

HENRI INGELMAN

Systems-level characterisation
and improvement of
Clostridium autoethanogenum
metabolism



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metabolism



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LIST OF ORIGINAL PUBLICATIONS

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- III. Ingelman, H., Shaikh, K.M., Valgepea, K. Reverse-engineered *Clostridium autoethanogenum* strains recover enhanced phenotypes from adaptive laboratory evolution. Manuscript.

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The author's contribution to each article is as follows:

- I. Second author in Publication, having participated in the design and execution of a substantial portion of the experiments, the analysis of data, and provided input to the writing of the manuscript.
- II. First author in Publication, played a major role in the conceptualisation of the methodology, was responsible for a substantial part of the experiments and data analysis, and wrote the manuscript, including the original draft.
- III. First author in Publication, played a major role in the conceptualisation of the methodology, was responsible for most of the experiments and resulting data analysis, and wrote the manuscript, including the original draft.

ABBREVIATIONS

2,3-BDO	– 2,3-butanediol
Adh	– alcohol dehydrogenase
AdhE	– acetaldehyde/alcohol dehydrogenase
ALE	– adaptive laboratory evolution
AOR	– acetaldehyde:ferredoxin oxidoreductase
C1	– one-carbon
CDS	– coding sequence
CO	– carbon monoxide
CO ₂	– carbon dioxide
CODH	– carbon monoxide dehydrogenase
CODH/ACS	– carbon monoxide dehydrogenase/acetyl-CoA synthase
COG	– Cluster of Orthologous Groups
D	– dilution rate
DEG	– differentially expressed gene
DEP	– differentially expressed protein
FBA	– flux balance analysis
Fd _{red}	– reduced ferredoxin
GEM	– genome-scale metabolic model
GO	– Gene Ontology
H ₂	– hydrogen
NTG	– N-methyl-N'-nitro-N-nitrosoguanidine
q _{2,3-BDO}	– specific 2,3-BDO production rate
q _{ace}	– specific acetate production rate
q _{CO}	– specific CO uptake rate
q _{CO2}	– specific CO ₂ production rate
q _{EtOH}	– specific ethanol production rate
q _{H2}	– specific H ₂ uptake rate
SNP	– single nucleotide polymorphism
WGS	– whole genome sequencing
WLP	– Wood-Ljungdahl pathway
YE	– yeast extract
μ	– specific growth rate

INTRODUCTION

Acetogens, a group of anaerobic bacteria, have emerged as promising biocatalysts for sustainable industrial applications, given their capacity to convert gaseous substrates such as carbon monoxide (CO), carbon dioxide (CO₂), and hydrogen (H₂) into valuable products including ethanol, acetate, and 2,3-butanediol (2,3-BDO). The growing interest in acetogen-based gas fermentation is driven by the pressing need to address climate change through the reduction of carbon emissions and the development of alternative, circular production routes for chemicals and fuels. Despite the potential, a number of significant challenges remain in fully harnessing the metabolic capabilities of acetogens for industrial biotechnology.

One of the primary challenges is the limited quantitative understanding of acetogen metabolism. For example, there is a lack of understanding regarding the relationship between specific growth rates (μ) and key bioprocess parameters, such as product yields and rates. This gap in knowledge impedes the ability to optimise industrial processes, where the precise control of metabolic fluxes is of critical importance for the achievement of economic viability. Moreover, the intricate regulatory mechanisms governing acetogen metabolism, including transcriptional mechanisms, remain largely uncharted territory, rendering it challenging to predict or engineer desired phenotypes and cellular behaviours.

In addition to the inherent metabolic complexity, the field of genetic engineering of acetogens remains in its relative infancy. In contrast to model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, where comprehensive gene libraries and metabolic engineering strategies are well-established, acetogens present distinctive challenges due to their strict anaerobic nature and slow growth. The advancement of robust microbial cell factories in acetogens is further constrained by the fact that the function for the majority of the genes in acetogens have yet to be characterised. This lack of understanding is a significant obstacle to targeted metabolic engineering.

Adaptive Laboratory Evolution (ALE) can be used as a powerful approach to overcome these constraints. ALE can be employed to subject acetogens to prolonged selection pressures, thereby revealing genetic mutations that confer advantageous traits, such as enhanced growth on minimal media or increased performance in industrially relevant conditions. Furthermore, reverse genetic engineering of mutations uncovered in ALE experiments supports identification of genotype-to-phenotype links.

This thesis seeks to address the aforementioned challenges by combining gas fermentation techniques with systems biology analyses, ALE, and reverse genetic engineering. The objective of this systematic investigation is to gain insight into the metabolic and genetic characteristics of acetogens and to enhance comprehension of genotype-phenotype relationships in acetogens. Ultimately, the findings are expected to facilitate the development of more efficient and industrially relevant acetogen cell factories.

LITERATURE REVIEW

I. Climate change challenge – shifting to carbon neutral circular bioeconomy

As the global community grapples with the urgent need to mitigate climate change and reduce greenhouse gas emissions, there has been a growing emphasis on developing sustainable methods for the production of renewable fuels and chemicals. The main contributors to climate change are one-carbon (C1) gases (CH₄, CO₂, CO) emitted from burning fossil fuels (International Energy Agency, 2022). This trend, if continued, could lead to irreversible climate change and a significant loss of biodiversity (Arias et al., 2021). Our linear take-make-waste economy must be replaced by a circular bioeconomy that includes decarbonised energy sectors, sustainable chemical and fuel production, and integrated waste management (Kircher, 2021). While solar and wind power are currently leading decarbonisation of energy generation, development of biocatalysts for biomanufacturing is considered an additional key strategy for achieving carbon neutrality through sustainable production of chemicals and fuels by ‘up-cycling’ waste C1 feedstocks (Zhang et al., 2022).

II. Role of acetogens and gas fermentation in the bigger picture

Gas fermentation is one attractive technology for recycling waste carbon into products, and bacteria known as acetogens are the ideal biocatalysts for this process as they can use gas as the sole carbon and energy source (Wood, 1991). This process using acetogens has the advantage of utilising variable gas mixtures, operating at near-ambient temperatures and pressures (Bengelsdorf et al., 2018), and being less sensitive to impurities in syngas compared to the chemical Fischer-Tropsch process (Liew et al., 2016b). Acetogens are capable of natively producing a range of compounds, including acetate, ethanol, butanediol, lactate, butanol, and hexanol (Drake et al., 2006). In addition to their relevance for biotechnology, acetogens play a significant role in both the global carbon and acetate cycles – it is estimated that they are responsible for fixing approximately 20% of CO₂ on Earth and for converting the CO₂ into billions of tons of acetic acid annually (Drake et al., 2006; Ljungdahl, 2009). Notably, the model-acetogen *Clostridium autoethanogenum* is already being used for commercial-scale ethanol production (Köpke and Simpson, 2020) and has been successfully piloted for the production of acetone and isopropanol (Liew et al., 2022).

III. C1-fixation in acetogens: the Wood-Ljungdahl pathway

Acetogens employ the Wood-Ljungdahl pathway (WLP) to fix carbon oxides (CO_2 and CO) into acetyl-CoA from a wide range of feedstocks (Pavan et al., 2022; Ragsdale, 2004). The WLP – also known as the reductive acetyl-CoA pathway – was identified and described in detail in acetogens by Harland G. Wood and Lars G. Ljungdahl, after whom the pathway is named (Ljungdahl, 1986). Alongside the reverse glycine reduction pathway, the WLP (Figure 1) is one of only two known linear autotrophic carbon fixation pathways (Pavan et al., 2022), while being the most energy-efficient CO_2 fixation pathway currently known (Cotton et al., 2018; Fast and Papoutsakis, 2012). It is notable that the WLP is the sole naturally occurring carbon fixation pathway that does not require additional ATP for operation (Fuchs, 2011; Ragsdale and Pierce, 2008). The WLP in acetogens reduces two CO_2 molecules in conjunction with several coenzymes and electron carriers into one central molecular intermediate – acetyl-CoA (Drake and Daniel, 2004). More precisely, acetyl-CoA is formed from the concurrent reduction of two CO_2 molecules via the methyl and carbonyl branches of the WLP: the methyl branch involves the serial reduction of CO_2 to a methyl group bound with a tetrahydropterin coenzyme as methyl-CoFeS; the carbonyl branch involves the reduction of the second CO_2 molecule to CO (Figure 1). In the presence of CO as the sole carbon source, CO is initially oxidised to CO_2 by carbon monoxide dehydrogenase (CODH) (Schuchmann and Müller, 2014), and then fed into the methyl branch. The combination of products from both branches is then catalysed by the central complex in the pathway – the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) enzyme complex (Lemaire and Wagner, 2021). This results in the return of one CoA molecule and formation of one acetyl-CoA molecule that can be used in downstream metabolism (e.g. for acetate or ethanol production, biomass synthesis). The CODH/ACS complex is essential for autotrophic growth of acetogens and also plays a significant role in heterotrophic growth (Liew et al., 2016a). See several comprehensive reviews for a detailed overview of the WLP pathway and its associated enzymes (Drake et al., 2006; Katsyv and Müller, 2020; Ragsdale, 2008, 2004; Ragsdale and Pierce, 2008; Schuchmann and Müller, 2014; Wood, 1991).

