionally, the copy number in *C. cellulovorans* of the plasmids pMTL82151, pMTL83151, pMTL87151, pMTL88151 and pMTL89151, containing respectively the replicons pBP1, pCB102, pUB110, p19 and pAMβ1 was examined. Real-time quantitative PCR (qPCR) was used to estimate the plasmid copy number. Primers for the target gene (*catP*) and reference gene (Clocel_3734) are listed in Table 7.5. Detailed information regarding plasmid copy number determination can be found in section 2.4.3.

Although there is no universal categorization, plasmids can be classified as low (1–10 copies), medium (11–20 copies) or high copy number (>20 copies) (Providenti et al., 2006). The plasmids pMTL82151 (copy number 7.21 ± 1.95), pMTL87151 (copy number 0.73 ± 0.31) and pMTL88151 (copy number 1.83 ± 0.37) were identified to be low copy number. The plasmid pMTL83151 (copy number 15.41 ± 3.83) and pMTL89151 (copy number 13.96 ± 1.88) can be classified as having a medium copy number (Fig. 3.15). Not surprisingly, the plasmid pMTL87151 showed the lowest copy number and was the most unstable. Also unsurprising, the plasmids pMTL83151 and pMTL89151 showed higher copy number and were very stable.

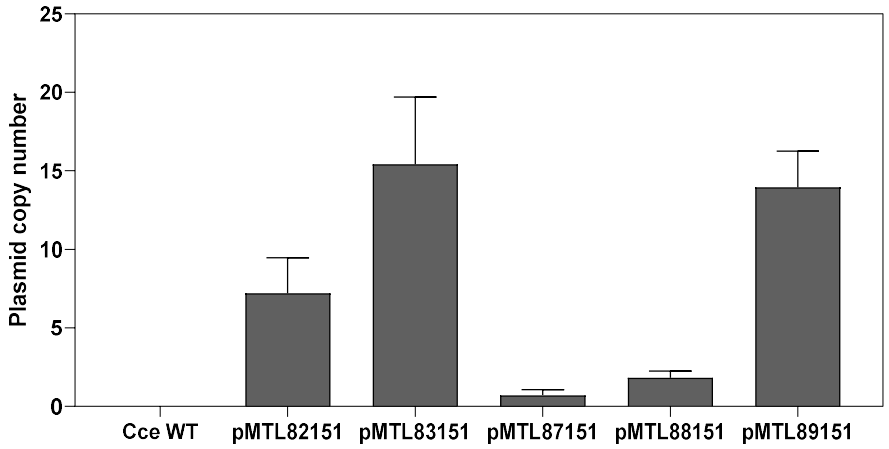

Figure 3.15 | Plasmid copy numbers relative to the assumed single-copy chromosome in *C. cellulovorans* as determined by qPCR. *C. cellulovorans* (Cce) WT was used as negative control. Values are averages of at least three biological replicates and error bars represent the propagated standard deviation. This figure is adapted from Almeida et al. (2024, submitted for publication).

3.2.5. Construction of restriction-less strains of *C. cellulovorans*

3.2.5.1. Construction of the deletion plasmids

RM systems allow the discrimination of self and non-self DNA, protecting bacteria against bacteriophage infections. However, the plasmid vectors used for targeted genomic modification are also recognized as non-self DNA and, therefore, are destroyed by this natural defense mechanism found in bacteria. Thus, overcoming the RM systems is an essential step to reach efficient introduction of the deletion vectors into bacterial host cells (Johnston et al., 2019; Minton et al., 2016; Riley & Guss, 2021).

Although *in silico* analysis revealed 12 theoretical restriction systems in *C. cellulovorans*, only 4 operons encode simultaneously a restriction endonuclease and methyltransferase, which according to the literature is a prerequisite for functional RM systems (Roberts et al., 2003). To knock out *C. cellulovorans* RM systems, deletion plasmids targeting the putative restriction endonucleases Clocel_1114 (type I), Clocel_4005 (type II), Clocel_4006 (type II), and Clocel_2651 (type III) were constructed.

The plasmids pLA_del_ori3 (Table 7.6), pLA_del_ori7, and pLA_del_ori8 were used as the plasmid backbone. The Gram-positive replicon pCB102 (also referred as “ori3”) in the plasmid pLA_del_ori3 was replaced by the Gram-positive replicons pUB110 (also referred as “ori7”) and p19 (also referred as “ori8”), present in the plasmids pMTL87151 and pMTL88151, respectively, via restriction-based cloning using the enzymes *Asc*I and *Fse*I. The plasmids were linearized via PCR using the primers pCN9_F_backbone and pCN9_R backbone.

The flanking region upstream of the ORF Clocel_1114 was amplified with the primers upflank_1113_fw2 and upflank_1113_rv, generating a fragment of 0.774 kb. The flanking region downstream of the target gene was amplified using the primers lowflank_1115_fw and lowflank_1115_rv2, generating a fragment of 1.056 kb. The primers Del_1114_fw_2 and Del_1114_rv_2 were used to verify the presence of the genomic deletion (Table 7.7).

The flanking region upstream of the ORF Clocel_4005 was amplified with the primers upflank_4004_fw and upflank_4004_rv, generating a fragment of 1.092 kb. The

flanking region downstream of the target gene was amplified using the primers lowflank_4006_fw and lowflank_4006_rv, generating a fragment of 1.099 kb. The primers Del_4005_fw and Del_4005_rv were used to verify the presence of the genomic deletion (Table 7.8).

The flanking region upstream of the ORF Clocel_4006 was amplified with the primers upflank_4005_fw and upflank_4005_rv, generating a fragment of 0.976 kb. The flanking region downstream of the target gene was amplified using the primers lowflank_4007_fw and lowflank_4007_rv, generating a fragment of 1.010 kb. The primers Del_4006_fw and Del_4006_rv were used to verify the presence of the genomic deletion (Table 7.9).

The flanking region upstream of the ORF Clocel_2651 was amplified with the primers upflank_2650_fw and upflank_2650_rv, generating a fragment of 0.901 kb. The flanking region downstream of the target gene was amplified using the primers lowflank_2652_fw2 and lowflank_2652_rv2, generating a fragment of 0.974 kb. The primers Del_2651_fw_2 and Del_2651_rv were used to verify the presence of the genomic deletion (Table 7.10).

All the PCR products for the upper and lower flanks contained an overlap sequence of 15–30 bp to the adjacent fragment. Vector and fragments were assembled in a one-step isothermal reaction using Gibson assembly (using the NEBuilder® HiFi DNA Assembly Master Mix). Subsequently, the plasmids were sent for sequencing and the presence of the insert was confirmed using the primers pLA_del_check_fw, pLA_del_check_rv, pLA_del_1114_check, pLA_del_4005_check, pLA_del_4006_check, pLA_del_2651_check, pLA_del_ori7_check_rv and/or pLA_del_ori8_check_rv. When required, the cloning primers were also used for sequencing to ensure that the whole insert was correct. In total, 12 deletion vectors were constructed, differing either in the homologous flanking region (target dependent) or Gram-positive replicon. The plasmids were constructed together with Jana Baumgartner and Selina Zenz (both at Chair of Microbiology, TUM), whose Bachelor's theses were co-supervised by me in the framework of this dissertation project (Baumgartner, 2023; Zenz, 2023). A schematic representation illustrating the deletion vectors is shown in Figure 3.16.

3.2.5.2. Determination of the 5-FC MIC for *C. cellulovorans* harboring the deletion vectors

Yeast extract is a complex supplement frequently used in medium for cultivation of bacteria. Since it contains nucleotides in its composition, it might interfere with the proper uptake of 5-FC during the counterselection. Therefore, the use of a less rich medium supplement such as tryptone is a more adequate option.

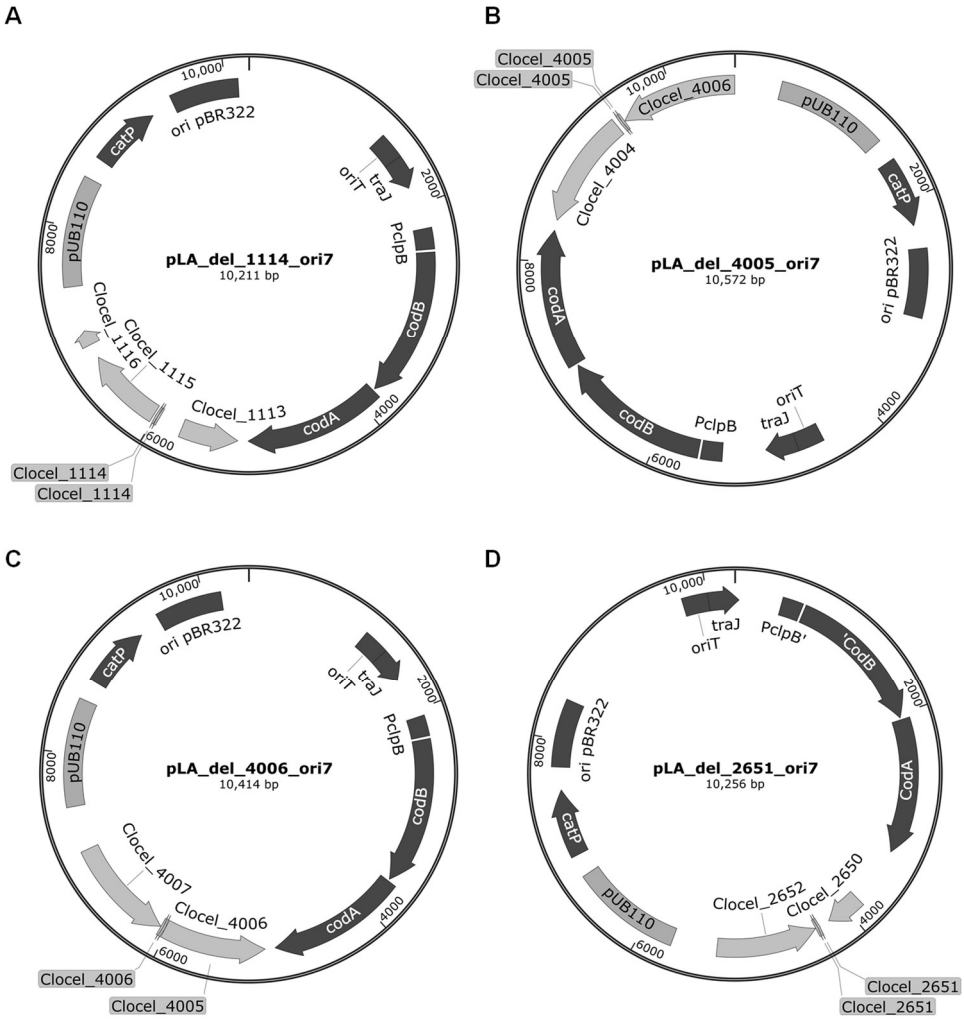

Figure 3.16 | Maps illustrating the deletion plasmidd used for the deletion of genes encoding putative restriction endonucleasesin *C. cellulovorans.* (A) Clocel_1114 (type I), (B) Clocel_4005 (type II), (C) Clocel_4006 (type II), and (D) Clocel_2651 (type III). P_{clpB}: promoter of the gene encoding the chaperone protein ClpB, *codB*: cytosine permease ORF, *codA*: cytosine deaminase ORF, pUB110: Gram-positive origin of replicon, *catP*: gene conferring chloramphenicol/thiamphenicol resistance, *traJ*: ORF encoding protein essential for regulating the expression of transfer gene involved in the conjugal transfer of DNA, *oriT*: origin of transfer necessary for the transfer of DNA.

According to the results for the determination of the 5-FC MIC for *C. cellulovorans* wild type, the use of medium containing 2 g/L tryptone as amino acid source efficiently supported growth. Therefore, to evaluate the effectiveness of the *codBA* counterselection technique, the determination of the 5-FC MIC for *C. cellulovorans* strains harboring the deletion vectors was performed. For that, 520 medium agar plates containing 2 g/L tryptone and 15 µg/mL thiamphenicol (to maintain the deletion plasmids) were used. However, it was observed that *C. cellulovorans* with all deletion vectors tested could grow normally even in the presence of 200 µg/mL 5-FC (Table 3.5), indicating a problem in the uptake of the counterselective compound.

To solve this problem, the tryptone component of the medium was replaced by casamino acids (which does not contain nucleic acid bases or nucleosides). The medium was supplemented with tryptophan (an amino acid missing in casamino acids) and a mix of vitamins to support better *C. cellulovorans* growth. Additionally, adenosine and guanosine were also added to the medium to ensure the presence of enough nucleosides to inhibit biosynthesis of nucleotides (Holger Edelmann, personal communication). With this modification, the 5-FC MIC was 200 µg/mL (Table 3.6). The media optimization was conducted together with Paul Walker during his research internship at the Chair of Microbiology under my co-supervision.

Table 3.5 | Growth of *C. cellulovorans* harboring the deletion vector 520 agar plates containing 5-fluorocytosine (5-FC) and 2 g/L tryptone.

Plasmid	**5-FC concentration (µg/mL)**				
	0	25	50	100	200
pLA_del_4005_ori8	+	+	+	+	+
pLA_del_4006_ori8	+	+	+	+	+
pLA_del_2651_ori8	+	+	+	+	+
pLA_del_1114_ori8	+	+	+	+	+

+ = growth - = no growth +/- = background growth

Table 3.6 | Growth of *C. cellulovorans* harboring various deletion vectors on 520 agar plates containing 5-fluorocytosine (5-FC) and 2 g/L casamino acids.

Plasmid	5-FC concentration (µg/mL)				
	0	25	50	100	200
pLA_del_4005_ori8	+	+	+	-	-
pLA_del_4006_ori8	+	+	+	+	-
pLA_del_2651_ori8	+	+	+	+	-
pLA_del_1114_ori8	+	+	+	+	-

+ = growth - = no growth +/- = background growth

3.2.5.3. Screening of the mutant strains

It was possible to conjugate all deletion plasmids with the replicon pCB102 and p19. However, as expected, the number of transconjugants using a replication-defective plasmid was lower. Only the plasmids pLA_del_1114_ori7 and pLA_del_2651_ori7 were successfully transconjugated into *C. cellulovorans*. After conjugation, the clones were streaked 3 to 5 times on plates containing 15 µg/mL thiamphenicol to remove the *E. coli* strains used for the mating since the selection with antibiotics was not possible. However, it was observed that the size of the *C. cellulovorans* colonies was smaller than usual under this condition. So, plates containing only 2.5 µg/mL thiamphenicol were used and the normal colony size was observed again. The MIC of thiamphenicol for *C. cellulovorans* is 2.5 µg/mL (data not shown).

Thereafter, colony PCR was performed to identify clones that had integrated the plasmid into the genome after the first homologous recombination event. To notice, using suicide vectors, clones that underwent the first homologous recombination event are selected on a selective medium. However, when using replicative plasmids, the application of this strategy is not possible, since potential integrants and cells with the autonomously replicating plasmid can both grow on the selective medium. Therefore, routinely, the screening of the clones after the first homologous recombination is performed via PCR using a primer specific to the plasmid and a primer specific to the genome outside of the flanking region. However, in this study, attempts to screen for the first homologous via PCR failed. It was observed that a PCR fragment can be generated due to the presence of the genomic DNA and primer in the template used for PCR (Fig. 3.17). Therefore, PCR analysis was performed only to identify clones

that potentially underwent the second homologous recombination event and have lost the targeted gene for the deletion.

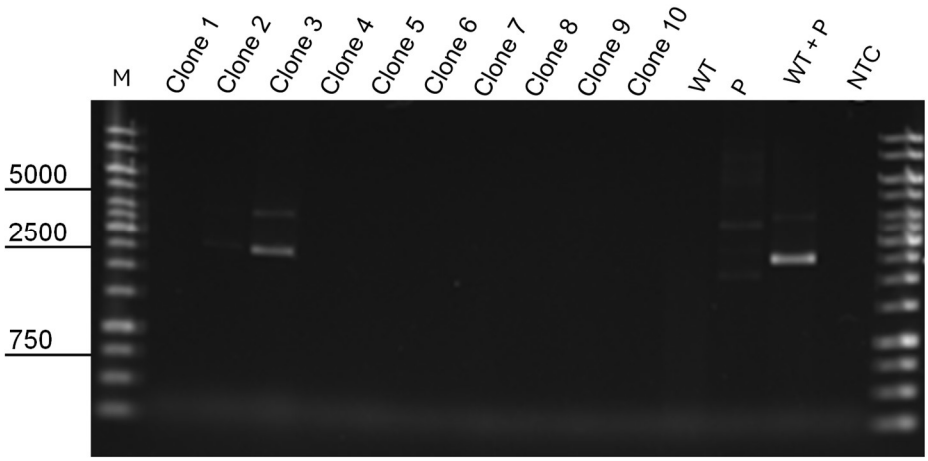

Figure 3.17 | PCR for the detection of integrant clones after first homologous recombination using a primer pair specific to the plasmid and one primer specific to the genome. PCR products were generated randomly due to the presence of plasmid and genomic DNA in the reaction tube. M = GeneRuler 1 kb DNA Ladder was used as marker (M) and bands are expressed in base-pairs (bp), WT = wild type, P = plasmid, NTC = non-template control. This PCR analysis was carried out together with Paul Walker during his research internship supervised by me.