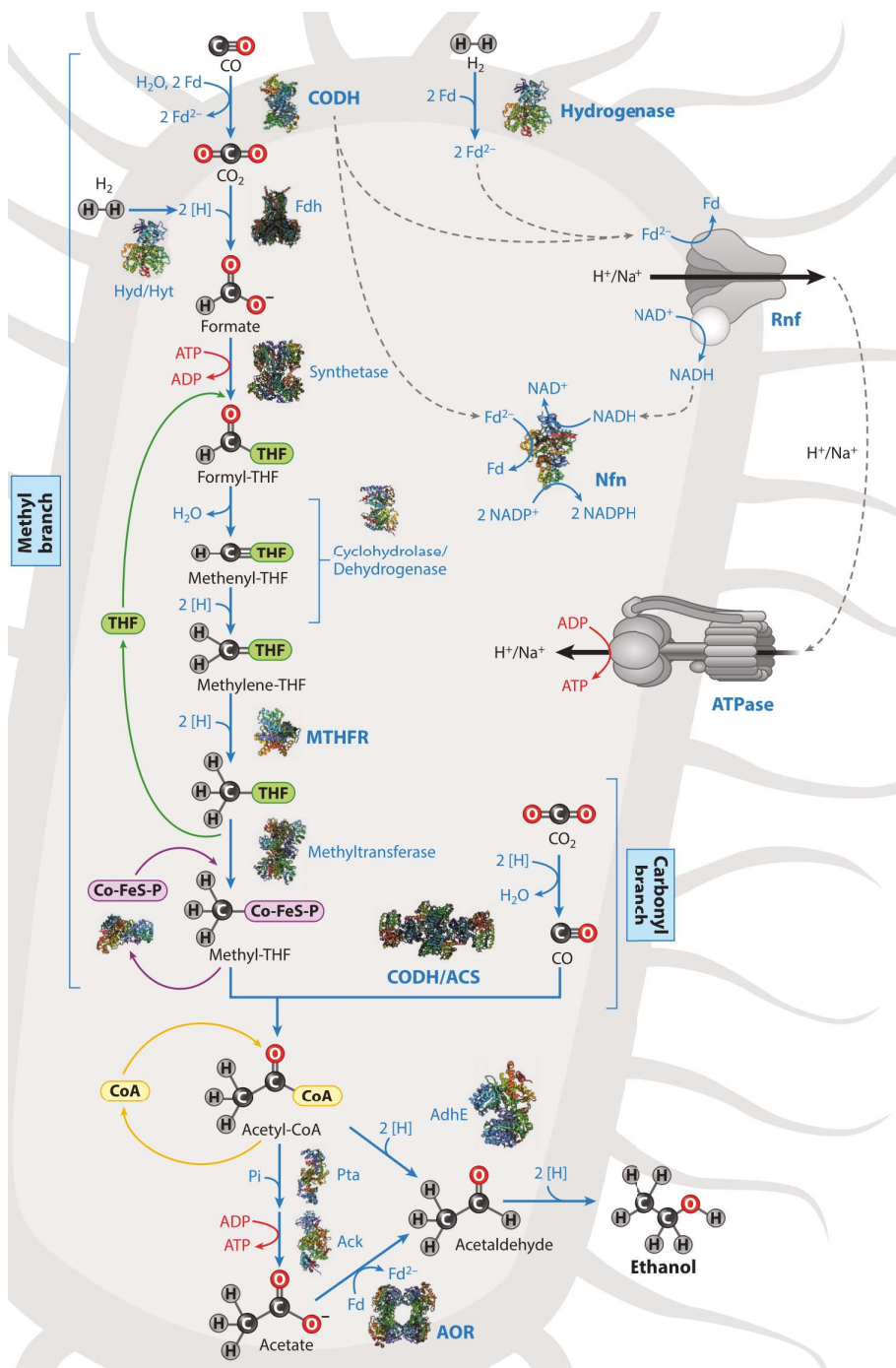


Figure 1. Overview of the Wood–Ljungdahl pathway in acetogens where acetyl-coA is formed via a series of enzymatically catalysed chemical reactions. Ack, acetate kinase; AdhE, bifunctional aldehyde/alcohol dehydrogenase; AOR, aldehyde:ferredoxin oxidoreductase; CODH/ACS, bifunctional CO dehydrogenase/Acetyl-CoA synthase complex; Co-FeS-P, corrinoid iron sulfur protein; Hyd/Hyt, NAD/NADP-specific electron-bifurcating hydrogenase; Fdh, formate dehydrogenase; MTHFR, methylene-THF reductase; Nfn, transhydrogenase; Pta, phosphotransacetylase; Rnf, ferredoxin:NAD⁺ oxidoreductase; THF, tetrahydrofolate. Figure adapted from Fackler et al., 2021.

IV. Challenges in accelerating the engineering of acetogen cell factories

Though significant steps have been recently taken towards better understanding of acetogen physiology, widespread deployment and expansion of the product spectrum of acetogen gas fermentation is hindered by multiple critical limitations (Pavan et al., 2022).

a) Limitations of non-commercial acetogen strains

Firstly, the lack of robust non-commercial acetogen strains has impeded the advancement of both understanding of acetogen metabolism and engineering of microbial cell factories. The strains publicly available exhibit slow growth, slow gas uptake, the requirement for yeast extract, and are on most cases extremely challenging to run in bioreactor continuous cultures (Martin et al., 2015). For instance, faster gas uptake and growth would support phenotyping and genetic engineering of acetogens (Bourgade et al., 2021; Pavan et al., 2022). Also, a minimal growth medium (e.g. without yeast extract) is essential for accurate carbon distribution quantification and metabolic modelling to gain deeper insights into metabolism and further predict acetogen phenotypes (Valgepea et al., 2018, 2017). Additionally, the implementation of a more robust strain in terms of operating continuous bioreactor cultures would ease the generation of high-quality steady-state datasets in higher throughput (Klask et al., 2020; Mahamkali et al., 2020). Consequently, the acetogen and gas fermentation communities would gain substantial benefits from the availability of public acetogen strains with the aforementioned enhanced characteristics.

b) Limited understanding of specific growth rate impact on acetogen metabolism

Secondly, our quantitative understanding of acetogen metabolism is limited. In particular, the effects of the key physiological parameter, specific growth rate (μ), on acetogen metabolism and on the gas fermentation bioprocess have not been determined. From an economic perspective, μ is highly relevant for industrial-scale acetogen gas fermentation since the process is operated as a continuous culture (Fackler et al., 2021; Köpke and Simpson, 2020) and generally μ of the cell culture significantly affects metabolic activity of cells, process rates, and economics (Lipson, 2015). Therefore, the selection of a suitable dilution rate (D) (i.e., μ at steady-state) is critical for optimal bioprocess performance (e.g., to reach high product selectivities, titres, rates, and yields) in continuous cultivation conditions. From the perspective of fundamental understanding, novel insights into μ -dependent metabolism of acetogens could be highly valuable. For instance, the data could shed the much-needed light on the energy-limited metabolism of acetogens (Molitor et al., 2017; Schuchmann and Müller, 2014) as faster growth

requires more energy. Moreover, studies of the impact of μ on cell metabolism in other microorganisms have revealed significant effects on product distribution and energy balance (Valgepea et al., 2010; Van Hoek et al., 1998), transcript and protein expression (Hackett et al., 2016; Ishii et al., 2007; Peebo et al., 2015; Valgepea et al., 2013, 2010), and stress responses (Regenberg et al., 2006). Thus, both the industrial gas fermentation process and fundamental understanding of acetogen physiology could notably improve from investigating the effects of μ .

c) Minimal description of acetogen genotype-phenotype relationships

Thirdly, description of genotype-phenotype relationships in acetogens is minimal. For instance, functions for the majority of genes even in the model-acetogen *C. autoethanogenum* remain uncharacterised, i.e. they are associated with unknown or unclear functions (Brown et al., 2014; Humphreys et al., 2015). While the majority of the genes responsible for C1-fixation through the WLP have confirmed biochemical functions, functions of four genes within the 20 kb C1-fixation gene cluster remain unknown (genes with proposed names *gcvH*, *cooC*, *lpdA*, *cooC2*). Furthermore, two genes (CLAU_1570 and CLAU_1571) adjacent to the WLP gene cluster, which exhibit comparable gene (Valgepea et al., 2017) and protein expression levels (Valgepea et al., 2022) to WLP genes in *C. autoethanogenum* during autotrophic growth on syngas, also remain functionally uncharacterised. Disruption of either gene changes the utilisation of complex nutrients and affects the expression of proteins in the WLP and those involved in ethanol synthesis (Nwaokorie et al., 2023). Interestingly, another study determined CLAU_1570 to be essential for autotrophic growth in *C. autoethanogenum* (Woods et al., 2022). Regarding ethanol synthesis, it is not clear which alcohol dehydrogenase(s) serve(s) primary functions and what are the roles of different acetaldehyde/alcohol dehydrogenase (AdhE) and aldehyde dehydrogenase isozymes (Valgepea et al., 2018), representing a significant knowledge gap that hinders the understanding and further engineering of ethanol production in *C. autoethanogenum*. Additionally, transcriptomic and proteomic data suggest that the acetaldehyde:ferredoxin oxidoreductase (AOR) route for ethanol synthesis might be primary as AOR isozymes are expressed higher than the genes in AdhE route (Richter et al., 2016; Valgepea et al., 2017). Furthermore, while *C. autoethanogenum* genome encodes for multiple hydrogenases (Di Leonardo et al., 2022) and their expression seems condition independent (Richter et al., 2016; Valgepea et al., 2018), it is not known which ones play a primary role in H_2 oxidation or redox balancing (Valgepea et al., 2017). A similar degree of uncertainty persists regarding the role of CODH encoding isogenes *cooS1* and *cooS2* (Liew et al., 2016a; Valgepea et al., 2022), further highlighting the potential widespread condition-specific isoenzyme utilisation in central metabolism. These observations are just a few examples for the need of large-scale genetic perturbation experiments

with *C. autoethanogenum* to unambiguously determine (condition-specific) *in vivo* enzyme functionalities for multiple key metabolic activities (Valgepea et al., 2022).

In comparison, extensive research of conventional model-organisms has generated vast knowledge and turned *E. coli* and *S. cerevisiae* into biotech workhorses (Gao et al., 2024; Nielsen and Keasling, 2016; Tullio, 2022). For instance, the *E. coli* Keio single-gene knockout collection (Baba et al., 2006; Yamamoto et al., 2009) has been employed as a valuable resource for reverse genetics and as a priceless starting point for engineering strains for chemical production (Gurdo et al., 2023). For example, Egoburo and co-workers (Egoburo et al., 2018) employed *cra* and *arcA* gene knockouts from the Keio collection to develop strains for succinate and 1,3-PDO production, whereas You and co-workers (You et al., 2024) utilised similar strategies to engineer an industrially relevant *E. coli* strain capable of producing 2'-fucosyllactose from fructose. Furthermore, the screening of *E. coli* knockout collections has enabled the identification of beneficial mutations for hydrogen synthesis using *E. coli* (Cao et al., 2022). Notably, Sáez-Sáez and colleagues (Sáez-Sáez et al., 2024) leveraged the existence of both Keio and yeast gene knockout (YKO)(Giaever and Nislow, 2014) collections to identify multiple transporter deletions that modulate the tolerance of the cells to various toxic aromatics and to construct a *S. cerevisiae* strain with improved 2-phenyl-ethanol production ability. These examples demonstrate the huge potential that improved understanding of acetogen metabolism, particularly with regard to genotype-phenotype relationships, through genetic and phenotypic characterisation holds for rational and targeted engineering of cell factories.

V. Towards a better understanding of acetogen metabolism

A detailed investigation of the functions of relevant genes (e.g., those that are highly expressed or under high or low evolutionary pressures) would be highly beneficial to understand and further unlock the potential of acetogen metabolism. Such studies could facilitate the utilisation of *C. autoethanogenum* in more industrial applications, potentially leading to their employment in a manner comparable to that observed in model organisms today. However, the current state of the field allows for genetic engineering of acetogens with moderate success and is very time consuming using homologous recombination, mobile elements, and CRISPR/Cas based methods (Bourgade et al., 2021; Jin et al., 2020; Pavan et al., 2022; Zhao et al., 2019). Furthermore, the selection of targets is often unclear due to a lack of knowledge about gene functions and their effect on metabolism (see above). One could accelerate the speed of learning of acetogen metabolism through generation of knockout or knockdown strain libraries with subsequent phenotyping as has been demonstrated for *E. coli* with the Keio collection and for *S. cerevisiae* with the YKO collection (see Chapter IV.c). The fact that acetogens are stringent anaerobes, however, makes the generation of such strain collections significantly more challenging. While LanzaTech has been able to establish

the world's first anaerobic biofoundry for automated genetic engineering of acetogens (Köpke and Simpson, 2020), it is costly and complex to operate, and largely inaccessible to academia for fundamental research. Therefore, until manual genetic engineering workflows are significantly improved and accelerated and automated workflows become more affordable and accessible, it would be beneficial to utilise adaptive laboratory evolution (ALE; also known as “evolutionary engineering”) to explore genotype-phenotype relationships more rapidly. This is because ALE generally yields strains with altered phenotypes coupled with changes in the genome without requiring *a priori* knowledge of the genetic alterations necessary to realise such changes (Sandberg et al., 2019).

VI. Adaptive laboratory evolution (ALE)

ALE is an evolutionary engineering approach in artificial conditions to facilitate organisms to increase fitness through the imitation of natural evolution. ALE is a powerful and elegant tool for obtaining strains with novel or improved traits, such as growth on minimal medium, faster growth, or tolerance towards a toxic compound in the growth environment. As in nature, ALE operates within a dynamic environment and is being influenced by various factors and depends largely on the genetic diversity within a population and fitness advantages of a certain genotype under selective conditions (Gibson et al., 2020).

a) Key factors involved in ALE experiments

The majority of genetic diversity in ALE experiments arises from the spontaneous mutation rate, but seldom physical mutagens are also employed (e.g. chemical mutagens, UV radiation). The source of genetic diversity exerts a significant influence on the diversity and complexity of mutations. For example, just relying on spontaneous mutations typically leads to the emergence of more single nucleotide polymorphisms (SNPs) and indels with fewer large-scale genomic rearrangements (such as deletions of larger chromosomal regions, which frequently result in the loss of gene functions). Importantly, increased mutation rates can vastly accelerate the diversification of bacterial strains as recently evidenced in a study conducted on samples from the International Space Station where increased solar radiation is present (Sengupta et al., 2024). One method for increasing mutation rates is to interfere with the DNA mismatch repair mechanism, which has been demonstrated in *E. coli* by deleting the *mutS* gene, leading to increased mutation rates (Choe et al., 2019). The study with International Space Station samples (Sengupta et al., 2024) is also an elegant example of adaptation by diversification (Fiegna et al., 2006; Rainey and Travisano, 1998; Zambrano et al., 1993) that often leads to multiple ecotypes/subpopulations, cross-feeding, and appearance of various other complex dynamics in evolving populations (Großkopf et al., 2016; Lenski and Travisano, 1994; McDonald, 2019; Rozen and Lenski, 2000; Tenaillon et al., 2016). As almost any attribute can be subject to selection in

evolving populations, the same applies to evolvability itself, which is influenced, for example, by epistatic effects and phenotypic heterogeneity (Healey et al., 2016; see Payne and Wagner, 2019 and Pigliucci, 2008 for further reading on evolvability).