No mutant genotype was observed using the deletion plasmids containing the pCB102 and p19 origins of replication. Fortunately, using the deletion plasmids containing the pCUB110 ori, it was possible to obtain clones with the ORFs Clocel_1114 (Fig. 3.18 A) and Clocel_2651 (Fig. 3.18 B) knocked out. Additionally, the plasmid pLA_del_2651_ori7 was transconjugated into the strain *C. cellulovorans ΔClocel_1114* and a mutant strain with both the type I and type III restriction systems knocked out was obtained (Fig. 3.18 C). The absence of the plasmid in the generated strains was confirmed by incubating the strains in medium with and without antibiotic, and via PCR using the primers catP_fw and catP_rv (Table 7.7). The strains *C. cellulovorans ΔClocel_2651* and *C. cellulovorans ΔClocel_1114 ΔClocel_2651* were generated during the Bachelor's thesis of Selina Zenz (Zenz, 2023) and the research internship of Daniel Köhler that both were carried out under my co-supervion.

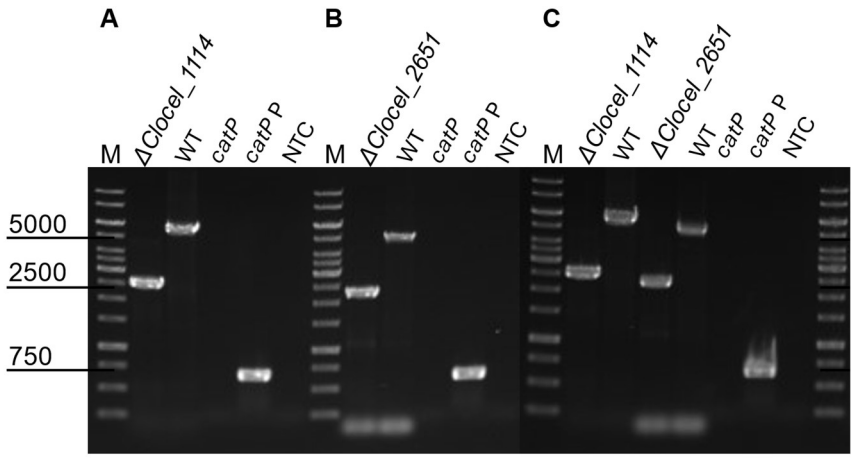

Figure 3.18 | PCR analysis for the confirmation of the chromosomal deletions in *C. cellulovorans*. PCR was performed using external primers annealing to the chromosome upstream and downstream of each flanking region. A) *C. cellulovorans ΔClocel_1114*, deletion of the type I RM system; expected amplicon sizes: ΔClocel_1114 = 2,510 bp and WT = 5,726 bp; B) *C. cellulovorans ΔClocel_2651*, deletion of the type III RM system; ; expected amplicon sizes: ΔClocel_2651 = 2,289 bp and WT = 5,130 bp; C) *C. cellulovorans ΔClocel_1114 ΔClocel_2651*, deletion of type I and III RM systems. M = GeneRuler 1 kb DNA Ladder was used as marker (M) and bands are expressed in base-pairs (bp), WT = wild type, *catP* = check for the absence of the *catP* resistance cassette in the generated strain, *catP* P = positive control using the plasmid as template, NTC = non-template control. This figure is adapted from Almeida et al. (2024, submitted for publication).

3.2.5.4. Evaluation of the role of *C. cellulovorans* restriction endonucleases in the conjugation efficiency

To investigate the role of the predicted RM systems, the conjugation efficiency using the restriction-less strains of *C. cellulovorans* was analyzed. For that, the pMTL83151 plasmid produced in *E. coli* 10-beta NEB® without the presence of the methylation plasmid was used as the donor plasmid. An increase of two orders of magnitude was observed for the *C. cellulovorans* recipient strains *ΔClocel_1114* and *ΔClocel_2651* (Table 3.7).

Surprisingly, the conjugation efficiency for the *ΔClocel_1114 ΔClocel_2651* double deletion strain as recipient was similar to the wild type using unmethylated plasmid. Additionally, conjugation experiments using as donor strain *E. coli* 10-beta NEB® bearing both pMTL83151 and the methylation plasmid (placORM) were also performed (Table 3.8). An increase of four and five orders of magnitude was observed for the strains *ΔClocel_1114* and *ΔClocel_2651*, respectively. Interestingly, an increase of three orders of magnitude was observed for the double mutant strain *ΔClocel_1114 ΔClocel_2651*. Still, either using methylated or unmethylated donor

plasmid, the conjugation of DNA molecules using the wild-type strain as recipient was always difficult.

Table 3.7 | Transconjugation efficiency analysis of the restriction-deficient strains of *C. cellulovorans*. Plasmid pMTL83151 propagated in the *E. coli* 10-beta NEB® without the presence of the methylation plasmid (placORM) was used as the donor plasmid. This table is adapted from Almeida et al. (2024, submitted for publication).

C. cellulovorans **strains**	**Tranconjugants (CFU/mL)**	**Recipients (CFU/mL)**	**Conjugation efficiency [a]**
WT	0	$4.89 \pm 4.58 \times 10^{6}$	0
ΔClocel_1114	$1.38 \pm 1.48 \times 10^{2}$	$3.75 \pm 1.63 \times 10^{6}$	$3.67 \pm 4.29 \times 10^{-5}$
ΔClocel_2651	$2.50 \pm 3.11 \times 10^{2}$	$7.59 \pm 3.57 \times 10^{6}$	$3.29 \pm 3.74 \times 10^{-5}$
ΔClocel_1114ΔClocel_2651	0	$3.87 \pm 1.5 \times 10^{6}$	0

[a] Conjugation efficiency was determined by the number of transconjugants produced per each initial recipient cell. Data represent the mean values and standard deviations from at least three independent experiments.

Table 3.8 | Transconjugation efficiency analysis of the restriction-deficient strains of *C. cellulovorans*. Plasmid pMTL83151 methylated by propagation in the *E. coli* 10-beta NEB® donor in the presence of the methylation plasmid (placORM) was used as the donor plasmid.

C. cellulovorans **strains**	**Tranconjugants (CFU/mL)**	**Recipients (CFU/mL)**	**Conjugation efficiency [a]**
WT	0	$4.89 \pm 4.58 \times 10^{6}$	0
ΔClocel_1114	$1.66 \pm 2.25 \times 10^{4}$	$3.75 \pm 1.63 \times 10^{6}$	$4.43 \pm 6.42 \times 10^{-3}$
ΔClocel_2651	$1.35 \pm 1.06 \times 10^{5}$	$7.59 \pm 3.57 \times 10^{6}$	$1.78 \pm 4.26 \times 10^{-2}$
ΔClocel_1114ΔClocel_2651	$7.11 \pm 7.01 \times 10^{3}$	$3.87 \pm 1.5 \times 10^{6}$	$1.84 \pm 1.47 \times 10^{-3}$

[a] Conjugation efficiency was calculated as the number of transconjugants produced per each initial recipient cell. Data represent the mean values and standard deviations from at least three independent experiments.

3.3. Metabolic engineering

3.3.1. Product formation pathways

In clostridia, ATP typically is synthesized by substrate-level phosphorylation during glycolysis (3-phosphoglycerate kinase and pyruvate kinase) and during acetate (acetate kinase) and butyrate (butyrate kinase) formation (Poehlein et al., 2017).

C. cellulovorans is an acetogenic clostridial species that produces lactate, formate, CO_2, H_2, acetate, and butyrate from cellobiose and cellulose (Sleat et al., 1984).

The end-product of glycolysis, pyruvate, can be reduced to lactate by lactate dehydrogenase (encoded by *ldh* gene). Two putative L-lactate dehydrogenases (the ORFs Clocel_1533 and Clocel_2700) are predicted in the annotated genome of *C. cellulovorans*. Clocel_2770 is predicted as an L-lactate dehydrogenase, while Clocel_1533 is potentially a lactate/malate dehydrogenase. L-lactate dehydrogenase is an important enzyme of the anaerobic metabolic pathway, catalyzing the reversible conversion of lactate to pyruvate with the reduction of nicotinamide adenine dinucleotide (NAD^+) to its reduced form NADH and vice versa.

Pyruvate can be irreversibly oxidized to acetyl coenzyme A (acetyl-CoA) via two different routes: (1) pyruvate:formate lyase (PFL) or (2) pyruvate:ferredoxin oxidoreductase (PFOR). PFL oxidizes pyruvate and CoA into acetyl CoA and formate. PFOR oxidizes pyruvate to generate acetyl CoA, CO_2, and reduced ferredoxin (Fd^{2-}). Reduced ferredoxins are the natural electron donors of the hydrogenase enzymes in anaerobes. However, to keep its reduced state, a reduction system has to continuously re-reduce ferredoxin. H_2 production can be catalyzed by three different types of ferredoxin-dependent hydrogenases. [NiFe], [FeFe], and [Fe] hydrogenases are distinguished by their metal composition. Two putative [Fe] hydrogenases (Clocel_2243 and Clocel_4097) are predicted in the annotated genome of *C. cellulovorans*. To balance redox cofactors ($NADH/NAD^+$), acetate formation is always accompanied by the formation of H_2.

The produced acetyl-CoA can be converted to acetate via phosphate acetyltransferase (encoded by *pta*) and acetate kinase (encoded by *ack*), resulting in the formation of one extra ATP. This is a key pathway for the production of ATP from excess acetyl-CoA. The *C. cellulovorans* 743B chromosome carries an operon encoding a phosphate acetyltransferase (phosphotransacetylase) (Clocel_1891, Pta) and an acetate kinase (Clocel_1891, Ack). Pta catalyzes the conversion of acetyl-CoA to acetyl phosphate. Subsequently, acetyl phosphate is converted to acetate by Ack, with the concomitant production of ATP through substrate-level phosphorylation.

Besides, two molecules of acetyl-CoA can also be further metabolized to synthetize crotonyl-CoA via condensation by thiolase, producing acetoacetyl-CoA which is reduced to β−hydroxybutyryl-CoA by a dehydrogenase. Then, water is eliminated via crotonase, resulting crotonyl-CoA. The crotonyl-CoA is further reduced to butyryl-CoA by the butyryl-CoA dehydrogenase. Butyryl-CoA finally is converted to butyrate by phosphate butyryltransferase (phosphotransbutyrylase) (encoded by *ptb* gene) and butyrate kinase (encoded by *buk* gene), with the production of ATP through substrate-level phosphorylation. For this, the *C. cellulovorans* chromosome carries an operon encoding a phosphate butyryltransferase (Clocel_3675, Ptb) and a butyrate kinase (Clocel_3674, Buk) (Fig. 3.19).

One of the main objectives of this study was to obtain strains of *C. cellulovorans* able to be part of a synthetic consortium for the continuous production of n-butanol using engineered clostridia. For that, three targets for chromosome modification were chosen: First, the *cat1* gene from *C. tyrobutyricum*, encoding a CoA-transferase, was used to replace in-frame the *ptb-buk* operon responsible for the last two steps of butyrate formation. This strain should couple butyrate formation to acetate re-consumption to produce acetyl-CoA. Second, the *pta-ack* operon should be deleted. A strain with this deletion can no longer produce acetate. It was planned that the strain should rely on the acetate produced by the solventogenic clostridia in co-culture for its growth. Besides, any acetyl-CoA produced in the strain should be directed to butyryl-CoA. Lastly, the main hydrogenase should be deleted, and thereby the NADH excess could be used for butyrate production.

3.3.2. Deletion of the *ptb-buk* operon and insertion of the *cat1* gene from *C. tyrobutyricum*

To knock out the *C. cellulovorans ptb-buk* operon, deletion plasmids targeting the putative phosphate butyryltransferase (Clocel_3675) and butyrate kinase (Clocel_3674) were constructed. In between the flanking regions, the *cat1* gene (CTK_RS03145) from *C. tyrobutyricum* was cloned to allow simultaneous knockout of the *ptb-buk* operon and knockin of the *cat1* gene.

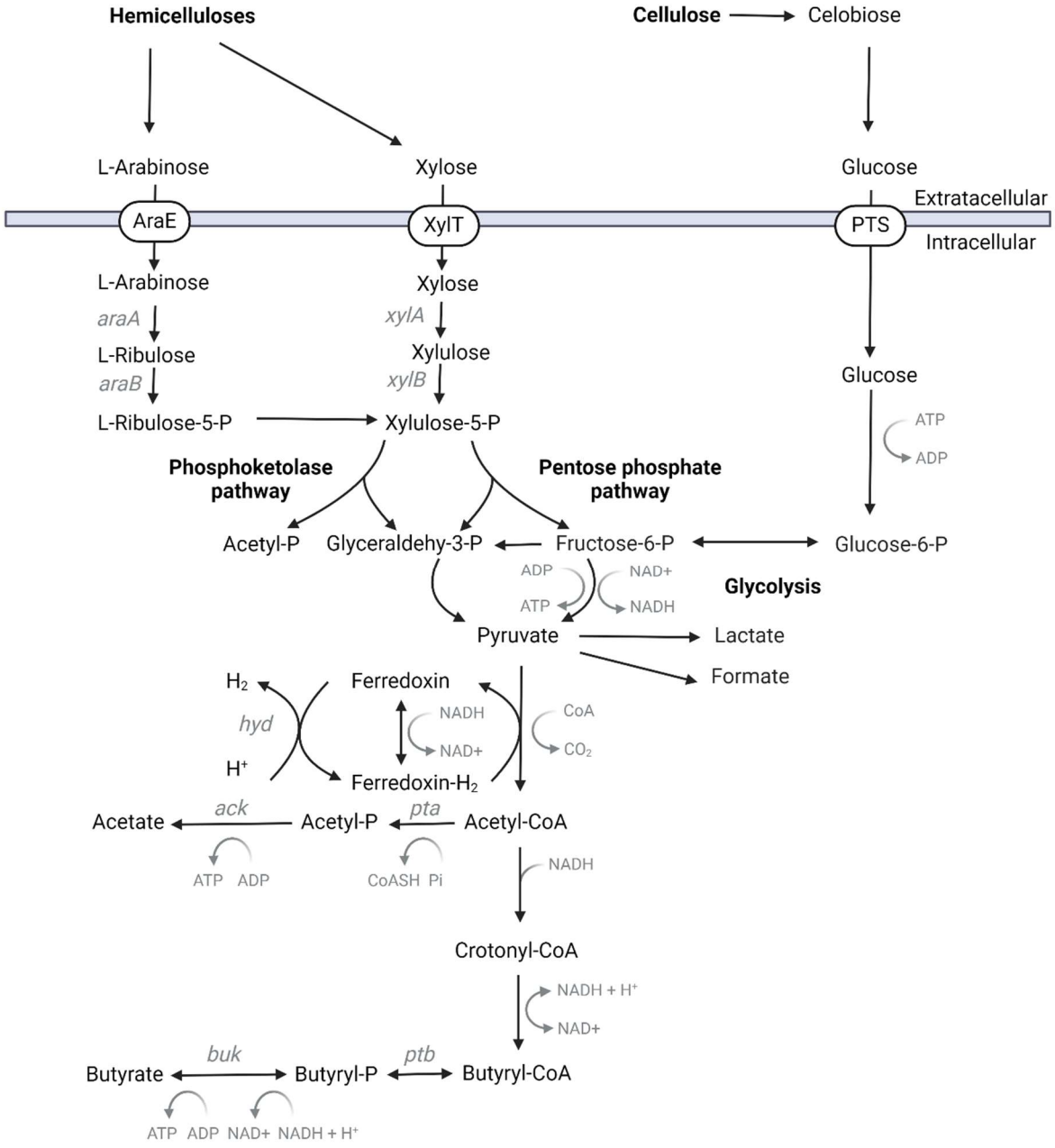

Figure 3.19 | *C. cellulovorans* central metabolism. L-arabinose and xylose are transported into the cell via the AraE and XylT transporters, respectively. The phosphotransferase system (PTS) mediates the uptake of glucose.Figure based on Tomita, H. (2019) and Wen et al. (2019).