Another crucial factor in ALE is the selectable trait and this can be, for instance, faster growth, prototrophy for a specific compound in the medium, increased survivability, shortened lag phase, or changes in physical parameters (such as increased or decreased sedimentation or buoyancy). It may be necessary to employ a smart experimental setup when the desired phenotype is not directly selectable, for example through coupling product formation to growth (von Kamp and Klamt, 2017). In practical terms, the selectable trait is frequently closely intertwined with the experimental design and the general choice of strategy: the experimentalist may opt to run ALE in either a batch or continuous culture setting, depending on the specific objective and the availability of equipment at hand. For instance, batch ALE experiments frequently experience significant population bottlenecks with each transfer, which results in a decreased mutation pool and alters the population mutation dynamics. This, in turn, may lead to extinction events that eliminate certain subpopulations (McDonald, 2019; Zeyl et al., 2001). In comparison, while continuous culture ALE experiments exhibit more stable conditions compared to batch cultures (including selective pressure), keeping conditions too stable may sometimes lead to trade-offs in applications with very variable environments (Carpenter et al., 2023). Interestingly, a dynamic selection pressure may result in the selection of (much more robust) constitutive adaptation mechanisms over induced adaptation, demonstrated, for example, by achieving constitutive tolerance of *S. cerevisiae* to acetic acid (González-Ramos et al., 2016). Additionally, differences in pH, nutrient availability, metabolite concentrations, and other environmental parameters between batch and continuous culture settings contribute to variations in ALE outcomes (Crook and Alper, 2012; Gresham and Hong, 2014). Conclusively, it is important to note that results from ALE experiments depend on the specific conditions selected – “you get what you select for” – and in some applications, a “generalist” isolate might be more beneficial than a “specialist”.

An additional notable feature is the dynamic nature of ALE experiments. In a recent study, Couce and colleagues demonstrated using *E. coli* long-term evolution experiment snapshots that, during experiments where numerous mutations are accumulated, some previously advantageous mutations may become deleterious (Couce et al., 2024). This illustrates the subjective nature of fitness and its dependence on the environment. It is crucial to acknowledge that the increase of fitness in ALE is typically rapid at the outset but adaptation decelerates during prolonged selection. At the same time, the number of mutations generally increases linearly in time (Barrick et al., 2009). However, increasing network complexity of interactions between mutations, genes, and metabolism results in a diminishing beneficial effect of additional beneficial mutations, which is commonly referred to as diminishing returns epistasis (Barrick et al., 2009; Chou et al., 2011; Hua et al., 2007; Khan et al., 2011; Kryazhimskiy et al., 2014).

b) Examples of relevant ALE studies

ALE has been used to engineer and enhance autotrophic growth capabilities in various microbes. For example, ALE has been used to convert the naturally heterotrophic workhorse microbes *E. coli* (Gleizer et al., 2019) and *Pichia pastoris* (Gassler et al., 2019) into CO₂-fixing organisms. Among acetogens, ALE has enhanced tolerance to methanol in *Sporomusa ovata* (Tremblay et al., 2015), cyanide in *Clostridium ljungdahlii* (Oswald et al., 2018), toluene, benzene and xylenes in *C. autoethanogenum* (Piatek et al., 2020), and acetate in *Clostridium sp. AWRP* (Kwon et al., 2022). ALE has also recently been used in *C. carboxidivorans* for acquiring a strain capable of producing hexanol from CO₂ and H₂ (Antonicelli et al., 2023) and in *Eubacterium limosum* for achieving faster growth, higher CO tolerance and improved growth on methanol mineral medium (Jin et al., 2022; Kang et al., 2020; Pregonon et al., 2022). Additionally, *C. autoethanogenum* has been adapted by continuous bioreactor cultivations towards improved growth on CO₂+H₂ (Heffernan et al., 2024). The results of abovementioned studies support exploitation of acetogens in biotechnological applications as has been the case with various industrial workhorse organisms. For example, ALE was involved in creating *Corynebacterium glutamicum* strains with faster growth on minimal media (Pfeifer et al., 2017), ability to utilise cellobiose (Lee et al., 2016) and D-xylose (Radek et al., 2017) as sole carbon source, improved methanol consumption and dependence on methanol (Tuyishime et al., 2018), high copy number plasmids (Choi et al., 2018), and various other traits, leading to industrially deployed microbial cell factories (Becker et al., 2011; Park et al., 2014; Stella et al., 2019). Using serial passaging on minimal media, ALE was also critical in developing *E. coli* strains capable of using either glycerol or sucrose as the sole carbon source (Kim et al., 2022; Mohamed et al., 2019). Similarly, ALE has improved ethanol yield and productivity and methanol assimilation in *S. cerevisiae* (Espinosa et al., 2020; Topaloğlu et al., 2023; Zhan et al., 2023) and performance of the high carotenoid and lipid producing yeast *Rhodotorula toruloides* (Wei et al., 2024; Yu and Shi, 2023) to realise commercial applications (www.cl6bio.com).

ALE studies have also revealed that different strategies aimed at achieving similar goals (e.g. autotrophic growth) can lead to distinct mutations with similar phenotypic outcomes, a phenomenon termed convergent evolution. This has been repeatedly seen in *E. coli* (Laehnemann et al., 2014), for example, when 115 *E. coli* populations were exposed to high temperature, a strong pattern of convergence emerged at the level of genes, operons, and functional complexes (Tenaillon et al., 2012). Furthermore, convergent evolution has been observed in *E. coli* ALE experiments aiming for increased acid tolerance (Du et al., 2020) and faster growth (Wytock et al., 2018). Additionally, ALE studies demonstrate that genes under stronger selection (i.e. acquiring more mutations) frequently overlap in analogous ALE experiments with comparable selection pressures. For example, Tenaillon and co-workers (Tenaillon et al., 2016) noted several parallel gene deletions and 57 genes exhibiting half of the nonsynonymous mutations during

the *E. coli* long-term evolution experiment. Recently, using the same dataset, Couce et al., 2024 also demonstrated the repeatability (and to some extent – predictability) of ALEs, genetic parallelism, and convergent evolution, as has been shown in various experimental conditions for different microbes (Good et al., 2017; Kryazhimskiy et al., 2014). The recent *C. autoethanogenum* ALE study on CO, CO₂ and H₂ also demonstrated genetic parallelism between isolates from parallel lineages (Heffernan et al., 2024). Taken together, the aforementioned examples underscore the fact that genes display disparate mutation rates that are highly dependent on the selection environment.

c) Using ALE for improving understanding of metabolism

As shown by the examples above, ALE has contributed greatly to metabolic engineering efforts and to obtaining strains with superior traits, such as faster growth and no need for complex nutrients, both of which are required for public acetogen strains. Notably, the phenomenon of variable mutation rates of genes in response to environmental stimuli may occasionally provide valuable insights into genotype-phenotype relationships. Additionally, genetic parallelism and convergent evolution are strong indicators for genes linked to particular phenotypic traits and this information can be utilised for choosing targets for reverse genetic engineering (see below). Furthermore, ALE may be combined with classical genetic engineering techniques in a cyclical manner: short rounds of artificial selection and *in vitro* genetic manipulation can be applied in repetitive cycles to obtain the desired phenotype more efficiently (Dragosits and Mattanovich, 2013). Additionally, integrating ALE studies with (genome-scale) metabolic modelling can yield valuable insights (Hansen et al., 2017; Lee and Kim, 2020; Sandberg et al., 2019), as demonstrated in both *E. coli* (Fong et al., 2005a; Großkopf et al., 2016; Sandberg et al., 2017) and *S. cerevisiae* (Otero et al., 2013). Importantly, creative application of ALE to unmapped genotype-phenotype relationships and various other knowledge gaps about metabolism has the potential to yield discoveries in both basic and applied microbiology (McDonald, 2019). For example, following laboratory evolution (including clone analysis and selection), a suitable isolate can be used to identify the genetic basis of the improved phenotype by reverse engineering the uncovered mutations into the starting strain of ALE (Wang et al., 2023). To conclude, while genes mutated during ALE indicate their relevance for the obtained phenotype and selection environment, combining ALE with reverse engineering enables to detect and quantify genotype-phenotype relationships.

d) Benefits of reverse genetic engineering

Metabolic engineering has been successfully applied in various acetogens, including *C. autoethanogenum* to enhance production of various non-native chemicals (Antonicelli et al., 2023; Bae et al., 2022; Daniell et al., 2012; Dykstra et al., 2022; Köpke and Simpson, 2020; Liew et al., 2022; Pavan et al., 2022).

However, creating microbial cell factories with robust phenotypes is a major challenge in industrial biotechnology, including identifying genotype-phenotype relationships for desirable traits (Fernández-Cabezón et al., 2019). Reverse genetic engineering, i.e. introduction of ALE mutations into wild-type strains (or the starting strain of ALE), is a powerful tool for metabolic engineering as it can propose targeted genetic modifications to reproduce desired genotypes and phenotypes. Additionally, combination of multiple mutations uncovered by ALE can lead to novel phenotypes (Sandberg et al., 2014).

Reverse engineering also enables researchers to circumvent the challenge of unannotated or poorly annotated genes that is often the case for non-model organisms. For instance, when integrated with next-generation sequencing and comprehensive omics analysis, ALE outcomes can be utilised to generate superior production strains by identifying novel targets for strain engineering (Wang et al., 2023; Warner et al., 2009). Furthermore, reverse engineering allows to determine the effects of mutating a specific gene, thereby facilitating investigations into its function. Ultimately, this approach can facilitate comprehensive understanding of biological pathway regulation (Stella et al., 2019). Importantly, reverse engineering allows to study effects of genes in isolation, providing clearer insights compared to the potential noise arising from studying ALE isolates with multiple mutations (Wang et al., 2023). It thus supports metabolic engineering that aims to use precise changes towards specific metabolic outcomes. Reverse engineering has been instrumental in both understanding gene functions and enhancing commercial biotechnological processes (Wang et al., 2023). Combination of reverse engineering of ALE strains and functional genomics has demonstrated considerable potential for elucidating genotype-phenotype relationships and identifying novel genetic engineering targets in both model organisms and unconventional microbes (Kim et al., 2023; Kwon et al., 2022; Long and Antoniewicz, 2018; Rychel et al., 2023; Wang et al., 2023; Wu et al., 2022; Xia et al., 2022). For instance, a combined effort of ALE, reverse engineering, and functional genomics contributed to better understanding of genotype-phenotype relationships for improved CO tolerance in the gas-fermenting hydrogenotroph *Cupriavidus necator* (Wickham-Smith et al., 2023) and to the design of improved *Saccharomyces cerevisiae* 2-phenylethanol producer strains through in-depth understanding of improved 2-phenylethanol tolerance mechanisms (Xia et al., 2022a).

VII. Understanding acetogen metabolism through phenotyping

Better understanding of genotype-phenotype relationships can be achieved through investigation of diverse genotypes but also through the characterisation of a single genotype under a range of environmental conditions. The latter has advanced our understanding of the gas fermentation bioprocess with recent studies highlighting how gas-liquid mass transfer, feed gas and media composition and pH shifts affect

biocatalyst performance, including gas uptake, biomass concentration, product profiles, yields, and rates (Abubackar et al., 2015, 2012; Cotter et al., 2009; Esquivel-Elizondo et al., 2017; Heffernan et al., 2020; Park et al., 2019; Valgepea et al., 2018, 2017). Many of the early studies to understand the bioprocess in depth were carried out in bottle batch fermentations. For example, higher gas-liquid mass transfer rates lead to greater product formation and faster growth of *C. autoethanogenum* in batch fermentation (Cotter et al., 2009). Subsequently, the impact of varying feed gases, pH, media components and yeast extract concentration on metabolite and biomass formation were described (Abubackar et al., 2015, 2012; Park et al., 2019; Valgepea et al., 2017, 2016).

Several findings regarding feed gas composition, pH and their effects on energy metabolism and product profiles have also been confirmed in bioreactor continuous chemostat cultures (Heffernan et al., 2020; Richter et al., 2016; Valgepea et al., 2018). For example, supplementation of CO cultures with H₂ or CO₂+H₂ cultures with CO had an industrially favourable effect on carbon distributions, significantly increasing carbon flux to ethanol (Heffernan et al., 2020; Valgepea et al., 2018). Furthermore, the shift towards increased ethanol production on lower pH values has been demonstrated in chemostat cultures by multiple studies (Klask et al., 2020; Martin et al., 2015). Studies on mixed cultures in continuous gas fermentation have also demonstrated beneficial outcomes, such as increased stability and higher ethanol production (Diender et al., 2019; Esquivel-Elizondo et al., 2017). Bioreactor continuous cultures are particularly relevant for characterisation of metabolism under various physico-chemical conditions as they provide more controlled and stable conditions. This yields higher quality data both for research and industrial processes, offering repeatability, and additionally supporting metabolic modelling (Adamberg et al., 2015). Moreover, the results obtained from laboratory-scale gas fermentation chemostat cultures exhibit operating conditions that are more similar to industrial fermentation systems than those of batch cultures (Köpke and Simpson, 2020).

Operating continuous gas fermentation cultures, however, is challenging due to several reasons. Firstly, unlike sugar-based fermentations where cells import the substrate using transporters, gaseous molecules freely enter cells that is fully dependent on gas-liquid mass transfer via physical forces. This is a critical aspect of bioreactor gas fermentation as CO becomes toxic to acetogens at high concentrations (Allaart et al., 2023). Therefore, it is necessary to gradually increase the gas-liquid transfer to achieve elevated gas uptake rates without compromising cell viability and causing the cultures to “crash”. Ramping of mass transfer is often highly dependent on the strain and gas composition and requires extensive manual troubleshooting and adjustment by the culture operator, as I experienced during my doctoral research (see Results and Discussion for details). Furthermore, strains that appear robust in bottle batch fermentations could struggle significantly in achieving stable high-density continuous cultures with acceptable CO uptake rates. This latter fact serves to illustrate the importance of extending the study of novel strains beyond bottle batch fermentations. Secondly, the feeding of cells with gaseous carbon (and H₂) is a more complex process in autotrophic

continuous culture chemostats given that the carbon source (and, consequently, the energy supply) is separate from the liquid medium containing other nutrients. This is because the carbon feed is decoupled from the culture dilution rate (D), which presents a challenge in operating a stable culture and achieving target biomass, titers, etc. in studies of acetogens.

AIMS OF THE THESIS

Acetogen gas fermentation has the potential to address two major global challenges: climate change and waste management. One of the extensively studied and industrially deployed acetogens is *C. autoethanogenum*. Despite the extensive research on the physiology and the C1-fixation pathway (WLP) in the model-acetogen, several knowledge gaps remain. For example, limited understanding of the effect of μ on acetogen metabolism and genotype-phenotype relationships are currently hindering the further deployment of microbial cell factories. Additionally, the acetogen and gas fermentation communities would benefit substantially from a publicly available *C. autoethanogenum* strain with improved growth characteristics.

The aims of the thesis are the following:

1. Quantitate the impact of the key physiological parameter, μ , on acetogen metabolism and the gas fermentation bioprocess.
2. Use ALE to acquire *C. autoethanogenum* strains with faster growth, capacity to grow on minimal medium, and be robust in continuous bioreactor culture operation (e.g. achieve high biomass, gas uptake rates, and μ).
3. Enhance the understanding of genotype-phenotype relationships in acetogens through employing a multi-disciplinary approach integrating ALE, -omics techniques, and reverse genetic engineering.

MATERIALS AND METHODS

Key details on materials and methods are provided within relevant chapters while detailed descriptions of all materials and methods used in this thesis can be found in the corresponding Publications. This section offers a brief overview of the approaches used in three Publications forming this thesis.