The plasmid pLA_del_ori7 was used as the plasmid backbone. The plasmids were linearized via PCR using the primers pCN9_F_backbone and pCN9_R backbone. The flanking region upstream of the ORF Clocel_3674 was amplified with the primers upflank_3673_G_fw and upflank_3673_G_rv, generating a fragment of 1.073 kb. The flanking region downstream of the ORF Clocel_3675 was amplified using the primers lowflank_3676_fw and lowflank_3676_G_rv, generating a fragment of 0.918 kb. The primers cat1_del_G_fw and cat1_del_rv were used to clone the *cat1* gene. Thus, the

plasmid pLA_del_ptbbuk_cat1_ori7 was constructed (Table 7.11). The primers ptbbuk_del_fw and ptbbuk_del_rv were used to verify the presence of genomic deletion and insertion. All the PCR products for the upper and lower flanks contained an overlap sequence of 15–30 bp to the adjacent fragment. Vector and fragments were assembled in a one-step isothermal reaction using Gibson assembly. Subsequently, the plasmids were sent for sequencing and the presence of the insert was confirmed using the primers pLA_del_check_fw, pLA_del_check_rv, cat1_check and cat1_check_2. When required, the cloning primers were also used for sequencing to ensure that the whole insert was correct.

At the same time as this study was conducted, a new genetic system based on the CRISPR/Cas nickase system was established in our laboratory (Aline Schöllkopf, unpublished). Thus, the deletion/insertion cassette present in the plasmid pLA_del_ptbbuk_cat1_ori7 was inserted in the plasmid pAS11 using the primers upflank_3673_AatII_fw and lowflank_3676_AscI_rv. The backbone and inserts were digested with the restriction enzymes *Aat*II and *Asc*I and ligated using T4 ligase. After sequencing, the seed sequence in the sgRNA was exchanged using the primers cat1_XhoI_J23119-sgRNA-fw_sgRNA1, cat1_XhoI_J23119-sgRNA-fw_sgRNA2 or cat1_XhoI_J23119-sgRNA-fw_sgRNA3. The primer XhoI_J23119_rv was used in all reactions. The primer S24_Cas9_ATG_out was used for sequencing of the seed region (Table 7.12). The CRISPR/Cas nickase plasmids were constructed by Selina Zenz in her Bachelor's thesis (Zenz, 2023) co-supervised by me. A schematic representation illustrating the deletion vectors is shown in Figure 3.20. *C. cellulovorans ΔClocel_1114* was used as the base strain due to its great conjugation efficiency. The deletion plasmids were transferred into *C. cellulovorans ΔClocel_1114* via conjugation. After conjugation, the clones were streaked 3 to 5 times on plates containing 2.5 µg/mL thiamphenicol and 2 g/L tryptone (no yeast extract) to remove the *E. coli* strains used for the mating. Yet, it was observed that the colonies were smaller than usual. Since the deletion of the *ptb-buk* operon and insertion of the *cat1* gene interfered with acetate and butyrate production, and consequently with the acetyl-CoA levels in the cells (Canganella et al., 2002), the medium supplementation with acetate could restore the acetyl-CoA pool. Therefore, the medium was supplemented with 40 mM sodium acetate. With this, the growth was restored. To notice, the expression of the CRISPR/Cas nickase present in the deletion plasmid is

controlled by a theophylline-responding riboswitch. However, even before the induction of the CRISPR/Cas nickase, it was possible to obtain two clones with the desired deletion using the pNickase_ptbbuk_cat1 plasmid (Fig. 3.21). Plasmid curing was achieved by cultivating the strain in medium lacking antibiotic for 3–6 passages. Notably, the cell morphology appeared atypical (with unusually long rods), even when the medium supplemented with 40 mM acetate.

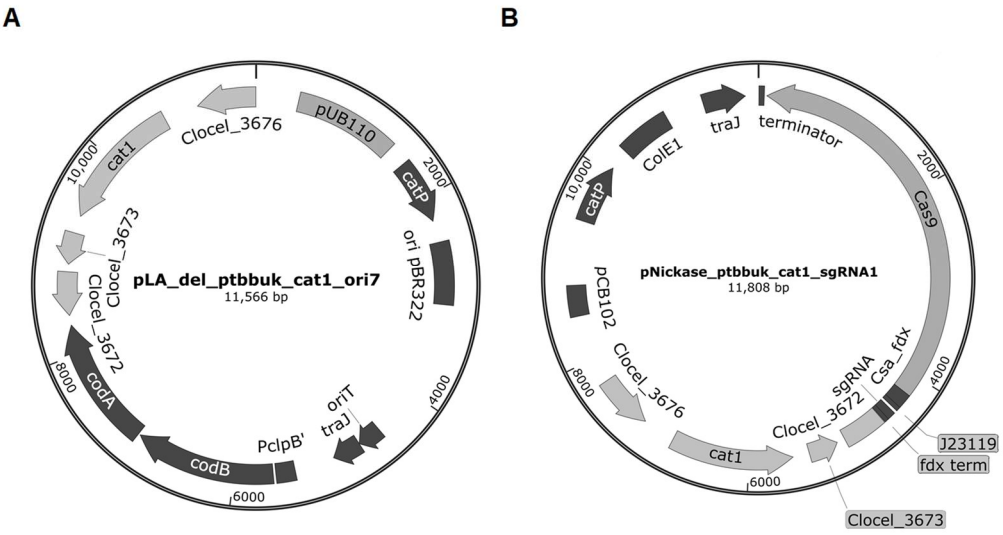

Figure 3.20 | Physical maps of the deletion plasmids used for the deletion of the putative *ptb-buk* operon (Clocel_3675 and Clocel_3674) and insertion of the *cat1* gene (CTK_RS03145) from *C. tyrobutyricum* based on the *codBA* (A) and in the CRISPR/Cas9 nickase (B) systems. P_{clpB}: promoter of the gene for chaperone protein ClpB, *codB*: cytosine permease ORF, *codA*: cytosine deaminase ORF, pUB110: Gram-positive replicon, *catP*: gene conferring chloramphenicol/thiamphenicol resistance, *traJ*: ORF for TraJ protein essential for regulating the expression of transfer gene involved in the conjugal transfer of DNA, oriT: origin of transfer necessary for the transfer of DNA, Cas9: CRISPR associated protein 9.

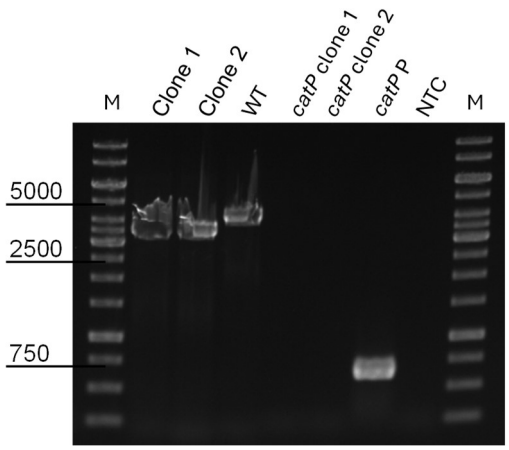

Figure 3.21 | PCR analysis to confirm chromosomal deletions and insertion in *C. cellulovorans*. PCR was performed using external primers annealing to the chromosome upstream and downstream of each flanking region. Two independent clones were obtained, generating the *C. cellulovorans ΔClocel_1114 Δptb-buk::cat1* strain. Deletion/insertion expected fragment = 3,477 bp and WT = 4,174 bp. M = GeneRuler 1 kb DNA Ladder was used as marker (M) and bands are expressed in base-pairs (bp), WT

= wild type, *catP* = check for the absence of the *catP* resistance cassette in the generated strain, *catP* P = positive control using the plasmid as template, NTC = non-template control.

3.3.3. Deletion of the *pta-ack* operon

To knock out the *C. cellulovorans pta-ack* operon, a deletion plasmid targeting the ORFs encoding putative phosphate acetyltransferase (Clocel_1891) and acetate kinase (Clocel_1891) should be created. However, it was not possible to assemble the fragment corresponding to the flanking region adjacent to the *ack* gene. Therefore, only the *pta* gene was selected as the target. The plasmid pLA_del_ori7 was used as the plasmid backbone. The plasmids were linearized via PCR using the primers pCN9_F_backbone and pCN9_R backbone.

The flanking region upstream of the ORF Clocel_1891 was amplified with the primers upflank_1890_G_fw and upflank_1890_G_rv_v2, generating a fragment of 1.145 kb. The flanking region downstream of the target gene was amplified using the primers lowflank_1892_fw and lowflank_1892_G_rv, generating a fragment of 0.904 kb. Thus, the plasmid pLA_del_pta_ori7 was constructed (Table 7.13). All the PCR products for the upper and lower flanks contained an overlap sequence of 15–30 bp to the adjacent fragment. Vector and fragments were assembled in a one-step isothermal reaction using Gibson assembly. Subsequently, the plasmids were sent for sequencing and the presence of the insert was confirmed using the primers pLA_del_check_fw, pLA_del_check_rv, upflank_1890_check_fw and lowflank_1892_check_fw. When required, the cloning primers were also used for sequencing to ensure that the whole insert was correct. The primers Del_pta_fw and Del_pta_rv were used to verify the presence of genomic deletion.

Later, the deletion cassette present in the plasmid pLA_del_pta_ori7 was inserted in the plasmid pAS11 using the primers upflank_1890_AatII_fw and lowflank_1892_AscI_rv. The backbone and inserts were digested with the restriction enzymes *Aat*II and *Asc*I and ligated using T4 ligase. After sequencing, the seed sequence in the sgRNA was exchanged using the primers pta_XhoI_J23119-sgRNA-fw_sgRNA1, pta_XhoI_J23119-sgRNA-fw_sgRNA2 or pta_XhoI_J23119-sgRNA-fw_sgRNA3. The primer XhoI_J23119_rv was used in all reactions. The primer S24_Cas9_ATG_out was used for sequencing of the seed region (Table 7.14). The CRISPR/Cas nickase plasmids were constructed by Anna Kimadze in a Bachelor's

thesis (Kimadze, 2024) planned in support of this research project and co-supervised by me. Physical maps illustrating the deletion vectors are shown in Figure 3.22.

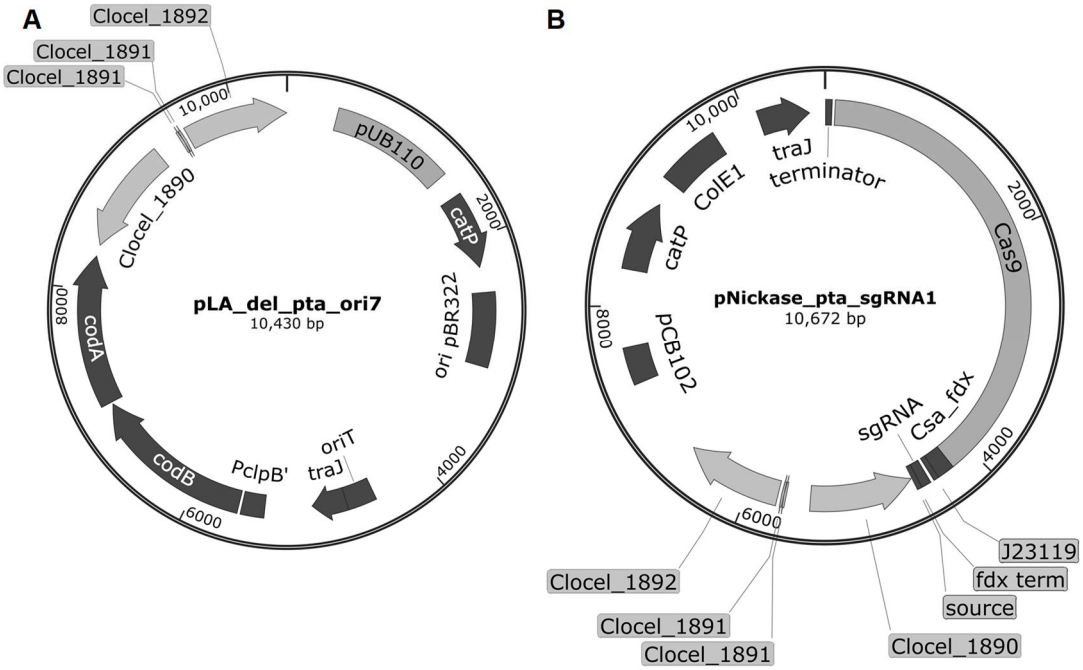

Figure 3.22 | Physical maps of the deletion plasmids used for the deletion of the putative *pta* gene (Clocel_1891) based on the *codBA* (A) and in the CRISPR/Cas9 nickase (B) systems. P_{clpB}: promoter of the gene for chaperone protein ClpB, *codB*: cytosine permease ORF, *codA*: cytosine deaminase ORF, pUB110: Gram-positive replication origin, *catP*: gene conferring chloramphenicol/ thiamphenicol resistance, *traJ*: ORF ecoding the TraJ protein essential for regulating the expression of transfer gene involved in the conjugal transfer of DNA, *oriT*: origin of transfer necessary for the transfer of DNA, Cas9: region encoding CRISPR associated protein 9.

C. cellulovorans ΔClocel_1114 was used as the base strain due to its great conjugation efficiency. The deletion plasmids were transferred into *C. cellulovorans ΔClocel_1114* via conjugation. After conjugation, the clones were streaked 3 to 5 times on plates containing 2.5 µg/mL thiamphenicol and 2 g/L tryptone (no yeast extract) to remove the *E. coli* strains used for the mating. The medium was supplemented with 40 mM sodium acetate to support the growth of the mutant strains. Again, even before induction, it was possible to obtain five clones with the desired deletion using the pNickase_pta plasmid (Fig. 3.23) and the plasmid was lost by cultivating the strain in medium lacking antibiotic for 3–6 passages. The use of the pLA_del_pta plasmid was also successful in obtaining the *pta* deletion. However, the plasmid curing was easier

using the pNickase_pta plasmid. *C. cellulovorans ΔClocel_1114 Δpta* was generated by Anna Kimadze in a Bachelor's thesis (Kimadze, 2024) planned in support of this research project and co-supervised by me.

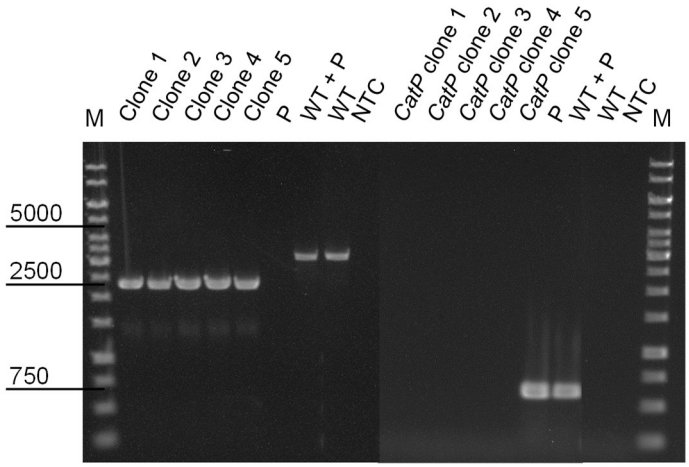

Figure 3.23 | PCR analysis for the confirmation of the chromosomal *ΔClocel_1114 Δpta* deletion in *C. cellulovorans*. PCR was performed using external primers annealing to the chromosome upstream and downstream of each flanking region. Five independent clones were obtained, generating the *C. cellulovorans ΔClocel_1114 Δpta* strain. Expected fragment size for deletion = 2,324 bp and WT = 3,281 bp. M = GeneRuler 1 kb DNA Ladder was used as marker (M) and bands are expressed in base-pairs (bp), WT = wild type, *catP* = control PCR to check for the absence of the *catP* resistance cassette in the generated strain, *catP* P = positive control using the plasmid as template, NTC = non-template control. Figure adapted from Bachelor's thesis of Anna Kimadze co-supervised by me (Kimadze, 2024).

3.3.4. Deletion of the hydrogenases

To knock out *C. cellulovorans* hydrogenases, deletion plasmids targeting the ORFs for two putative hydrogenases (Clocel_4097 and Clocel_2243) were constructed. The plasmid pLA_del_ori7 was used as the plasmid backbone. The plasmids were linearized via PCR using the primers pCN9_F_backbone and pCN9_R backbone.

The flanking region upstream of the ORF Clocel_4097 was amplified with the primers upflank_4096_G_fw and upflank_4096_G_rv, generating a fragment of 1.016 kb. The flanking region downstream of the target gene was amplified using the primers lowflank_4098_fw and lowflank_4098_G_rv, generating a fragment of 0.980 kb. Thus, the plasmid pLA_del_hyd_4097_ori7 was constructed (Table 7.15).

The flanking region upstream of the ORF Clocel_2243 was amplified with the primers upflank_2242_G_fw and upflank_2242_G_rv, generating a fragment of 0.981 kb. The

flanking region downstream of the target gene was amplified using the primers lowflank_2244_fw and lowflank_2244_G_rv, generating a fragment of 0.940 kb. Thus, the plasmid pLA_del_hyd_2243_ori7 was constructed (Table 7.16).