In Publication 1, a bioreactor continuous culture set-up is employed to investigate the impact of μ on the metabolism of *C. autoethanogenum* DSM 23693 and the gas fermentation bioprocess using CO and syngas as a feed gas. The assessment of systems-level metabolic responses was conducted using off-gas analysis, extracellular metabolomics, transcriptomics, and genome-scale metabolic modelling.

In order to study the wild-type *C. autoethanogenum* strain JA1-1 (Publications 2 and 3), a multi-disciplinary approach was utilised, integrating ALE, -omics techniques, and reverse genetic engineering. Briefly, classical ALE techniques were used to obtain evolved isolates, which were then subjected to growth in autotrophic batch bottles and bioreactor continuous cultures, in conjunction with extracellular metabolomics, proteomics, transcriptomics and whole genome sequencing (WGS). Furthermore, the effects of three mutations from ALE isolates were reverse engineered into the starting strain of ALE (wild-type strain JA1-1) and strains were characterised using gas fermentation and systems biology tools.

RESULTS & DISCUSSION

I. Characterisation of steady-state gas-fermenting chemostat cultures of *Clostridium autoethanogenum* (Publication 1)

To investigate and unambiguously quantify μ -dependent bioprocess performance of the acetogen *C. autoethanogenum* at a systems-level, we employed continuous chemostat cultivation by controlling μ of cells via D of the liquid medium in the bioreactor system. This approach is superior to batch cultures where, for example, variable culture parameters (e.g. growth media) have to be used to adjust the μ of the cells, which could significantly confound the results (Adamberg et al., 2015). However, as briefly stated in the literature review, achieving stable high biomass conditions in gas fermentation chemostat cultures can be challenging. For instance, in our first bioreactor experiments conducted in the GasFermTEC laboratory (using the industrial strain DSM 23693) we were forced to rerun some experiments due to culture crashes or other anomalies. In addition to “teething issues” regarding the technical setup (for example, unexplained volume fluctuations, overpressure in the system or wrong tubing materials that degraded during first experiments), we experienced difficulties with maintaining H_2 uptake in numerous syngas experiments. For instance, H_2 uptake was lost on various situations (e.g. during gas-liquid mass-transfer ramping or after sampling) and often even reducing gas flow to pre-crash values did not trigger recovery. On some occasions, the H_2 uptake appeared to recover spontaneously with the underlying mechanisms remaining unclear. Furthermore, in two experiments, H_2 uptake appeared to reach a specific plateau value. We even observed unstable H_2 production (~ 400 mmol/gram of dry cell weight [gDCW]/day) at Ds above the highest D where a stable culture was obtained (see below). Eventually, stable and significant H_2 uptake was observed (see below) when using more conservative ramping strategies for mass-transfer and D. Notably, these ramping strategies later turned out to be ineffective with less robust ALE isolate strains, necessitating the development of alternative approaches through trial and error, which consumed a significant amount of time of my doctoral research (see Chapter II for more details).

In this study, the acetogen *C. autoethanogenum* strain DSM 23693 was grown in six different growth conditions, resulting in total 23 chemostats where cells reached steady-states. The cultures were studied using a chemically defined PETC-MES minimal medium at Ds ~ 1.0 , ~ 2.0 , and ~ 2.8 day⁻¹ ($\mu \sim 0.04$, 0.08 , and 0.12 h⁻¹, respectively) using either CO (60% CO, 40% Ar) or syngas (50% CO, 20% H_2 , 20% CO₂, and 10% Ar) as feed gas (Table 1). We adjusted gas-liquid mass transfer conditions (e.g., gas flow rates and stirring speed) between different Ds to maintain comparable biomass concentrations ($\sim 1.6 \pm 0.1$ gDCW/L; average $\pm$ standard deviation) (Table 1; Figure 2A), as it can affect carbon distribution (Valgepea et al., 2017). Gas uptake and production rates of the cultures

were continuously quantified using online off-gas analysis and cultures were sampled for extracellular metabolome and transcriptome analysis. In addition, genome-scale metabolic modelling was used to estimate intracellular metabolic flux rates of the steady-state cultures.

Table 1. Variable operational parameters of 23 steady-state chemostat cultures of *C. autoethanogenum*.

Feed gas	Gas flow rate (mL/min)	Agitation (RPM)	μ (day ⁻¹)	Biomass concentration (gDCW/L)	Number of bioreplicates
CO	50	690	1.02 ± 0.01	1.58 ± 0.06	4
	72	815	2.03 ± 0.06	1.65 ± 0.01	3
	72	1175	2.79 ± 0.03	1.65 ± 0.02	3
Syngas	50	675	1.01 ± 0.02	1.59 ± 0.03	6
	72	800	2.01 ± 0.07	1.57 ± 0.08	3
	72	1160	2.79 ± 0.07	1.43 ± 0.03	4

μ , specific growth rate; gDCW, gram of dry cell weight. Data are average ± standard deviation between bioreplicates.

a) Faster growth supports higher yields and productivities for reduced by-products ethanol and 2,3-BDO

We detected secretion of acetate, ethanol, and 2,3-BDO across the chemostats by the cells (Figure 2A). While there was no clear trend in the specific acetate production rate (q_{ace} ; mmol/gDCW/h) profiles in response to an increase in μ between the two gases, the specific ethanol production rates (q_{EtOH}) more than doubled on both gas mixtures (Figure 2B). It is notable that this also results in significantly higher ethanol productivity values (g/L/h) due to comparable biomass levels. Increasing trends for specific 2,3-BDO production rates ($q_{\text{2,3-BDO}}$) were also observed at higher μ that was particularly pronounced for syngas fermentations. In addition, increasing the D_s (and therefore μ) caused the molar acetate/ethanol ratios to decrease from ~1.5 to 0.9 and 0.6 for CO and syngas, respectively. This was strongly driven by a large decrease in acetate levels at comparable ethanol concentrations (Figure 2A). These trends are very advantageous for an industrial gas fermentation process, as higher productivity is not associated with lower selectivity and furthermore, faster growth and higher biomass levels complimentary lead to higher product yields and volumetric productivities (g/L/h). This in turn allows to save time and resources on downstream processes, for example, for ethanol purification.

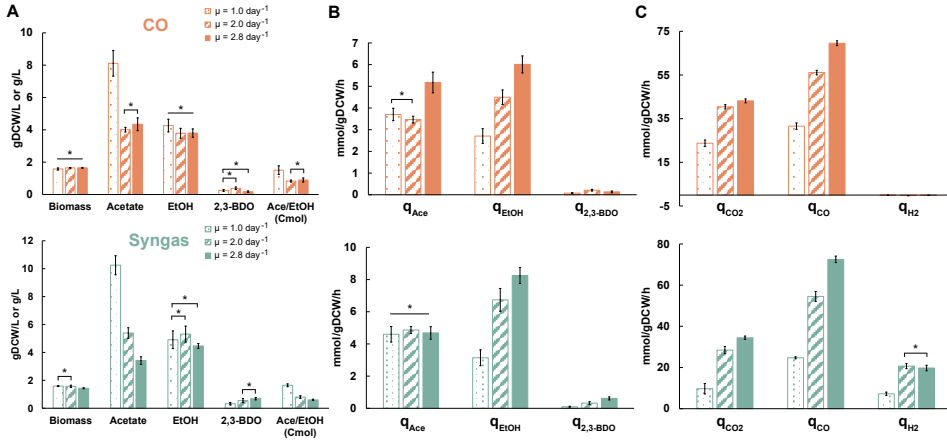


Figure 2. Specific growth rate-dependent growth characteristics in CO (top) and syngas (bottom) grown *C. autoethanogenum* chemostats. **(A)** Biomass and by-product concentrations. **(B)** Specific by-product production rates. **(C)** Specific gas uptake and production rates. Data are average $\pm$ standard deviation between bio-replicates (see **Table 1**). Asterisk denotes values statistically not different according to t-test (p -value < 0.05). μ , specific growth rate; $gDCW$, gram of dry cell weight; *EtOH*, ethanol; 2,3-BDO, 2,3-butanediol; *Ace*, acetate; q , specific rate; q_{CO2} , specific CO_2 production rate; q_{CO} and q_{H2} , specific CO and H_2 uptake rates, respectively.

b) Non-linear gas uptake rate trends with increasing μ

The chemostats were CO limited under these conditions, as by varying the gas-liquid mass transfer rates, we were able to maintain similar steady-state biomass concentrations across studied D_s . Therefore, the specific CO uptake rate (q_{CO} ; mmol/gDCW/h) is expected to increase proportionally with μ . However, we detected a different, non-proportional increase of q_{CO} on both gas mixtures: 1.8-fold and 2.2-fold for CO; 2.2-fold and 2.9-fold for syngas, with 2-fold and 2.8-fold faster growth, respectively (Figure 2C). This means that in CO chemostats the biomass yield (per CO consumed) is higher on elevated μ and in syngas chemostats the biomass yield (per CO consumed) is slightly higher on $\mu = 1 \text{ day}^{-1}$ compared to higher μ values. Interestingly, when comparing $\mu = 2$ and 2.8 day^{-1} , q_{CO} differences (1.2-fold for CO and 1.3-fold for syngas) are smaller than the D differences ($2.8 / 2 = 1.4$) for both gases, indicating increased biomass yields at $\mu = 2.8 \text{ day}^{-1}$. It is noteworthy that while the specific H_2 uptake rate (q_{H2} ; mmol/gDCW/h) showed a 2.9-fold (7 ± 1 to 21 ± 1) increase between $\mu = 1.0$ and 2.0 day^{-1} on syngas, no further change was observed with accelerated growth, despite the gas-liquid mass transfer being increased concurrently with D . The specific CO_2 production rates (q_{CO2} ; mmol/gDCW/h) increased by 1.8-fold (24 ± 1 to 43 ± 1) and 3.4-fold (10 ± 2 to 34 ± 1) with 2.8-fold faster growth for the CO and syngas cultures, respectively (Figure 2C). This indicates a strong effect for H_2 uptake on CO oxidation, as previously observed for *C. autoethanogenum* growing on different gas mixtures (Valgepea et al., 2018).

c) Faster growth leads to significant changes in carbon balances

By integrating bioreactor off-gas data from the mass spectrometer with extra-cellular metabolite and biomass measurements, it is possible to carry out carbon balancing, i.e. quantification of carbon flows from substrates to products. Consistent with data on Figure 2, the carbon flux to acetate decreased with faster growth by 24% ($19.2 \pm 2.2\%$ to $14.6 \pm 1.3\%$) for CO and by 57% ($32.3 \pm 2.5\%$ to $13.9 \pm 1.1\%$) for syngas (Figure 3). While for CO cultures, carbon flux to 2,3-BDO showed no clear μ -dependent trend, on syngas cultures cells diverted up to 140% more carbon into 2,3-BDO ($1.5 \pm 0.3\%$ to $3.6 \pm 0.4\%$) at increased μ values. Overall, on syngas we observed higher carbon flux towards both ethanol and 2,3-BDO with faster growth, as seen previously at higher steady-state biomass concentrations for syngas-fermenting *C. autoethanogenum* cultures (Valgepea et al., 2017). The fact that no clear trend between μ and carbon flux to 2,3-BDO was observed for CO-grown cultures (Figure 3) confirms that the composition of the feed gas has a significant effect on acetogen product distribution (Diender et al., 2016; Heffernan et al., 2020; Jack et al., 2019; Valgepea et al., 2018; Xu et al., 2017). Doubling of q_{EtOH} with faster growth (Figure 2B) was supported by up to 21% increased carbon flux to ethanol on CO (Figure 3). As faster growth requires more energy, these observations are not trivial. In addition, reduced products – such as ethanol and 2,3-BDO – consume redox cofactors that acetogens could otherwise use for ATP production (Schuchmann and Müller, 2014). Remarkably, the cells were even able to simultaneously channel more carbon into biomass formation (increase of 48 and 24% for CO and syngas, respectively; Figure 3). We note that high losses of substrate carbon as CO_2 are close to theoretical stoichiometric and thermodynamic calculations for CO_2 dissipation with ethanol production (Molitor et al., 2016), especially from CO (67%) at $\mu = 2.0 \text{ day}^{-1}$.

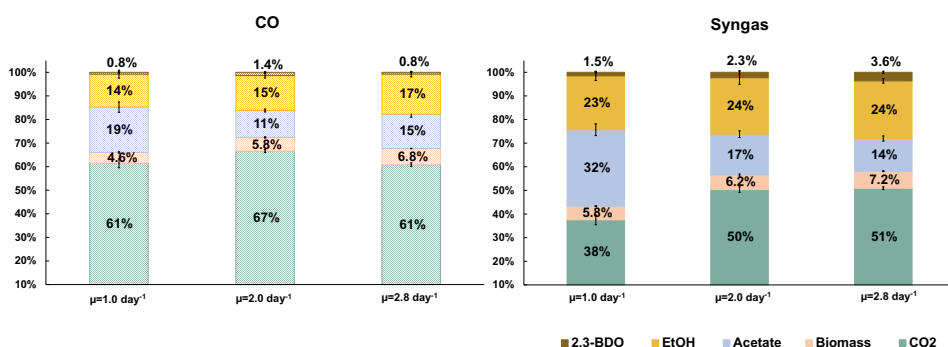


Figure 3. Specific growth rate-dependent carbon balances in CO (left) and syngas (right) grown *C. autoethanogenum* chemostats. Carbon recoveries were $110 \pm 16\%$, $103 \pm 6\%$, $92 \pm 1\%$ for CO and $122 \pm 6\%$, $107 \pm 3\%$, $101 \pm 3\%$ for syngas at $\mu = 1.0, 2.0, 2.8 \text{ day}^{-1}$, respectively. Carbon recoveries were normalised to 100% to have a fair comparison of carbon distributions between different conditions. Data are average $\pm$ standard deviation between bioreplicates (see **Table 1**). μ , specific growth rate; 2,3-BDO, 2,3-butanediol.

d) Significantly increased metabolic fluxes for faster growing cells

As the bioreactor setup enabled the collection of quantitative steady-state data on carbon and redox flows entering and leaving the cells (see Chapter I.c), we took the opportunity to estimate intracellular metabolic fluxes using a genome-scale metabolic model (GEM). The GEM iCLAU786 of *C. autoethanogenum* (Valgepea et al., 2018) coupled with flux balance analysis (FBA) (Orth et al., 2010) was employed in this study. As anticipated, the flux through the WLP was significantly increased with faster growth, although the increase was less pronounced than that observed in CO uptake for syngas cultures since more of the fixed CO was oxidised and dissipated as CO₂ (Figure 4). Additionally, faster growth was supported by increased ATP production via the ATPase with increases of 82% and 125% observed in CO and syngas conditions, respectively (Figure 4). It is noteworthy that the ratio between the ATP production fluxes of the ATPase and the acetate kinase (acetyl-P → acetate) changed minimally (9% in average; Supplementary Table S8 in Publication 1), despite a notable shift in product distributions (Figure 3). Concurrently, maintenance ATP costs increased by 80 and 66% on CO and syngas, respectively (Figure 4; Supplementary Tables S8, S9 in Publication 1), averaging at 30% of total ATP production, as observed in previously studied autotrophic *C. autoethanogenum* cultures (Valgepea et al., 2018, 2017).

In addition to ATP, an elevated supply of NADPH is also required for biomass synthesis during faster growth. For *C. autoethanogenum* cultures of this work, additional NADPH was provided by additional flux through the Nfn transhydrogenase (increase of 66 and 190% on CO and syngas, respectively) and not by higher flux through the electron-bifurcating hydrogenase HytA-E complex (Figure 4). No increase in flux through HytA-E with accelerated growth on syngas indicated that the production of the essential reducing power co-factor, reduced ferredoxin (Fd_{red}), was presumably supported by CO oxidation. Indeed, the model exhibited an increase in the proportion of Fd_{red} generated by CO oxidation with elevated μ (from $63 \pm 2\%$ to $74 \pm 1\%$; Supplementary Table S8 in Publication 1). The entire CO₂ content of the WLP was reduced directly to formate through the formate-H₂ lyase activity of the HytA-E/FdhA complex (Wang et al., 2013) as also seen in previous CO+H₂ or CO₂+H₂-fermenting *C. autoethanogenum* steady-state cultures (Heffernan et al., 2020; Valgepea et al., 2018, 2017). This saves valuable redox for the cells compared to growth on CO alone and allows increased production of reduced compounds (e.g. ethanol and 2,3BDO) when H₂ is available. The majority of the H₂ supplied within syngas was used by the cells to directly reduce CO₂ to formate in the WLP (Figure 3). Furthermore, the H₂ supply had a direct effect on intracellular redox homeostasis as H₂ oxidation provided up to 10% of the total Fd_{red} generated by the cells (Supplementary Table S8 in Publication 1) that is also consistent with previous results (Valgepea et al., 2018, 2017).