All the PCR products for the upper and lower flanks contained an overlap sequence of 15–30 bps to the adjacent fragment. Vector and fragments were assembled in a one-step isothermal reaction using Gibson assembly. Subsequently, the plasmids were sent for sequencing and the presence of the insert was confirmed using the primers pLA_del_check_fw, pLA_del_check_rv, upflank_4096_check_fw, lowflank_4098_check_fw, upflank_2242_check_fw and lowflank_2244_check_fw. When required, the cloning primers were also used for sequencing to ensure that the whole insert was correct. The primers Del_4097_fw and Del_4097_rv were used to verify the presence of the chromosomal deletion in the ORF Clocel_4097. The primers Del_2243_fw and Del_2243_rv were used to verify the presence of the chromosomal deletion in the ORF Clocel_2243.

Later, the deletion cassette present in the plasmids pLA_del_hyd_4097_ori7 and pLA_del_hyd_2243_ori7 were inserted in the plasmid pAS11 using either the primers upflank_4096_AatII_fw and lowflank_4098_AscI_rv, or upflank_2242_AatII_fw and lowflank_2244_AscI_rv. The backbone and inserts were digested with the restriction enzymes *Aat*II and *Asc*I and ligated using T4 ligase. After sequencing, the seed sequence in the sgRNA was exchanged using the primers hyd_XhoI_J23119-sgRNA-fw_sgRNA1, hyd_XhoI_J23119-sgRNA-fw_sgRNA2 or hyd_XhoI_J23119-sgRNA-fw_sgRNA3 for the plasmid pNickase_hyd_4097 (Table 7.17). The seed sequence in the sgRNA was exchanged using the primers hyd2243_XhoI_J23119-sgRNA-fw_sgRNA1, hyd2243_XhoI_J23119-sgRNA-fw_sgRNA2 or hyd2243_XhoI_J23119-sgRNA-fw_sgRNA3 for the plasmid pNickase_hyd_2243 (Table 7.18). The primer XhoI_J23119_rv was used in all reactions. The primer S24_Cas9_ATG_out was used for sequencing of the seed region. The CRISPR/Cas nickase plasmids were constructed by Anna Kimadze in a Bachelor's thesis (Kimadze, 2024) planned in support of this research project and co-supervised by me. Physical maps illustrating the deletion vectors are shown in Figure 3.24. *C. cellulovorans ΔClocel_1114* was used as the base strain due to its great conjugation efficiency. The deletion plasmids were transferred into *C. cellulovorans ΔClocel_1114* via conjugation. After

conjugation, the clones were streaked 3 to 5 times on plates containing 2.5 µg/mL thiamphenicol and 2 g/L tryptone (no yeast extract) to remove the *E. coli* strains used for the mating. The medium was supplemented with glycine or gluconic acid to support the growth of the mutant strains. However, even after laborious screening, no mutant clone could be obtained.

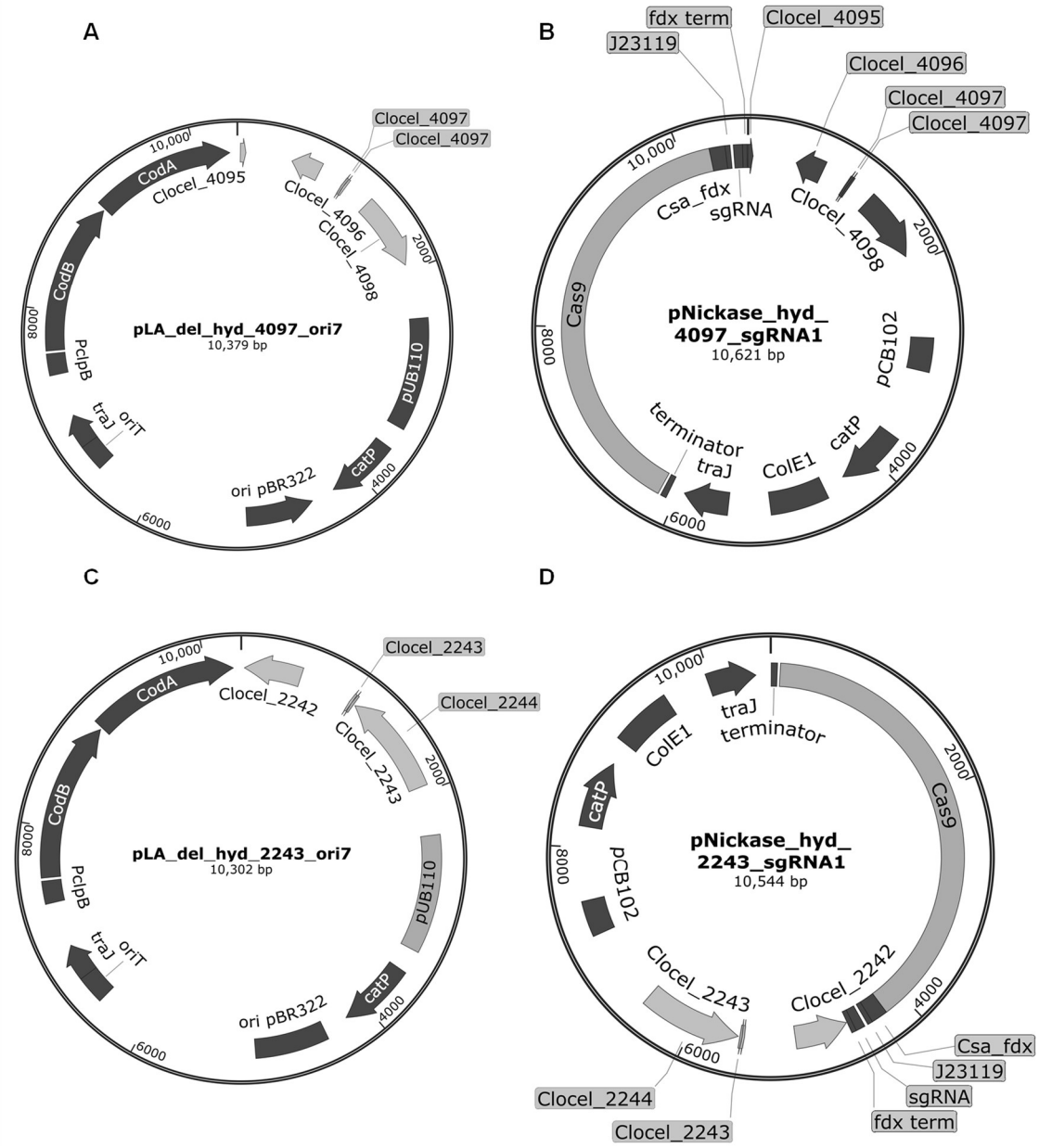

Figure 3.24 | Physical maps of the deletion plasmids used for the deletion of the putative hydrogenase genes (Clocel_4097 and Clocel_2243) based on the *codBA* (A and C) and in the CRISPR/Cas9 nickase (B and D) systems. P_{clpB}: promoter of the gene for chaperone protein ClpB, *codB*: cytosine permease ORF, *codA*: cytosine deaminase ORF, pUB110: Gram-positive replication origin, *catP*: gene conferring chloramphenicol/thiamphenicol resistance, *traJ*: ORF ecoding the TraJ protein essential for regulating the expression of transfer gene involved in the conjugal transfer of DNA, *oriT*: origin of transfer necessary for the transfer of DNA, Cas9: region encoding CRISPR associated protein 9.

4. Discussion

The decrease in the oil reserves has increased the interest in using renewable resources for the production of fuels and chemicals (García et al., 2011). In this context, lignocellulosic biomass, the most abundant feedstock found on earth, has been considered a promising feedstock (Ibrahim et al., 2017). It can be obtained from dedicated energy crops or residues such as agricultural and municipal waste (Bharathiraja et al., 2017). It is composed of cellulose, hemicellulose, and lignin (Ilić et al., 2023; Tayyab et al., 2018). The cellulose and hemicellulose parts can be converted into sugars that can be further fermented by microorganisms to produce valuable chemicals. For instance, *C. acetobutylicum* can produce acetone, butanol, and ethanol through a process called ABE fermentation using glucose as C-source (Dürre, 2008). Among these solvents, butanol is considered a “superior solvent” due to its high energy content, and, most importantly, butanol is a valuable bulk chemical used in the production of paints, synthetic rubber, and waxes.

However, using lignocellulosic biomass as a feedstock for the production of butanol presents several challenges. First, its complex structure makes it difficult to break down into fermentable sugars (Procentese et al., 2017). Therefore, effective pretreatment methods are necessary to increase the accessibility of cellulose to enzymatic hydrolysis, increasing the costs of obtaining lignocellulosic chemicals and fuels (Guo et al., 2015). Fortunately, cellulolytic microorganisms can be used for biomass degradation. However, no microorganism in nature can efficiently degrade cellulose and produce valuable chemicals, such as butanol, in competitive yields.

In this context, *C. cellulovorans*, an anaerobic, mesophilic, and cellulolytic bacteria has been considered a promising candidate for the production of lignocellulosic fuels and chemicals. It can efficiently degrade cellulose at rates comparable to those of thermophilic bacteria (Sleat et al., 1984). However, wild-type *C. cellulovorans* cannot produce valuable chemicals at high yields. Interestingly, although this bacterium is not able to produce solvents such as butanol, it produces acetyl-CoA and butyryl-CoA as intermediates during carbohydrate fermentation, which are two important nodes in the central carbon metabolism and butanol synthesis.

Several strategies have been attempted to achieve butanol production by *C. cellulovorans*. For instance, Wen and co-workers (2019) obtained the production of n-butanol by introducing the butanol metabolic pathway from *C. acetobutylicum* into *C. cellulovorans*. The alcohol aldehyde dehydrogenase gene (*adhE1*) from *C. acetobutylicum* ATCC 824 on a recombinant plasmid could be successfully overexpressed in *C. cellulovorans*. Thus, the engineered strain was able to produce 4.96 g/L of butanol (Wen et al., 2019). However, such an approach has two drawbacks. First, butanol displays high cell toxicity (Ingram, 1976), leading to a metabolic burden on the cell. Second, strategies that rely on plasmids require constant supplementation of the medium with antibiotics, which is costly.

In the recent years, it has been demonstrated that co-culturing cellulolytic and solventogenic *Clostridium* species can result in the degradation of complex lignocellulosic substrates with efficient production of specific products (Du et al., 2020). However, the growth in co-cultures is usually unstable, since one strain might overgrow the other. This study aimed to engineer a strain of *C. cellulovorans* able to be part of a synthetic co-culture with a solventogenic clostridium. The engineered strain should be metabolic dependent on the solventogenic clostridial strain, but the lack of genetic tools has hampered the metabolic engineering of *C. cellulovorans*. Therefore, it was necessary to first develop a system to allow targeted chromosome modification in *C. cellulovoarans*. It is worth mentioning that significant research has been conducted on the cellulosomal and non-cellulosomal enzymes produced by *C. cellulovorans* (Aburaya et al., 2019; Eljonaid et al., 2022; Matsui et al., 2013; Tamaru et al., 2000) in the last years. However, since its initial description there has been no subsequent research published regarding other aspects of *C. cellulovorans* physiology. Thus, the first aim of the present thesis was to investigate selected features of *C. cellulovorans* physiology and its previously described sporulation capability, which will be discussed in the following sections.

4.1. *C. cellulovorans* **characterization**

4.1.1. Growth and metabolic product characteristics

C. cellulovorans can use cellulose, xylan, pectin, cellobiose, glucose, maltose, galactose, sucrose, lactose, and mannose as substrates for growth (Sleat et al., 1984).

In this study, its growth was analyzed in 0.5 % cellobiose, 0.5 % PASC, 0.3 % PASC, 0.5 % Avicel and 0.3 % Avicel. Due to the insolubility of PASC and Avicel, which makes it impossible to use optical density as a measure for growth assessment on these substrates, the growth was estimated by measuring the protein concentration using the Bradford assay. The highest protein concentration (µg/mL) was achieved in 0.5 % PASC (19.72 ± 0.40) and 0.5 % Avicel (17.94 ± 0.24) after 6 days of incubation. Not surprisingly, the growth of *C. cellulovorans* 743A in cellobiose was faster, achieving 12.44 ± 0.15 µg/mL after 1 day of incubation. Cellobiose is a major intermediate in cellulose degradation and can readily be used as an energy source. Tang and co-workers (2023) investigated *C. cellulovorans* growth in glucose (40 g/L) or Avicel (40 g/L). The authors estimated the growth in both cases by measuring the optical density (OD_{600nm}). The highest OD_{600nm} was achieved with glucose (3.5 ± 0.2) after 2 days of incubation. After 5 days, an OD_{600nm} of 2.6 ± 0.3 was obtained during cellulose fermentation. However, this method is not accurate for estimating the growth on cellulose. In the present study, a protocol to analyze growth performance on insoluble substrates was established based on the determination of protein concentration via the Bradford assay. Prior to the protein concentration measurements, it was important to resuspend the cell pellet in TE buffer and incubate for 30 min at 95 °C to obtain complete cell disruption. As observed in the present study, growth of *C. cellulovorans* on cellulose was much slower than that on “ready to use” soluble substrates such as glucose.

As reported before (Sleat et al., 1984; Tang et al., 2023), the main fermentation products of *C. cellulovorans* are acetate and butyrate. Tang and co-workers (2023) reported that *C. cellulovorans* produced fewer acids when growing on cellulose. However, in the present study, it was observed that despite the growth as well as the production of acid was slower on cellulose, the final yields were similar in all tested conditions. In terms of substrate utilization, *C. cellulovorans* utilized almost all cellulose in the medium, which was added at 5 g/L, as an energy source. When growing in cellobiose, the fast production of acids leads to an abrupt drop in the pH (Fig. 3.3 A). At a pH of 5, *C. cellulovorans* is not able to grow. To obtain an efficient fermentation, continuous adjustment of the pH in the medium is required if cellobiose is used. For instance, during the fermentation process in Tang and co-workers (2023) study, pH was automatically adjusted to 7.0 with 2M KOH and all the glucose was used.

Surprisingly, only 33 % of cellulose was used during the period of fermentation (8 days). To notice, the lack of available literature and discrepancies in the methods used make it difficult to compare the findings observed in different research groups.

4.1.2. Sporulation

Bacterial endospores are dormant forms of bacteria, exhibiting minimal metabolism. They serve as a survival strategy during unfavorable conditions such as the lack of nutrients, desiccation, heat, radiation, and the presence of antibiotics (Russell, 1990; Setlow, 2011). From a medical perspective, endospores can either make bacterial infections difficult to treat (Mallozzi et al., 2010), or may help in the treatment of diseases such as cancer (Bahl & Dürre, 2001; Staedtke et al., 2016). Endospore-forming bacteria can also be of significant concern in the food industry, due to spoilage originated from the proliferation of vegetative bacterial cells after spore germination (Wohlgemuth & Kämpfer, 2014). On the other hand, in the context of probiotic production, endospores can be used to reduce the inoculum added to the food (Bader et al., 2012).

Clostridium is one of the most important endospore-forming genera (Russell, 1990). The model organism to study molecular mechanisms involved in endospore formation in clostridia is *C. acetobutylicum* (Al-Hinai et al., 2015). It is known that nutrient deprivation triggers sporulation in many endospore formers (Sorg & Sonenshein, 2008; Stephens, 1998), but this is not the case for clostridia. In *C. acetobutylicum*, sporulation happens during exponential growth and is associated with the accumulation of organic acids, such as butyrate and acetate, even in the presence of excess nutrients (Long et al., 1984; Sauer et al., 1995). Based on the investigation performed in *C. acetobutylicum*, it is known that while consuming glucose and producing organic acids, the pH of the medium drops, and the environment becomes increasingly toxic to the cells. In response to this stress, the cells initiate two survival mechanisms: solventogenesis and sporulation (Al-Hinai et al., 2015). During solventogenesis, the low pH is neutralized by converting organic acids into solvents. Sporulation enables cells to survive until a more suitable environment is established.

The formation of endospores is a very complex process. During sporulation, cells undergo morphological changes while the endospore develops. First, the cells obtain a distinct form characterized by a swollen appearance with accumulating granulose

vesicles made of an amylopectin-like polysaccharide. Thereafter, an asymmetric septum is formed. This is the first morphologically distinct state that indicates that the cells have initiated sporulation. The smaller compartment is destined to become the endospore. Then, the engulfment process starts, where membranes formed inside the mother cell grow around the smaller compartment. Thereafter, a thick layer of peptidoglycan, known as the cortex, forms between the two membranes surrounding the forespore. A coat composed of various proteins is formed around the cortex. The last step is the mother cell lysis, comminating in the release of the mature spore (Al-Hinai et al., 2015).

From the biotechnological point of view, sporulation is not a desirable trait. It has been reported that cellulose catabolism was improved in *C. cellulolyticum* by sporulation abolishment (Li et al., 2014). Thus, obtaining a strain of *C. cellulovorans* with an abolished sporulation process could significantly enhance cellulose utilization. In order to conduct further research in this area, knowing the conditions favorable for spore formation is essential. Although *C. cellulovorans* has been described as an endospore-forming bacterium (Sleat et al., 1984), after routine microscopic analysis and standard pasteurization procedures (80 ºC, 10 min), no endospores could be observed from *C. cellulovorans* cultures.

Since studies regarding the environmental triggers of sporulation have not been documented for *C. cellulovorans*, in the present study *C. cellulovorans* was cultivated on 520 agar plates with yeast extract, tryptone, carbohydrate-free tryptone, and casamino acids to motivate sporulation. Although starvation is not the most common trigger for sporulation in clostridia, 520 medium with no vitamin or yeast extract supplementation and low cysteine concentration (0.05 g/L) were also prepared. Cellobiose was used as C-source. Cells were incubated for 5, 7, or 15 days at 37 ºC. Prolonged incubation seems to be required for spore maturation. As can be seen in Figure 3.4, only after 15 days of incubation, cells showed signs of release of the mature spore.

Applying a sublethal heat treatment is a common procedure to geminate spores, also called heat activation (Krawczyk et al., 2017; Nasuno & Asai, 1960; Wen et al., 2022). The heat treatment also ensures that the growth is prevenient from spores and not vegetative cells. Thus, *C. cellulovorans* cell suspension samples containing vegetative

cells and potential spores were heat-shocked at 70 or 80 ºC for 0, 1, 2, 11, and 30 min. Then, cells were incubated in 520 liquid media or 520 agar plates containing yeast extract as vitamin source and cellobiose as C-source (germination medium). However, no growth was observed under all tested conditions. The absence of growth is an indication that *C. cellulovorans* spores might not be heat resistant. An additional attempt supplementing the germination medium with L-alanine, L-asparagine, D-glucose, D-fructose, and K (Ala-AGFK) was also tested since it was reported that this mixture can trigger germination receptors (Yi & Setlow, 2010; Cruz-Mora et al., 2015; Bressuire-Isoard et al., 2018). But still, no growth could be obtained after heat treatment.

The C-source used in the sporulation experiment could play an important role. In *C. thermocellum* it has been noticed that sporulation occurs while cells are attached to the biomass fibers (Wiegel & Dykstra, 1984). It has been reported that high concentrations of cellulose (35 % w/v) trigger sporulation in *C. cellulolyticum* (Desvaux & Petitdemange, 2002). Additionally, the pH also had a significant impact on spore formation in *C. cellulolyticum*. Desvaux and Petitdemange (2002) observed that the level of sporulation was higher under unregulated pH fermentation. Thus, further investigation using cellulose as C-source should be performed. pH adjustments will not be required in the cultivation on Avicel or PASC. As shown in Figure 3.3 A, the pH of the medium will not drop below the limit tolerated by *C. cellulovorans* (6.4–7.8). The difficulty found in this study to sporulate *C. cellulovorans* might be due to the fast acidification of the medium when cellobiose is used as C-source (after 3 days of incubation, a pH of 5.5 was measured, which is lower than the optimal pH for *C. cellulovorans* growth). A longer incubation time should also be considered. Li and co-workers (2014) incubated *C. cellulolyticum* cells in medium containing 50 g/L cellulose for 18 days before harvesting.

4.1.3. Prophage induction

Bacteriophages, also called phages, are viruses that replicate exclusively in bacteria. Their genetic material is found as prophages in many bacterial genomes. Bacteriophages can possess two life cycles, lysogenic and lytic. During the lysogenic cycle, phages replicate together with the bacterial chromosome. During the lytic cycle, the host cells are lysed after the intracellular production of phage particles, which can

infect new host cells (Zhang et al., 2022). Virulent phages replicate using only the lytic cycle, while temperate phages replicate using both cycles. Prophages represent the genomes of temperate phages present in bacterial cells. They can be found either integrated directly into the host chromosome or in an extrachromosomal element, such as a plasmid (Canchaya et al., 2003). Environmental stressors such as starvation, temperature changes, oxidative stress, pH changes, and antibiotic exposure can trigger the lytic cycle (Howard-Varona et al., 2017).

During the course of this research, the growth of *C. cellulovorans* was found to be extremely unpredictable. Therefore, it was hypothesized that maybe potential prophage induction led to growth instability. *In silico* prediction using the database PHASTER identified 4 intact prophage regions in the chromosome of *C. cellulovorans* 743A. Obtaining a phage-free (cured) strain would be a great advancement for the handling of *C. cellulovorans* and avoid unwanted genetic changes.

The use of chemical agents (e.g. mitomycin C or acridine orange), physical methods (e.g. heat treatment and UV radiation), and targeted gene editing (e.g. use of the CRISPR-Cas systems to specifically delete phage regions) can be employed to obtain the loss of phages from bacterial cells (Feyereisen et al., 2019; Madera et al., 2009; Meijer et al., 1998). In the present study, UV light and mitomycin C (MMC) were used to cause DNA damage. DNA damage often causes an SOS response in bacterial cells. Initially, the SOS response was recognized as regulating DNA damage repair (Podlesek & Žgur Bertok, 2020), but it has a broader role. It also triggers the lytic cycle, leading to the excision and replication of prophages (Lemire et al., 2011). However, 10 min UV exposure did not lead to any visible cell lysis cell lysis *C. cellulovorans* or *Latilactobacillus sakei* (positive control). This is potentially due to the ineffectiveness of the method employed. For *Lat. sakei,* phage induction was observed using UV light exposure (312 nm) before (Ambros & Ehrmann, 2022). The wavelength and the duration are important criteria for phage induction. Feyereisen and co-workers (2019) obtained phage induction in *Lactobacillus brevis* by UV light exposure (254 nm) for 30 min. In the same way, the cultivation of *C. cellulovorans* with MMC did not lead to cell lysis, but it induced cell lysis in the *Lat. sakei* with intact prophages. Not surprisingly, *C. cellulovorans* stopped growth in the cultures with 3 and 30 µL/mL of MMC due to the MMC toxicity. The lack of research on the induction of prophages in clostridia makes it difficult to define the appropriate parameters. Thus, additional experiments

are still necessary to identify the proper conditions for prophage induction in *C. cellulovorans*. Fortunately, with the development of the genetic tools developed in this work (discussed in the following paragraphs), it should be possible to obtain a phage-free strain of *C. cellulovorans* by deleting specific prophage regions.

4.1.3. Restriction/modification system

Clostridial species are usually difficult to transform due to the presence of restriction/modification systems (RM systems). RM systems are a form of innate immunity defense found in many bacteria. They protect the organism against foreign DNA, mainly from bacteriophages and archaeal viruses (Rusinov et al., 2018). These systems typically consist of a methyltransferase (MTase), to methylate the own DNA, and a restriction endonuclease (REase), to digest foreign DNA. The genetic engineering of clostridial species is hindered by the activity of REases, as the plasmid DNA used for genetic modification is recognized as foreign DNA and digested. Some exceptions are *C. botulinum* and *C. perfringens*, which only encode copies of MTase genes. Such organisms can be readily transformed with plasmid DNA, facilitating strain engineering approaches (Minton et al., 2016). This is not the case with *C. cellulovorans*, which possess 12 putative RM systems.

A common way to overcome host RM systems involves *in vivo* methylation of plasmid DNA in *E. coli* cells by expressing the host MTases before introduction of the DNA into the host. Usually, the MTase genes are expressed in, preferably, a low copy number plasmid. The “co-residence” of the methylation plasmid with the the recombinant plasmid of interest in *E. coli* cells yields methylated plasmid DNA, which then can be successfully introduced into the clostridial host, either via transformation with isolated methylated plasmid DNA or via conjugation. The plasmid protection can also be obtained by the expression of MTase from a different organism. For instance, the *in vivo* methylation using the *B. subtilis* phage phi 3T I MTase protected plasmids from restriction digest after transformation in *C. acetobutylicum* (Mermelstein et al., 1993). In an attempt to overcome *C. cellulovorans* RM systems, Yang and co-workers (2014) established an *in vivo* methylation system, expressing the native MTases M. Cce743I and M. Cce743II in *E. coli*. To evaluate the effectiveness of the methylation plasmid, the authors incubated plasmids that underwent previous *in vivo* methylation and unmethylated plasmids in *C. cellulovorans* cell free extract (CFE). Even after 20 h

incubation in the CFE, *in vivo* methylated plasmids were not digested (Wen et al., 2014). This is very important because after electroporation or conjugation, the plasmid needs to replicate efficiently during the recovery time (1–24 h). In the present work, the same experiment was repeated, but a complete digestion of the plasmid was observed even after 1h incubation with the CFE. In Yang and co-workers (2014), the protein concentration of the CFE was not informed, which makes it difficult to compare both studies. Besides, buffer conditions needed for *C. cellulovorans* endonucleases activity were unknown. The unspecific digestion patterns could be due to inappropriate conditions. Another hypothesis is that in *C. cellulovorans* the type I RM system is the most important. This type of RM system recognizes at specific sequences but cuts in an unspecific manner. Although the type II RM systems are seen as the most important barrier in Clostridial transformation (Pyne et al., 2014), in *C. saccharobutylicum*, for instance, only type I RM systems were found (Huang et al., 2018).

Plasmid *in vitro* methylation has also been described (Donahue et al., 2000; Lin et al., 2010; Alegre et al., 2004). In this approach, the plasmid is incubated with the CFE, but the appropriate reaction conditions must be employed to avoid the activity of REase. Since most restriction endonucleases need the presence of Mg^{2+} ions to cut DNA, by omitting Mg^{2+} in buffer and simultaneously adding S-adenosyl methionine (SAM), a common cosubstrate involved in methyl group transfers, *in vitro* methylation can be achieved, with the absence of REase activity (Lin et al., 2010; Alegre et al., 2004).

In a different approach, Bao and co-workers (2019) constructed a new shuttle plasmid (the plasmid pYL001, based on the pMTL83151 plasmid) where all restriction sites of two RM systems of *C. cellulovorans*, Cce743I and Cce743II, were removed. Indeed, in this work, a 100 % transformation was obtained (in 10 out of 10 experiments transformants were obtained). However, this plasmid does not contain the *traJ* gene. This gene is essential for conjugation between *E. coli* and Clostridia species. Therefore, this approach limits the methods of DNA introduction. However, obtaining a restriction-deficient strain is seen as an efficient way to increase the transformation rates without any limitation (Huang et al., 2018; Lesiak et al., 2014), but for the success of this strategy genetic manipulation tools need to be available. This is not the case for *C. cellulovorans*. Thus, the second aim of the present thesis was to develop genetic

manipulation tools for *C. cellulovorans*, which will be discussed in the following sections.

4.2. Genetic manipulation

Clostridial genetics has been revolutionized over the last few decades partially thanks to the Next-generation sequencing (NGS). With this technology, thousands of genes or even whole genomes can be sequenced in a short period. The rise of NGS led to the availability of annotated whole-genome sequences which is a prerequisite for the development of sophisticated genetic tools because it allows precise genetic manipulation (Kuehne et al., 2019). *C. cellulovorans* genome was sequenced in 2010. It consists of about 5.1 Mbp (megabase pairs), encoding 4254 proteins (Tamaruet al., 2010). With this, it is now possible to precisely select genes and identify the putative RM systems. However, it should not be assumed that the sequence is correct. For instance, the resequencing of *C. acetobutylicum* ATCC 824 showed 175 single nucleotide variations (SNVs) and 48 insertions/deletions (Indels) (Minton et al., 2016), while the resequencing of *C. autoethanogenum* DSM 10061 showed surprisingly 243 SNVs (Ehsaan et al., 2016). In the present study, *C. cellulovorans* from two different culture collections were resequenced. The whole genome sequence analysis showed 10 genome alterations compared to the genome sequence available in NCBI. Additionally, it proved that the cultures from different collections were not identical (*C. cellulovorans* from ATCC possess in the genome 5 additional alterations compared to the genome sequence *C. cellulovorans* from DSMZ, although the growth behavior was similar (Fig. 3.2).

Another important aspect of performing genetic manipulation is having an efficient procedure that allows gene transfer since Clostridia are not naturally competent. DNA transfer in Clostridia is achieved via electroporation and conjugative transfer. Electroporation has been reported as feasible for *C. cellulovorans* (Yang et al., 2016), but in the present work, it was not possible to reproduce it despite numerous attempts. Bao and co-workers (2019) reported the same issue. It was only possible to obtain transformants when most of the potential restriction sites were removed in the shuttle plasmid used (Bao et al., 2019). Fortunately, a protocol for triparental conjugation was established in our group while the present study was being conducted (Aline

Schöllkopf, unpublished). The conjugation protocol was proved to be reliable and was used to introduce all the plasmid DNA constructed in the present work.

Nowadays, many tools for the genetic manipulation of pathogenic and solventogenic Clostridia are available, allowing precise gene deletion, point mutations, overexpression, complementation, or even integration of foreign DNA into genomes. Genetic manipulation strategies can be divided into two main categories: forward and reverse genetics. In forward genetics, random screens are used to identify genes associated with a particular phenotype. In this case, the phenotype is studied, and the gene mutated is linked to a function. Forward genetics tools available for Clostridia include the random insertion of a transposon to create a random library of mutants and later analyze their phenotypes (Cartman & Minton, 2010). For the success of this strategy, is crucial that mutants have a single insertion. A successful example is the study developed by Cartman and Minton (2010), where a mariner-based transposon system allowed the *in vivo* random mutagenesis of *C. difficile*.

On the other hand, in reverse genetics, a gene is inactivated to study the phenotype it produces. In this approach, it is required that the target gene must be identified first. For Clostridia, the ClosTron system was a breakthrough technology. The ClosTron is based on the insertion of a mobile group II intron from the *ltrB* gene of *L. lactis* (Ll.ltrB) in the target gene (Heap et al., 2007). An erythromycin (*ermB*) resistance cassette is present inside the intron to select for intron insertion. However, it limits accumulated insertions in the same organism. To overcome this, the *ermB* resistance cassette can be removed from the insertion site by introducing an additional plasmid expressing the flippase (FLP) recombinase enzyme (Heap et al., 2010). This second plasmid must be lost by segregational instability before another gene disruption can be introduced (Heap et al., 2010), requiring laborious effort.

Another technique to achieve targeted genomic modification in Clostridia is the Allele-Coupled exchange (ACE). ACE is based on the natural process of homologous recombination (HR), where two DNA molecules with identical or similar sequences are exchanged (Court et al., 2002). Genomic modification via ACE is a two-step process. In the first step, a plasmid vector containing homology regions flanking the target sequence is integrated into the genome. After, the plasmid is excised in a second homologous recombination event, generating a mutated locus or the wild type.

Contrary to the ClosTron system, this strategy allows markerless deletion of genes, which is fundamental to investigating the role of single genes. The success of this strategy has been proved for *C. saccharobutylicum* (Huang et al., 2018). However, for the implementation of this strategy in a new organism, several factors such as the selection of the counterselection marker and identification of a suitable origin of replication that functions as a “pseudo-suicide replicon” must be considered and will be discussed in the following paragraphs.

4.2.1. Counter-selection markers

The selection of a counter-selection marker is an important aspect to enable genomic modification based on the HR (Reyrat et al., 1998). Counter-selection markers allow the selection of the second homologous recombination event. A common counter-selection strategy used for Clostrdia involves the disruption of the *pyrE* gene (encoding an orotate phosphoribosyltransferase) to create uracil auxotrophic mutants (Minton et al., 2016). The mutant requires supplementation with exogenous uracil for growth but is also resistant to the antimetabolite 5-fluoroorotic acid (5-FOA) (Minton et al., 2016). The drawback of this approach is that an initial mutant, which is a difficult step, is required in the first place. On the other hand, the *codBA* counterselection technique can be readily employed.

The use of the *codA* gene as a negative selection marker was initially exploited to allow genomic modification in *Rhodococcus equi* (Van Der Geize et al., 2008) and *Streptomyces* spp. (Dubeau et al., 2009). The *codA* gene encodes a cytosine deaminase, an enzyme broadly found in many bacteria and fungi. This enzyme catalyzes the deamination of cytosine to uracil as a part of the pyrimidine salvage pathway (Mullen et al., 1992; O’donovan & Neuhard, 1970). Similarly, this enzyme also converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU). 5-FU is highly cytotoxic since it can be directly incorporated into RNA, interfering with RNA transcription (Papanastasopoulos & Stebbing, 2014). Clinically, 5-FC is an antifungal agent used in the treatment of *Candida* spp. and *Cryptococcus* spp. infections (Billmyre et al., 2020; Pfaller et al., 2002), while 5-FU is used as an anticancer drug (Miura et al., 2010). However, 5-FC can be used as a counterselective compound to select for both plasmid excision and curing.

Although the presence of the cytosine permease is not a requirement for the success of *codA*-base counterselection, it has been reported that the expression of a cytosine permease, contributes to the uptake of the counterselective compound 5-FC (Kostner et al., 2013). The *codB* gene, encoding a cytosine permease, an enzyme also broadly found in many organisms, has been used to promote the uptake of 5-FC during the counterselection. The *codBA*-base counterselection system has been successfully employed for Gram-positive bacteria, including *Bacillus licheniformis* (Kostner et al., 2017) and *C. saccharobutylicum* (Huang et al., 2018). This system relies on the presence of the *codBA* cassette in the deletion plasmid and the use of the 5-FC as the counterselective compound. Therefore, in the presence of 5-FC, only wild-type or cells that lost the deletion plasmid after the second homologous recombination can grow. The greatest advantage of this method is that no prior mutations are required, therefore the wild-type strain can be directly used for targeted mutations. Although bioinformatic analysis has shown that many *Clostridium* species contain *codA* homologs, which restricts the applicability of the *codBA* strategy (Al-Hinai et al., 2012), *C. cellulovorans* does not contain it.

Thus, the *codBA* cassette from *C. ljungdahlii*, used for genomic modification *C. saccharobutylicum* expressed under the functional control of the constitutive P_{clpB} promoter from *Staphylococcus aureus* showed to be suitable for our application, despite the mutations in the promoter sequence. The arabinose promoter/repressor system could also be suitable for our application. Although *C. cellulovorans* can grow on L-arabinose as sole carbon source (after 2 days of incubation), it does not have important arabinose transporters (Zhang et al., 2012). Which is potentially responsible for the delayed uptake of arabinose, which is not a desirable aspect for the counterselection.

CRISPRP/Cas is a gene editing methodology that revolutionized molecular biology. It is also considered a counterselection marker, but instead of using chemicals for the selection of successful events, it uses double or single-strand brakes for the selection. In nature, the CRISPR/Cas system is a system found in prokaryotes to protect them against foreign DNA from bacteriophage infection. The system comprises clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated genes (Cas). Sequencing analysis revealed that two different CRISPR loci in

Streptococcus thermophilus strains were homologous to some bacteriophage, leading to the hypothesis that CRISPR was a defense mechanism. To test this hypothesis, a phage-sensitive wild-type *S. thermophilus* strain was challenged with two different virulent bacteriophages. It was found the bacteria submitted to viral stress integrated new spacers from phage genomic sequences that can lead to a diverse phage resistance phenotype of the bacteria. The authors also noticed that in the proximity of CRISPR sites a particular set of CRISPR-associated (cas) genes that coded Cas proteins (Barrangou et al., 2007). The Cas proteins have different functions, such as recognizing and inserting new spacers (e.g., Cas1 and Cas2) into the CRISPR array in the chromosome of the bacteria (called the adaptation or spacer acquisition phase), transcribing and processing (e.g., Cas6) the CRISPR array into crRNAs (called expression or crRNA Biogenesis) and with the help of crRNAs, other Cas proteins (e.g., Cas9, Cas12 and Cas13) cleaves bacteriophage DNA at specific site (Makarova et al., 2011). However, from the biotechnological point of view, it allows precise genetic manipulation.

Homologous recombination-based strategies could take advantage of the specificity of the Cas9 enzyme to direct the double-strand break necessary for homologous recombination. Thus, this study also investigated the applicability of the newly developed system CRISPR/Cas for *C. cellulovorans* (Aline Schöllkopf, unpublished). Differently from the traditional Cas9 that introduces double-strand breaks, which is frequently lethal, the Cas-Nickase (a variant of the Cas9 differing by a point mutation in the nuclease domains - D10 in the RuvC domain or H840 in the HNH domain) introduces only one strand break, which is sufficient for the recognition of the DNA damage by proteins involved in the repairing mechanism (Lee et al., 2023). As shown in in 3.3.2, the Cas-Nickase plasmid allowed the simultaneous deletion *ptb-buk* genes and insertion of the *cat1* gene from *C. tyrobutyricum* in *C. cellulovorans*. No mutant strain was obtained using the *codBA*-based system in this case. Interestingly, both *codBA*-based and Cas-Nickase systems allowed the successful deletion of the *pta* gene. However, plasmid curing was more laborious with the *codBA*-based. Potentially, the Cas-Nickase present in the deletion plasmid brings a burden to the cells, and only the cells that have lost the plasmid during segregation can grow.

4.2.2. Origin of replication

Plasmids are extrachromosomal DNA molecules that replicate independently of the host cellular division found in prokaryotes and some eukaryotes (Woese et al., 1990). They are normally circular, but also some linear forms were described in *Streptomyces rochei* (Hirochikal et al., 1984), *Borrelia* spp. (Barbour & Garon, 1987), and *Thiobacillus versutus* (Wlodarczyk & Beata, 1988). Many bacteria contain plasmids that provide new phenotypic characteristics to their host. Although plasmids are usually nonessential to their hosts, from an evolutionary point of view, plasmids allow rapid short-term adaptation of bacteria to changing environmental conditions. For instance, plasmids provide an advantage to the host, such as antibiotic resistance, but they also impose an energy burden that can slow cell growth. Therefore, in the absence of selective pressure, plasmids can be lost segregationally. However, plasmids can be stably replicated and be maintained in a bacterial population even under nonselective conditions due to plasmid stability or host fitness (San Millan & MacLean, 2017; Wein et al., 2019).

Since plasmids can carry genetic information, they can be used as cloning vectors for clinical and biotechnological applications. Plasmids can be transferred within one or between many species. Promiscuous plasmids are adapted to a wide range of bacteria and can be stably inherited in distantly related hosts. In addition to plasmid-inherited determinants, replication initiation is dependent on various host-encoded enzymes. Many plasmids can therefore replicate only in one or a few closely related hosts. The ability to replicate independently to the host chromosome is due to the presence of the origin of replication in the plasmid. The origin of replication, or ori, is the specific location where plasmid replication starts.

The knowledge about the compatibility of the origin of replication in different hosts is useful for the development of genetic systems. Both replicative and suicide plasmids have been used as vectors in genetic engineering systems. The use of suicidal plasmids offers an advantage for the development of tools for genetic engineering. This type of plasmid only replicates when the plasmid is integrated into the host chromosome. However, suicide plasmids require high transformation rates. The low efficiency of DNA transfer by transconjugation from *E. coli* to *C. cellulovorans* wild type rendered the use of suicide vectors for genetic modifications. Therefore, in the present

work, the compatibility of several origins of replication present in the pMTL80000 plasmid series was investigated. 5 origins of replication were identified as suitable for *C. cellulovorans*. Interestingly, for Yang and co-workers the ori found in the plasmid pMTL82151 (pBP1) was not replicative in *C. cellulovorans*. However, it was possible in our hands, potentially, this is due to the great DNA transfer efficiency obtained with the use of the triparental conjugation method.

Besides identifying a compatible ori, for the ACE vectors, it is also important that this replicon is replication defective, also called “pseudo-suicide” replicon. Pseudo-suicide describes a segregationally unstable plasmid that replicates at a rate slower than the host chromosome. The “pseudo-suicide” plasmid can replicate autonomously, but the segregation into daughter cells is defective (Minton et al., 2016). Through the plasmid stability assay, it was identified that some replicons, such as pCB102 (pMTL83151) were extremely stable. Therefore, this replicon should not be used in the ACE vectors. The replicon pUB110 (pMTL87151) is a better candidate to be part of the ACE vectors used for *C. cellulovorans*. This knowledge is also relevant for optimizing plasmid gene expression.

Plasmid replication occurs in three stages: initiation, elongation, and termination (Kuest & Stahl, 1989). The initiation process depends on host-encoded (e.g. DNA polymerase) and plasmid-encoded proteins (replication initiation protein, also called Rep proteins). In general, initiation is catalyzed by various Rep proteins, which generally recognize plasmid-specific DNA sequences and determine the point from which replication starts (the origin of replication). Rep proteins often bind to the ori and cause localized unwinding of the DNA, facilitating the recruitment of other necessary replication machinery. Plasmid elongation is carried out by DNA polymerases. DNA polymerases are essential enzymes to create DNA molecules by assembling nucleotides, but they are unable to initiate *de novo* replication, making necessary the independent generation of a primer by primases. Primases generate short RNA primers to provide a starting point for DNA polymerases. Termination occurs when the two forks meet and fuse, resulting in two separate double-stranded DNA molecules (Kuest & Stahl, 1989; Solar et al., 1998). In the present work, the ori pCB102, from *C. butyricum*, was transconjugated more efficiently, while the ori pUB110, from

S. aureus, was transconjugated less efficiently. It is possible to speculate that this results from discrepancies in host-encoded proteins.

In terms of the replication mechanism of the origin of replication compatible with *C. cellulovorans*, nothing is known. There are two common replication mechanisms for circular plasmids: theta type and rolling circle. The theta type is often a bidirectional replication mechanism. It starts in the ori region and then, a DNA helicase unwinds the DNA, creating a replication bubble that is seen as a θ ("theta")-shaped molecule under electron microscopy. After, the synthesis of a primer RNA (pRNA) allows the initiation of DNA synthesis by the DNA polymerase. The two replication forks move away from the ori in opposite directions. DNA synthesis is continuous on one of the strands (leading strand, adding nucleotides to the 3') and discontinuous on the other (lagging strand, adding nucleotides to the 5' end). Replication continues until the forks meet and two new circular DNA molecules are synthesized (Solar et al., 1998). Rolling circle replication starts with a nick in one strand of the circular DNA at the origin. DNA polymerase extends the 3' end of the nicked strand using the intact circular strand as a template. The 5' end of the nicked strand is displaced as the 3' end is extended, resulting in a continuous elongation. The displaced single-stranded DNA can circularize and serve as a template for complementary strand synthesis. This mechanism is very efficient for producing large quantities of DNA quickly (Wawrzyniak et al., 2017).

Concerning the plasmid copy number, some plasmids occur in only one, a few copies, or several copies per cell. In the present work, the plasmid copy number was for the first time investigated in *C. cellulovorans*. The plasmid pMTL87151 had the lowest copy number and was the most unstable. The plasmid pMTL83151 had the highest copy number and was very stable. Thus, the results obtained indicate a correlation between stability and copy number for *C. cellulovorans*.

4.2.3. Generation of a 'domesticated' strain

Bacterial conjugation is one of the three major horizontal gene transfer mechanisms found in bacteria, the other two are transduction and bacterial transformation (Villa et al., 2019). During conjugation, a DNA molecule is transferred from a donor to an acceptor cell by direct contact. Conjugation plays an important role in synthetic biology

approaches. The plasmids used for genetic engineering applications can be delivered into the acceptor host via conjugation. However, most often the RM systems but sometimes also CRISPR/Cas and other systems represent critical barriers in bacteria making the delivery of the plasmid vectors difficult. Therefore, the knockout of restriction endonucleases that are part of the RM systems should make the strain more accessible to DNA transfer via conjugation or transformation via electroporation. In the present work, the deletion of the type I and III RM systems was accomplished and showed an increase in two orders of magnitude in the conjugation efficiency (Table 3.7). Thus, the generated strains could be used for further metabolic engineering.

4.3. Metabolic engineering

To date, the metabolic engineering studies for *C. cellulovorans* focused on the over-expression of heterologous enzymes, such as the butyraldehyde/butanol dehydrogenase (*adhE2*) from *C. acetobutylicum* to enable butanol production from crystalline cellulose (Yang et al., 2017; Yang et al., 2016). *C. cellulovorans* full biotechnological potential has not so far been exploited due to the lack of genetic tools available. However, with the genetic tools developed in the present work and our working group, *C. cellulovorans* is now genetically accessible.

As part of the project SynConsor4Butanol *C. cellulovorans* strains were generated to be part of a synthetic co-culture with the solventogenic clostridia. *C. cellulovorans* is a butyrate-producing bacteria, and as reported before, the addition of butyrate to the medium significantly induces butanol production (Kao et al., 2013; Lee et al., 2008). Thus, it was hypothesized that establishing a co-culture of *C. cellulovorans* and a solventogenic Clostridia would lead to increased butanol production. However, it was suggested that *ptb-buk* channel is reversibly functional in *C. cellulovorans* (Wen et al., 2020a), which is not efficient for the co-culture setup. To prevent Butyryl-CoA generation, Wen and co-workers (2020) expressed the *cat1* gene (encoding a butyryl-CoA−acetate CoA transferase) from *C. tyrobutyricum*. The authors observed a higher butyrate production in *C. cellulovorans* (10.7 g/L). Thus, in the present work, the *ptb-buk* genes were successfully deleted and replaced by the *cat1* gene from *C. tyrobutyricum*. The *cat1* gene is also responsible for the conversion of acetate to acetyl-CoA. Thus, a high butyrate formation is expected for such strain since it coupled butyrate formation to acetate re-consumption.

However, the growth of two different organisms in a co-culture is typically unstable, but generating metabolically dependent strains could resolve this issue. To obtain metabolic-dependent strains, in the SynConsor4Butanol, it was aimed to generate a strain *C. cellulovorans* with the acetate metabolism abolished and a strain of a solventogenic clostridia with the butyrate metabolism abolished. Both strains need external acetate or butyrate, respectively, to grow. Thus, in the synthetic co-culture, *C. cellulovorans* needs the acetate provided by the solventogenic clostridia, and the solventogenic clostridia needs the butyrate provided by *C. cellulovorans*. The present study focused on the metabolic engineering of *C. cellulovorans*. To obtain the deletion of the *pta-ack* operon, responsible for the acetate production, deletion plasmids must be constructed. However, the first challenge encountered was the impossibility of cloning the fragment corresponding to the flaking region adjacent to the *ack* gene. The flaking region covered three ORFs Clocel_1893 (encoding a Zn-finger-like protein, nucleic acid binding), Clocel_1894 (encoding a ribosomal L32p protein), and Clocel_1895 (encoding a fatty acid/phospholipid synthesis protein PlsX). Potentially, it was encoded in the flanking region a protein with function in *E. coli*. Therefore, only the *pta* gene was deleted. For the success of the proposed co-culture, it is important to obtain a single strain with both metabolic modifications. However, it was not possible during the present study. Notice, that one metabolic modification can lead to a significant metabolic burden in the organism and two metabolic modifications are potentially lethal.

The third metabolic engineering approach exploited in the present study was to enhance butyrate synthesis by enhancing the NADH pool. During the butyrate synthesis, NADH is oxidized generating NAD^+. In the present study, to maximize butyrate production in *C. cellulovorans*, it was hypothesized that the pool of NADH in the cells could be enhanced with the deletion of the main hydrogenase since in such co-culture, molecular hydrogen (H_2) is an undesired byproduct. Thus, with the abolishment of the H_2 production, the strain needs to re-oxidize the NADH produced in the glycolytic pathway through the production of butyrate. As a result, the generated strain should only produce butyrate and CO_2. However, despite several attempts, the deletion of one of the two putative hydrogenases (Clocel_2243 and Clocel_4097) was not possible. In Clostridia, H_2 production is synchronized with the reduction of ferredoxin. Ferredoxin is reduced by the pyruvate:ferredoxin oxidoreductase (PFOR)

to oxidize the NADH produced in excess during glycolysis. Oxidized ferredoxin is regenerated by the hydrogenase. Hydrogenases are enzymes responsible for producing H_2, catalyzing the reversible oxidation of redox equivalents accumulated during fermentation. Hydrogenases transfer electrons from physiological electron donors, such as ferredoxin, to protons (2 reduced ferredoxin + 2 $H^+ \rightleftharpoons H_2$ + 2 oxidized ferredoxin) (Calusinska et al., 2010; Demuez et al., 2007). To notice, Fd_{red} and NADH are the main reducing equivalents produced during Clostridia fermentation. They cannot be accumulated in cells and must be oxidized immediately to maintain the redox balance in cells (Tang et al., 2023). Thus, since H_2 formation is the main pathway for Fd_{red} oxidization, its abolishment might not be compatible with cell survival. Potentially, a redox imbalance caused by the deletion of hydrogenases is lethal to the cells, and no mutant strain can be obtained.

Additionally, the strains generated in this work could also be used to produce butanol directly from *C. cellulovorans*. In previous work, Wen and co-workers (2020a) proposed that pushing the carbon flow from butyrate and acetate back to butyryl-CoA improved the butanol production in *C. cellulovorans*. For that, the authors overexpressed the *cat1* gene (encoding a butyryl-CoA−acetate CoA transferase) from *C. tyrobutyricum* in *C. cellulovorans*. To ensure that the activity observed is prevenient from the expression of the *cat1* gene, it is required that the native butyrate pathway is abolished in *C. cellulovorans*. However, despite several attempts, it was not possible to delete the *ptb-buk* operon in *C. cellulovorans* using the ClosTron system (Wen et al., 2020a). In the present study, the conjugation of deletion vectors was facilitated due to the construction of restriction-less strains of *C. cellulovorans*. Using the strains and techniques developed in this work, it was possible not only to delete the *ptb-buk* operon in *C. cellulovorans*, but also the *cat1* gene could be inserted into the genome, which is a more stable strategy for biotechnological use. Additionally, the acetate pathway was partially abolished. As proved for *C. acetobutylicum* and *C. tyrobutyricum* the disruption of the acetate pathway reinforced the solvent formation pathway (Jang et al., 2012; Yu et al., 2011). With the deletion of *C. cellulovorans* hydrogenases, the increased intracellular NADH could potentially enhance butanol production when *adhE2* is expressed. As demonstrated for *C. thermocellum*, the abolishment of hydrogen production led to increased alcohol production (Biswas et al., 2015). Thus, future studies should explore this possibility.

5. Conclusion

C. cellulovorans bacteriology has been little explored so far. In the present study, insights into its growth on different substrates, sporulation, the presence of prophages, and RM systems have been provided. As expected, on cellobiose, the growth is faster than on a complex substrate such as crystalline cellulose. This led to a faster acidification of the medium, creating an environment that is toxic to the cells due to the low pH. However, for genetic work, it is important to use a C-source that allows fast growth for convenience. Thus, cellobiose is the best candidate for *C. cellulovorans* cultivation. Nevertheless, the use of fresh cultures (16–24 h) is extremely important. After this point, most of the cells in the culture are no longer viable due to the accumulation of acids in the medium.

Regarding sporulation, although *C. cellulovorans* has been reported as a sporulating organism, no endospores were detected during this study. Frequently, swollen bright cells were observed under phase contrast microscopy. However, this is only an indicator of the accumulation of storage material, potentially amylopectin.

In regard to the presence of prophages in *C. cellulovorans,* no report has been found in the literature. However, *in silico* analyses revealed the presence of 4 intact prophages in its genome. The induction of prophages is potentially the reason behind the difficult handling of the organism. The triggers for the prophage induction are still unknown. Attempts to obtain a cured strain by inducing the prophages using mitomycin C and UV light were not successful, potentially due to the use of suboptimal conditions.

In terms of the RM systems, *C. cellulovorans* has 12 putative RM systems. Frequently, the presence of RM systems makes DNA transfer in *Clostridium* spp. intractable. Contrary to what was reported in the literature, the type I restriction system is likely to play a significant role in *C. cellulovorans*. In this study, the complete digestion of plasmid by the cell free extract showed an unspecific cutting pattern, which is a characteristic of type I RM systems.

Additionally, a new gene editing system was developed in the present study. The method of choice was the Allele-Coupled Exchange using the *codBA*-system for counterselection. For the success of this system, it was fundamental that the promoter

in front of the *codBA* cassette provide a moderate expression. The arabinose inducible systems, *araBAD* and Arai, are not suitable for gene expression in *C. cellulovorans*. Fortunately, using the P_{clpB} promoter in front of the *codBA* cassette allowed molecular cloning in *E. coli* and successful counterselection in *C. cellulovorans*. However, medium supplementation with purines was critical for the proper function of the counterselective compound 5-FC. Another key aspect was the selection of a "pseudo-suicide" Gram-positive replicon. In this study, new suitable Gram-positive replicons were identified for *C. cellulovorans*. The evaluation of the plasmid segregational stability revealed that the replicon pUB110 (present in the plasmid pMTL87151) is replication defective and the best candidate for use in the deletion vectors. With the developed gene editing system, two putative RM systems were successfully knocked out. Three strains were obtained: *C. cellulovorans ΔClocel_1114*, *C. cellulovorans ΔClocel_2651*, and *C. cellulovorans ΔClocel_1114 ΔClocel_2651*. The conjugation efficiency evaluation showed an increase of two orders of magnitude conjugation efficiency for the single mutants. Interestingly, although the type II RM system is the most common and frequently considered the most important in other clostridial species, the type I and III RM systems are of major importance in *C. cellulovorans*. The high conjugation efficiency demonstrated by the newly generated strains was a breakthrough allowing the metabolic engineering approaches aimed in this study.

As is widely recognized, lignocellulosic biomass (LB), the most abundant renewable feedstock on Earth, is a promising feedstock for the production of eco-friendly chemicals and fuels. However, its natural recalcitrance makes it difficult to explore. In this context, microorganisms can be utilized for the production of lignocellulosic chemicals and fuels. Unfortunately, there is no single organism, described so far, that can efficiently break down the LB and produce chemicals in concentrations useful for the chemical industry. In recent years, it has been demonstrated that establishing synthetic co-cultures of cellulolytic and solventogenic bacteria is more promising than engineering a single organism with both traits. However, these different organisms have varying growth rates, which results in an unstable co-culture. In the present study, *C. cellulovorans* strains were generated to be metabolically dependent on the solventogenic clostridia. The chosen strategy was to create strains with the acetate pathway deactivated and a strengthened butyrate pathway. Thus, the strain requires acetate from the solventogenic clostridia to survive and will provide great amounts of

butyrate, which is an intermediate in the butanol production in the solventogenic clostridia.

In this study, the *C. cellulovorans ΔClocel_1114 Δptb-buk::cat1* strain was generated. The *ptb* and *buk* genes, responsible for the production of butyrate are assumed to reversibly convert butyrate to butyryl-CoA. For the efficient production of butyrate, these genes were replaced by the *cat1* gene from *C. tyrobutyricum*, which simultaneously converts butyryl-CoA to butyrate and acetyl-CoA to acetate. Interestingly, without acetate supplementation in the medium, the organism cannot grow as usual. This indicates the *cat1* gene is active in *C. cellulovorans* and catalyzes the conversion of acetate to acetyl-CoA. This finding supports the hypothesis that *C. cellulovorans* will only be able to grow in the presence of the external acetate provided by the solventogenic clostridia, and this strain is a good candidate to establish a synthetic co-culture. The other strain generated was *C. cellulovorans ΔClocel_1114 Δpta*. In the present work, it was not possible to completely abolish the acetate pathway by deleting the *pta* and *ack* genes, since it was not possible to clone in *E. coli* the flanking regions for the deletion of the *ack* gene. The strain *C. cellulovorans Δpta* can still grow in medium without the supplementation of acetate. Indicating that the deletion of the *ack* gene is essential to completely shut down acetate production. Besides, in an attempt to maximize butyrate production by raising the NADH pool, *C. cellulovorans* hydrogenases should be deleted. Unfortunately, it was not possible to obtain the desired strains in this work. The deletion of hydrogenases disrupts the redox balance in the cells which may not be compatible with the survival of the organism.

6. References

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7. Appendixes

Table 7.1 | Primers for the construction of the plasmid pMTL83151_codBA. This plasmid was constructed to clone the codBA operon from *C. ljungdahlii* under the control of P_{fdx} promoter from *C. sporogenes* in the vector pMTL83151. The overhangs are marked in bold. The sequencing primers CodBA_Seq2, CodBA_Seq3 and CodBA_Seq4 were kindly provided by Holger Edelmann (unpublished). The primers AS11 and AS12 were kindly provided by Aline Schöllkopf (unpublished).

Name	Sequence (5' → 3')	Use
pMTL83151_backbone_fw	**GAATTACCTTTGATATAAATAAGTAG**GCATAAAAATAAGAAGCCTGCATTTG	Amplification of the backbone
pMTL83151_backbone_rv	**TTCACAGGCTACTACACG**CAGCGGCCGCGGTCATAG	Amplification of the backbone
CodBA_S_pMTL83151_fw	**CTATGACCGCGGCCGCTG**CGTGTAGTAGCCTGTGAA	Amplification of the insert
CodBA_S_pMTL83151_rev	**CAAATGCAGGCTTCTTATTTTTATGC**CTACTTATTTATATCAAAGGTAATTC	Amplification of the insert
seq_pMTL83151_codBA_fw	AAGGCAAGACCGATCG	Sequencing of the insert
seq_pMTL83151_codBA_rv	TTCTTTCTATTCAGCACTGTTATG	Sequencing of the insert
CodBA_Seq2	ATTTGGGCTTCCTGCTTATG	Sequencing of the insert
CodBA_Seq3	TGCTGTAGAACAATTTGATG	Sequencing of the insert
CodBA_Seq4	AGAATATGGCGTGGATTCTG	Sequencing of the insert
AS11	ACAGAGCTCATGCAGACTACAACAGAAATG	Amplification of the ORFs Clocel_3986 and Clocel_398 to verify *C. cellulovorans* identity
AS12	CACCTCGAGCTACTCACATTTGGCATAATAATTTATGC	Amplification of the ORFs Clocel_3986 and Clocel_398 to verify *C. cellulovorans* identity

Table 7.2 | Primers for the construction of the plasmid pMTL83151CodBA_araBAD. This plasmid was constructed to clone the *araC* repressor and araBAD promoter from *E. coli* in front of the *codBA* operon from *C. ljungdahlii*. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pMTL83151CodBA_backbone_S_fw	**CATACCCGTTTTTTGGGCTAA**TAAGGAGGTGTGTTACATATGG	Amplification of the backbone
pMTL83151CodBA_backbone_S_rv	**CAGACAATTGACGGCTTGACGG**CATAGCTGTTTCCTGATAAAAAAATTG	Amplification of the backbone
araBAD_S_fw	**CAATTTTTTTATCAGGAAACAGCTATG**CCGTCAAGCCGTCAATTGTCTG	Amplification of the insert
araBAD_S_rv	**CCATATGTAACACACCTCCTTA**TTAGCCCAAAAAACGGGTATG	Amplification of the insert

Name	Sequence (5' → 3')	Use
check_pMTL83151codBA_araBAD_fw	TTCGCGGAGCTGGTGAAGTACATC	Sequencing of the insert
check_pMTL83151_codBA_araBAD_rv	ACAGTTTCAAGGGCAGCATATTCC	Sequencing of the insert
Seq_araBADP_codB	AACCAATTGTCCATATTGCATC	Sequencing of the insert

Table 7.3 | Primers for the construction of the plasmid pMTL83151codBA_ARAi. This plasmid was constructed to clone the arabionose repressor (*araR*) and *ptk* promoter (P_{ptk}) from *C. acetobutylicum in* front of the *codBA* operon from *C. ljungdahlii*. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pMTLcodBA_fw	**ATAAATTTAGGAGGAATATTATG**ATGGATAACAAGGAAACAAATG	Amplification of the backbone
pMTLcodBA_rv	CTGTTTCCTGATAAAAAAATTG	Amplification of the backbone
araR_fw	ATATGATCTTCCATAACTTAAC	Amplification of the insert
araR_rv	**CAATTTTTTTATCAGGAAACAG**TTATGAAAGCGATTACCTATA	Amplification of the insert
ptk_fw	**GTTAAGTTATGGAAGATCATAT**CAGTTGAGCAAGTTTATGAC	Amplification of the insert
ptk_rv	CATAATATTCCTCCTAAATTTAT	Amplification of the insert
check_pMTL83151codBA_ARAi_fw	CTGCAGGATAAAAAAATTGTAG	Sequencing of the insert
check_pMTL83151codBA_ARAi_rv	CCAGGTTCCGTTGTTTGCATTG	Sequencing of the insert
Seq_ptk_codB	ACTAAAAAGTCATTGACATATGC	Sequencing of the insert

Table 7.4 | Primers for the construction of the plasmid pMTL8x151_pE194(ts). The restriction sites are underlined.

Name	Sequence (5' → 3')	Use
pE194ts_AscI_fw	ATATATATGGCGCGCCATTTAGGGGAGAAAACATAGGG	Amplification of the insert
pE194ts_FseI_rv	ATATATATGGCCGGCCCTATCGGTCAGATTTATACCGATT	Amplification of the insert

Table 7.5 | Primers used for by Real-time quantitative PCR (qPCR).

Name	Sequence (5' → 3')	Use
qPCR_Clocel_3734_fw	AAGGTTGCTGGTCCAGTG	Reference gene used for qPCR
qPCR_Clocel_3734_fw	GAGTTCTGATTTCGAATTGCTCTC	Reference gene used for qPCR

Name	Sequence (5' → 3')	Use
qPCR_catP_fw	GCCTTTGGACTGAGTGTAAG	Target gene used for qPCR
qPCR_catP_rv	GGAGCATTTGGCTTTCCT	Target gene used for qPCR

Table 7.6 | Primers for the construction of the plasmid pLA_ori3 (also referred as pHE1pCB102). This plasmid was constructed to clone the Gram-positive replicon pCB102 (also referred as "ori3") into the plasmid pHE1 containing the P_{clpb} promoter from *S. aureus in* front of the *codBA* operon from *C. ljungdahlii*. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pHE1backb_fw	**CAGTGGGCAAGTTGAAAAATTCA**GGAGTG CAGTCGAAGTGGGCAAG	Amplification of the backbone
pHE1backb_rv	CTTAATAACTTCTTGAACACTTG	Amplification of the backbone
oripCB102_fw	**CAAGTGTTCAAGAAGTTATTAAG**TGCAGG CTTCTTATTTTTATGG	Amplification of the insert
oripCB102_rv	TGAATTTTTCAACTTGCCCACTG	Amplification of the insert
check_oripCB102	TTTTAAATAAATATAGTGTTATTTC	Sequencing of the insert
check2_oripCB102	GTTTATTAAAGATACTGAAATATGC	Sequencing of the insert

Table 7.7 | Primers for the construction of the plasmid pLA_del_1114. This plasmid contains homologous flanks to the ORF Clocel_1114. The overhangs are marked in bold. The primers pCN9_F_backbone and pCN9_R_backbone were kindly provided by Ching-Ning Huang (unpublished). The primers catP_fw and catP_rv were kindly provided by Aline Schöllkopf (unpublished).

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
upflank_1113_fw2	**CTACTTAATTGTGTGTAAGATAAG**TTTTATA GATTTTCTGCCACGAG	Amplification of the upper flank
upflank_1113_rv	**CTAGCCTATGCTTTCGAACAT**TAAAAAGGT GAAGTTACTCATTC	Amplification of the upper flank
lowflank_1115_fw	ATGTTCGAAAGCATAGGCTAG	Amplification of the lower flank
lowflank_1115_rv2	**CTTGAACACTTGCCGAAA**AATTTATCTTCT GCACCTGTTTC	Amplification of the lower flank
Del_1114_fw_2	AAGTAACACCTTTATCGTAAGC	Verification of the deletion
Del_1114_rv_2	AACTCTTGGGGTAAAATATTGAC	Verification of the deletion
Del_1114_check_seq_fw	TACAAGGAGATTATACTATAAG	Verification of the deletion
Del_1114_check_seq_rv	ACATTTGCATTTCTATATTG	Verification of the deletion

Name	Sequence (5' → 3')	Use
pLA_del_1114_check	TTCATTGTGTATACCTCCTGAAG	Sequencing of the insert
pLA_del_check_fw	CTCTCAAAAACTGAACCTAAAAAG	Sequencing of the insert
pLA_del_check_rv	TTCTTTTCTGTAAATTTCTTTCTATTC	Sequencing of the insert
pLA_del_ori7_check_rv	TCCCTGATCTCGACTTCGTTCT	Sequencing of the insert
pLA_del_ori8_check_rv	TGGCTAAGCGATAGAGGAC	Sequencing of the insert
catP_fw	TTTGAGAGGGAACTTAGATGG	Amplification of the *catP* cassette
catP_rv	ACTATTTATCAATTCCTGCAATTCG	Amplification of the *catP* cassette

Table 7.8 | Primers for the construction of the plasmid pLA_del_4005. This plasmid contains homologous flanks to the ORF Clocel_4005. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
upflank_4004_fw	**CTACTTAATTGTGTGTAAGATAAG**GCAGCAATAGCAGATACC	Amplification of the upper flank
upflank_4004_rv	**GGAATGAAGATAGTTTCAAGTTAC**ATAGGGTTGCTAGAATTATAAGAG	Amplification of the upper flank
lowflank_4006_fw	GTAACTTGAAACTATCTTCATTCC	Amplification of the lower flank
lowflank_4006_rv	**CTTGAACACTTGCCGAAA**CGATGATAGAGGCTGAAC	Amplification of the lower flank
Del_4005_fw	AATGTTTGGTTCTTTCCCAATGTTC	Verification of the deletion
Del_4005_rv	AAAGTTACAGAATCCTATAATGGCAC	Verification of the deletion
pLA_del_4005_check	ACTGTGGCACGAATCTCC	Sequencing of the insert
pLA_del_check_fw	CTCTCAAAAACTGAACCTAAAAAG	Sequencing of the insert
pLA_del_check_rv	TTCTTTTCTGTAAATTTCTTTCTATTC	Sequencing of the insert
pLA_del_ori7_check_rv	TCCCTGATCTCGACTTCGTTCT	Sequencing of the insert
pLA_del_ori8_check_rv	TGGCTAAGCGATAGAGGAC	Sequencing of the insert

Table 7.9 | Primers for the construction of the plasmid pLA_del_4006. This plasmid contains homologous flanks to the ORF Clocel_4006. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone

Name	Sequence (5' → 3')	Use
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
upflank_4005_fw	**CTACTTAATTGTGTGTAAGATAAG**TCAACTGACCGAGTGTAG	Amplification of the upper flank
upflank_4005_rv	**CCGTGTATCATTATTTGTTTACA**TTTTACGATAAGGAAGATTAGG	Amplification of the upper flank
lowflank_4007_fw	TGTAAACAAATAATGATACACGG	Amplification of the lower flank
lowflank_4007_rv	**CTTGAACACTTGCCGAAA**TTGGTGGATGGGAAGATG	Amplification of the lower flank
Del_4006_fw	AAGCGTATCTGCAGCATCC	Verification of the deletion
Del_4006_rv	GAATGGCTTAAACCGCAAGTAAAC	Verification of the deletion
pLA_del_4006_check	TCACCGCTTTCAGCTGTCTC	Sequencing of the insert
pLA_del_check_fw	CTCTCAAAAACTGAACCTAAAAAG	Sequencing of the insert
pLA_del_check_rv	TTCTTTTCTGTAAATTTCTTTCTATTC	Sequencing of the insert
pLA_del_ori7_check_rv	TCCCTGATCTCGACTTCGTTCT	Sequencing of the insert
pLA_del_ori8_check_rv	TGGCTAAGCGATAGAGGAC	Sequencing of the insert

Table 7.10 | Primers for the construction of the plasmid pLA_del_2651. This plasmid contains homologous flanks to the ORF Clocel_2651. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
upflank_2650_fw	**CTACTTAATTGTGTGTAAGATAAG**ACTTAACCGACCAACCAG	Amplification of the upper flank
upflank_2650_rv	**CCATGAAAGTAGAATTATTTCCA**GGCTTTTTTTGTAATTACTAAATG	Amplification of the upper flank
lowflank_2652_fw2	TGGAAATAATTCTACTTTCATGGTTACCTC	Amplification of the lower flank
lowflank_2652_rv2	**CTTGAACACTTGCCGAAA**GGAAGCGAGCATCAGAAGC	Amplification of the lower flank
Del_2651_fw_2	CCGCTGAGCTTTTGATTGG	Verification of the deletion
Del_2651_rv	ATCAACCTTATAATTTCGCATACG	Verification of the deletion
pLA_del_2651_check	TGAATGCTGCCAAGATTTAGACC	Sequencing of the insert
pLA_del_check_fw	CTCTCAAAAACTGAACCTAAAAAG	Sequencing of the insert
pLA_del_check_rv	TTCTTTTCTGTAAATTTCTTTCTATTC	Sequencing of the insert

Name	Sequence (5' → 3')	Use
pLA_del_ori7_check_rv	TCCCTGATCTCGACTTCGTTCT	Sequencing of the insert
pLA_del_ori8_check_rv	TGGCTAAGCGATAGAGGAC	Sequencing of the insert
catP_fw	TTTGAGAGGGAACTTAGATGG	Amplification of the *catP* cassette
catP_rv	ACTATTTATCAATTCCTGCAATTCG	Amplification of the *catP* cassette

Table 7.11 | Primers for the construction of the plasmid pLA_del_ptbbuk_cat1. This plasmid contains homologous flanks to the genes *ptb* and *buk* and the *cat1* gene in between the flanks. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
cat1_del_G_fw	**GAATAAAAAAAGAGGTGCAGTTGG**ATGAG TTTTGAGGAATTGTATAAG	Amplification of the *cat1* gene
cat1_del_rv	TCAATATTTACATCCAAATCTTTTTTC	Amplification of the *cat1* gene
upflank_3673_G_fw	**GTTCTTCTACTTAATTGTGTGTAAGATAAG**C AACTGGACTTTGAATTTCATC	Amplification of the upper flank
upflank_3673__G_rv	**GAAAAAAGATTTGGATGTAAATATTGA**TAG ATATAGAAAAAACGCTAGCAAT	Amplification of the upper flank
lowflank_3676_fw	CCAACTGCACCTCTTTTTTTATTC	Amplification of the lower flank
lowflank_3676_G_rv	**CTTCTTGAACACTTGCCGAAA**CTGCTTCAG AGGAAAATAGATTAG	Amplification of the lower flank
cat1_check	TGACTTCATTAATAGTTGCTATGT	Verification of the integrity of the *cat1* gene
cat1_check_2	CTGTAAGATGGTTTGCATAAACT	Verification of the integrity of the *cat1* gene
upflank_3673_check	GTGCTGTATTCCATAAGAAAGG	Verification of the integrity of the upper flank
lowflank_3676_check	GAAGGAACATCCTTCATAAAGTC	Verification of the integrity of the lower flank
ptbbuk_del_fw	GCAGCACCATTAGCACAATC	Verification of the deletion
ptbbuk_del_rv	GAGTAAGAATTGAGCATAGTCAAG	Verification of the deletion

Table 7.12 | Primers for the construction of the plasmid pNickase_ptbbuk_cat1. The restriction sites are underlined. The seed sequences are marked in italics. The primers XX_XhoI_J23119_rv and S24_Cas9_ATG_out were kindly provided by Aline Schöllkopf (unpublished).

Name	Sequence (5' → 3')	Use
upflank_3673_AatII_fw	ATATATATGACGTCCAACTGGACTTTGAATTTCATC	Amplification of the insert
lowflank_3676_AscI_rv	ATATATATGGCGCGCCCTGCTTCAGAGGAAAATAGATTAG	Amplification of the insert
cat1_XhoI_J23119-sgRNA-fw_sgRNA1	ATAATCTCGAG*ATCGTATGTCATATGGGCGG*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
cat1_XhoI_J23119-sgRNA-fw_sgRNA2	ATAATCTCGAG*AACTTAATCGTATGTCATAT*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
cat1_XhoI_J23119-sgRNA-fw_sgRNA3	ATAATCTCGAG*GCTTTTTCTCCAGAAAGAA G*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
XX_XhoI_J23119_rv	CTCGAGATTATACCTAGGACTGAGCTAGC	Seed sequence exchange – general primer
S24_Cas9_ATG_out	ATCTAAGCCTATTGAGTATTTCTTATCCAT	sgRNA sequencing

Table 7.13 | Primers for the construction of the plasmid pLA_del_pta. This plasmid contains homologous flanks to the *pta* gene. The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
upflank_1890_G_fw	**GTTCTTCTACTTAATTGTGTGTAAGATAAG**TTCTGTTTATTCTCGTCAATG	Amplification of the upper flank
upflank_1890_G_rv_v2	**TATTAAATTCCTTTTTGTGCTTG**AATCTTCTGCATAAATTCCAT	Amplification of the upper flank
lowflank_1892_fw	CAAGCACAAAAAGGAATTTAATA	Amplification of the lower flank
lowflank_1892_G_rv	**CTTCTTGAACACTTGCCGAAA**TCTCTAAAATCACTACTTAATCC	Amplification of the lower flank
upflank_1890_check_fw	GCAGAATTTACTGCATATACTGTT	Verification of the integrity of the upper flank
lowflank_1892_check_fw	TAACAAAGAATACGGTGTAATAG	Verification of the integrity of the lower flank
Del_pta_fw	TAAGTTGGAGGTTAGAATTC	Verification of the deletion
Del_pta_rv	CAAAAGCTATACAGTCAAC	Verification of the deletion

Table 7.14 | Primers for the construction of the plasmid pNickase_pta. The restriction sites are underlined. The seed sequences are marked in italics.

Name	Sequence (5' → 3')	Use
upflank_1890AatII_fw	ATATATATGACGTCTTCTGTTTATTCTCGTCAATG	Amplification of the insert
lowflank_1892_AscI_rv	ATATATATGGCGCGCCTCTCTAAAATCACTACTTAATCC	Amplification of the insert
pta_XhoI_J23119-sgRNA-fw_sgRNA1	ATAATCTCGAG*TAAGTCACCGGTTGTATGAA*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
pta_XhoI_J23119-sgRNA-fw_sgRNA2	ATAATCTCGAG*CAAATAATTAAAACGGCTCC*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
pta_XhoI_J23119-sgRNA-fw_sgRNA3	ATAATCTCGAG*AAAGATAACATCGCAACCTT*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
XX_XhoI_J23119_rv	CTCGAGATTATACCTAGGACTGAGCTAGC	Seed sequence exchange – general primer
S24_Cas9_ATG_out	ATCTAAGCCTATTGAGTATTTCTTATCCAT	sgRNA sequencing

Table 7.15 | Primers for the construction of the plasmid pLA_del_hyd_4097. This plasmid contains homologous flanks to the putative main hydrogenase gene (ORF Clocel_4097). The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
upflank_4096_G_fw	**GTTCTTCTACTTAATTGTGTGTAAGATAAG**GTAAAGCAGTCAATAATAAAC	Amplification of the upper flank
upflank_4096_G_rv	**CACCAATGAAAACTATAGTTATTGAT**CATGTAAAATATAAAAAATAAATTG	Amplification of the upper flank
lowflank_4098_fw	ATCAATAACTATAGTTTTCATTGGTG	Amplification of the lower flank
lowflank_4098_G_rv	**CTTCTTGAACACTTGCCGAAA**ATTTCAGTGATATTCTTTCGATAG	Amplification of the lower flank
upflank_4096_check_fw	TTTGCACCTTAATACACATCTC	Verification of the integrity of the upper flank
lowflank_4098_check_fw	AATAAGAGTGATTTGTTCGTTTAC	Verification of the integrity of the lower flank
Del_4097_fw	CGTAGGAAATTCTGTAGGTCTC	Verification of the deletion
Del_4097_rv	AAAAGAACTGCTTCTAAATGAGG	Verification of the deletion

Table 7.16 | Primers for the construction of the plasmid pLA_del_hyd_2243. This plasmid contains homologous flanks to the putative main hydrogenase gene (ORF Clocel_2243). The overhangs are marked in bold.

Name	Sequence (5' → 3')	Use
pCN9_F_backbone	TTTCGGCAAGTGTTCAAGAAG	Amplification of the backbone
pCN9_R_backbone	CTTATCTTACACACAATTAAGTAGAAGAAC	Amplification of the backbone
upflank_2242_G_fw	**GTTCTTCTACTTAATTGTGTGTAAGATAAG**A ATATCAGATAATCCTATTAAG	Amplification of the upper flank
upflank_2242_G_rv	**GCTATGGAATTAATTCAGTTAACT**TATAAAA ATAGACATAGTTAAA	Amplification of the upper flank
lowflank_2244_fw	AGTTAACTGAATTAATTCCATAGC	Amplification of the lower flank
lowflank_2244_G_rv	**CTTCTTGAACACTTGCCGAAA**GTAGAGGA GAACCAACTACTAAAC	Amplification of the lower flank
upflank_2242_check_fw	TATACCTTGCATAGGATAAAATTC	Verification of the integrity of the upper flank
lowflank_2244_check_fw	GAGCCCATCATAGACCCTAC	Verification of the integrity of the lower flank
Del_2243_fw	CATCAAACAATTCTGGTCTATG	Verification of the deletion
Del_2243_rv	AGAAATTGGTGTAATAGGAGAAG	Verification of the deletion

Table 7.17 | Primers for the construction of the plasmid pNickase_hyd_4097. The restriction sites are underlined. The seed sequences are marked in italics.

Name	Sequence (5' → 3')	Use
upflank_4096_AatII_fw	ATATATATGACGTCGTAAAGCAGTCAATAATA AAC	Amplification of the insert
lowflank_4098_AscI_rv	ATATATATGGCGCGCCATTTCAGTGATATTCT TTCGATAG	Amplification of the insert
hyd_XhoI_J23119-sgRNA-fw_sgRNA1	ATAATCTCGAG*GGCGGATGCGTGAACGGA GG*GTTTTAGAGCTAGAAATAGCAAGTTAAAA TAAGG	Seed sequence exchange
hyd_XhoI_J23119-sgRNA-fw_sgRNA2	ATAATCTCGAG*CCTCCGTTCACGCATCCGC C*GTTTTAGAGCTAGAAATAGCAAGTTAAAAT AAGG	Seed sequence exchange
hyd_XhoI_J23119-sgRNA-fw_sgRNA3	ATAATCTCGAG*TCAGCTTGTCTCACCCAAC C*GTTTTAGAGCTAGAAATAGCAAGTTAAAAT AAGG	Seed sequence exchange
XX_XhoI_J23119_rv	CTCGAGATTATACCTAGGACTGAGCTAGC	Seed sequence exchange – general primer
S24_Cas9_ATG_out	ATCTAAGCCTATTGAGTATTTCTTATCCAT	sgRNA sequencing

Table 7.18 | Primers for the construction of the plasmid pNickase_hyd_2243. The restriction sites are underlined. The seed sequences are marked in italics.

Name	Sequence (5' → 3')	Use
upflank_2242_AatII_fw	ATATATATGACGTCAATATCAGATAATCCTATTAAG	Amplification of the insert
lowflank_2244_AscI_rv	ATATATATGGCGCGCCGTAGAGGAGAACCAACTACTAAAC	Amplification of the insert
hyd2243_XhoI_J23119-sgRNA-fw_sgRNA1	ATAATCTCGAG*ACTTCTCTCAGACCATCCTC*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
hyd2243_XhoI_J23119-sgRNA-fw_sgRNA2	ATAATCTCGAG*AGCAGTATGATTAGGAGCG**C**GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
hyd2243_XhoI_J23119-sgRNA-fw_sgRNA3	ATAATCTCGAG*ATAGATTTTCAGGCTGTTAG*GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Seed sequence exchange
XX_XhoI_J23119_rv	CTCGAGATTATACCTAGGACTGAGCTAGC	Seed sequence exchange – general primer
S24_Cas9_ATG_out	ATCTAAGCCTATTGAGTATTTCTTATCCAT	sgRNA sequencing

8. Supplementary data

Supplementary data, Table 1 | Comparative genome sequence analysis of *C. cellulovorans* 743B obtained from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and American Type Culture Collection (ATCC). ID: identification; POS: position; REF: reference; ALT: alteration; VR: variation.The gray shadow highlights the exclusive differences between *C. cellulovorans* strains from different culture collections.

ID	POS	TYPE	REF	ALT	EVIDENCE	TYPE	EFFECT	LOCUS	PRODUCT
C. cellulovorans from ATCC collection									
1	246833	ins	G	GA	GA:195 G:0				
2	986110	del	TA	T	T:74 TA:0				
3	988232	snp	T	C	C:50 T:1				
4	1064776	snp	A	T	T:421 A:0	CDS	missense	Clocel_0871	transport system permease protein
5	2391118	del	TA	T	T:76 TA:0				
6	2474247	ins	G	GT	GT:242 G:0				
7	2623754	ins	C	CT	CT:138 C:0	CDS	frameshift	Clocel_2208	siroheme synthase
8	2952673	snp	T	C	C:18 T:0	VR	missense	Clocel_2482	
9	2961350	snp	T	C	C:51 T:0	CDS	missense	Clocel_2482	amino acid adenylation domain protein
10	2974581	ins	C	CT	CT:46 C:0	CDS	frameshift	Clocel_2487	amino acid adenylation domain protein
11	3209624	ins	T	TA	TA:233 T:1				
12	3826454	snp	G	A	A:32 G:1	CDS	missense	Clocel_3117	hemolysin-type calcium-binding region
13	4062297	del	TA	T	T:180 TA:0	CDS	frameshift	Clocel_3315	regulator of chromosome condensation RCC1
14	4249782	snp	A	G	G:137 A:6				
15	5226578	ins	A	AT	AT:497 A:5				

ID	POS	TYPE	REF	ALT	EVIDENCE	TYPE	EFFECT	LOCUS	PRODUCT
C. cellulovorans from DSMZ collection									
1	246833	ins	G	GA	GA:210 G:0				
2	986110	del	TA	T	T:68 TA:0				
3	988232	snp	T	C	C:49 T:0				
4	2391118	del	TA	T	T:179 TA:1				
5	2474247	ins	G	GT	GT:230 G:1				
6	2623754	ins	C	CT	CT:171 C:0	CDS	frameshift	Clocel_2208	siroheme synthase
7	2974581	ins	C	CT	CT:33 C:0	CDS	frameshift	Clocel_2487	amino acid adenylation domain protein
8	3209624	ins	T	TA	TA:230 T:0				
9	4062297	del	TA	T	T:176 TA:0	CDS	frameshift	Clocel_3315	regulator of chromosome condensation RCC1
10	5226578	ins	A	AT	AT:338 A:5				