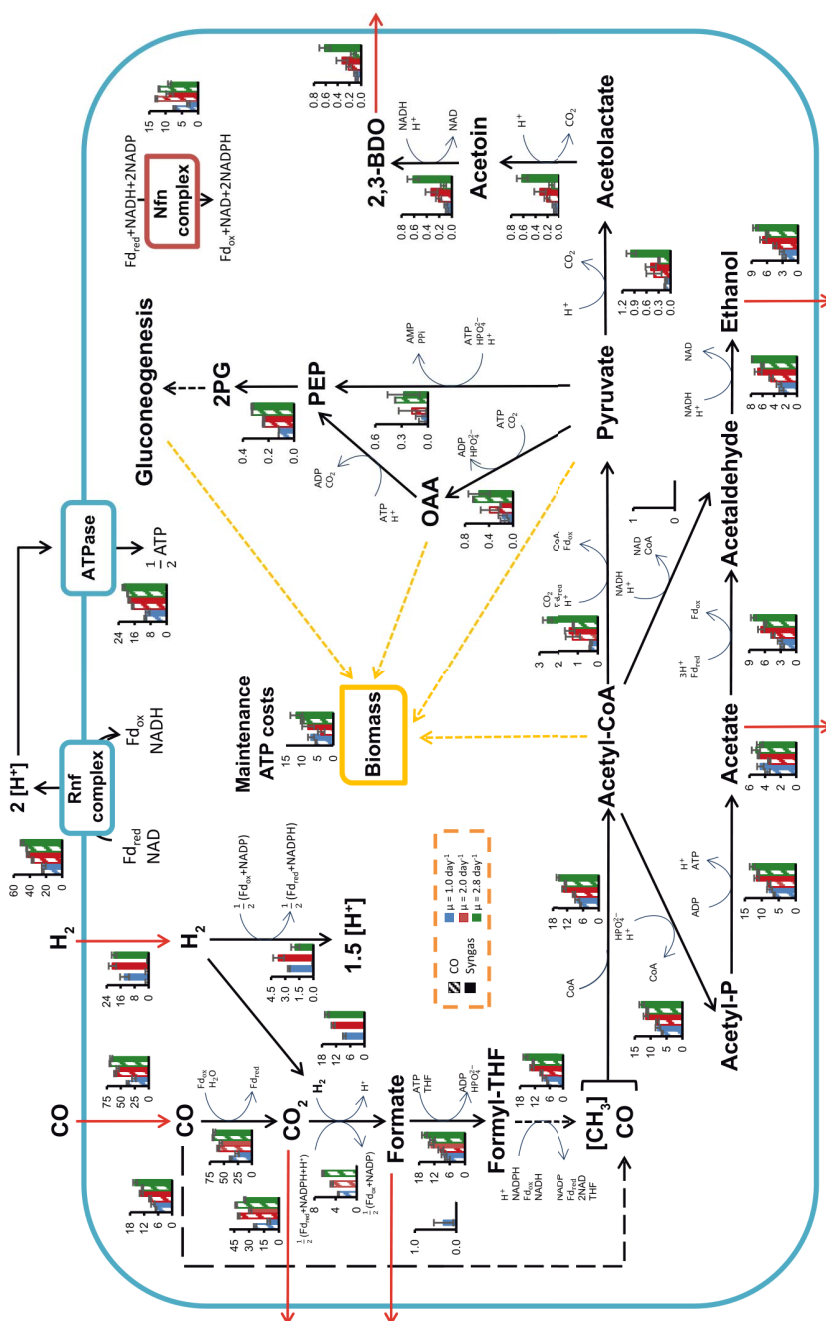


Figure 4. Specific growth rate-dependent central metabolism flux levels in CO and syngas grown *C. autoethanogenum* chemostats. See dashed inset for bar chart details. Fluxes (mmol/gDCW/h) are represented as average $\pm$ standard deviation between bioreplicates (see Table 1). Arrows show direction of calculated fluxes; red arrows denote uptake or secretion; dashed arrows denote a series of reactions. Cofactors used in the GEM iCLAU786 are shown. Flux into PEP from oxaloacetate and pyruvate is merged. gDCW, gram of dry cell weight; μ , specific growth rate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; THF, tetrahydrofolate; 2PG, 2-phosphoglycerate; 2,3-BDO, 2,3-butanediol. See SIM1–23 in Tables S8, S9 in Publication 1 for data and co-factor abbreviations.

GEMs can also be utilised for the prediction of growth phenotypes (O'Brien et al., 2015) and the accuracy of the model can be evaluated beforehand. For example, by comparing experimental product profiles with those predicted by the model by maximising biomass yield in FBA when only substrate uptake rates and maintenance ATP costs are constrained. Regarding ethanol and 2,3-BDO production, our initial predictions were inaccurate (SIM24–46 in Supplementary Tables S8, S9 in Publication 1), as seen before (Valgepea et al., 2018, 2017). However, when the model was additionally constrained by coupling carbon and redox metabolism from H₂ utilisation for syngas cultures (see Materials and Methods in Publication 1 for additional details) and disabling the predicted formate production (as no formate was detected in metabolomics), ethanol production was observed to be in closer agreement with the experimental values (on average 28% off from the experimental values; SIM47–59 in Supplementary Tables S8, S9 in Publication 1). Additionally, our simulations align with the previously published *C. autoethanogenum* datasets and gene knockout experiments (Liew et al., 2017), which demonstrate that ethanol is synthesised using the AOR activity through acetate, coupling it with ATP production (Figure 4).

e) Significant transcriptome changes with increasing μ

Global transcriptome trends

Subsequently, RNA sequencing was performed on the continuous culture dataset to quantify μ -dependent gene expression changes on both gas mixes at the transcriptome level. As my personal contribution to transcriptomics was minimal, I hereby only highlight key findings linked to previously described fermentation growth characteristics and metabolic modelling results (see Publication 1 for all results). The data exhibited high reproducibility, as evidenced by the clear clustering of bio-replicates (Figures 5A, B) and an average Pearson correlation coefficient of $R = 0.96$ between bio-replicates (Figure 5C). Additionally, considerable μ -dependent expression differences were observable for a multitude of genes in both gases (Figure 5B). A total of 1,337 differentially expressed genes (DEGs) were identified, with a fold-change (FC) > 1.5 (up- or down-regulation) and a q -value < 0.05 after FDR correction in at least one pairwise comparison within the three pairwise μ -dependent comparisons ($\mu = 2.0$ vs. 1.0 , 2.8 vs. 1.0 , and 2.8 vs. 2.0 day^{-1}) of both gas mixes (Supplementary Tables S4, S5 in Publication 1). As anticipated, many DEGs were shared between the various comparisons conducted with both gas mixes (Figure 5D). While 442 DEGs were shared between the comparison of CO and syngas at of $\mu = 2.8$ vs. 1.0 day^{-1} , hundreds of unique DEGs were also identified. This illustrates the impact of the feed gas mixture on μ -dependent gene expression patterns. The identification of over a thousand DEGs suggests that the acetogen *C. autoethanogenum* employs transcriptional regulation to a significant extent, at least for the purpose of facilitating faster growth. This is in contrast to the findings of previous omics studies, which have proposed a limited role for hierarchical regulation of gene expression in autotrophic metabolism (Al-bassam et al., 2018; Mahamkali et al., 2020; Richter et al., 2016; Valgepea et al., 2018, 2017).

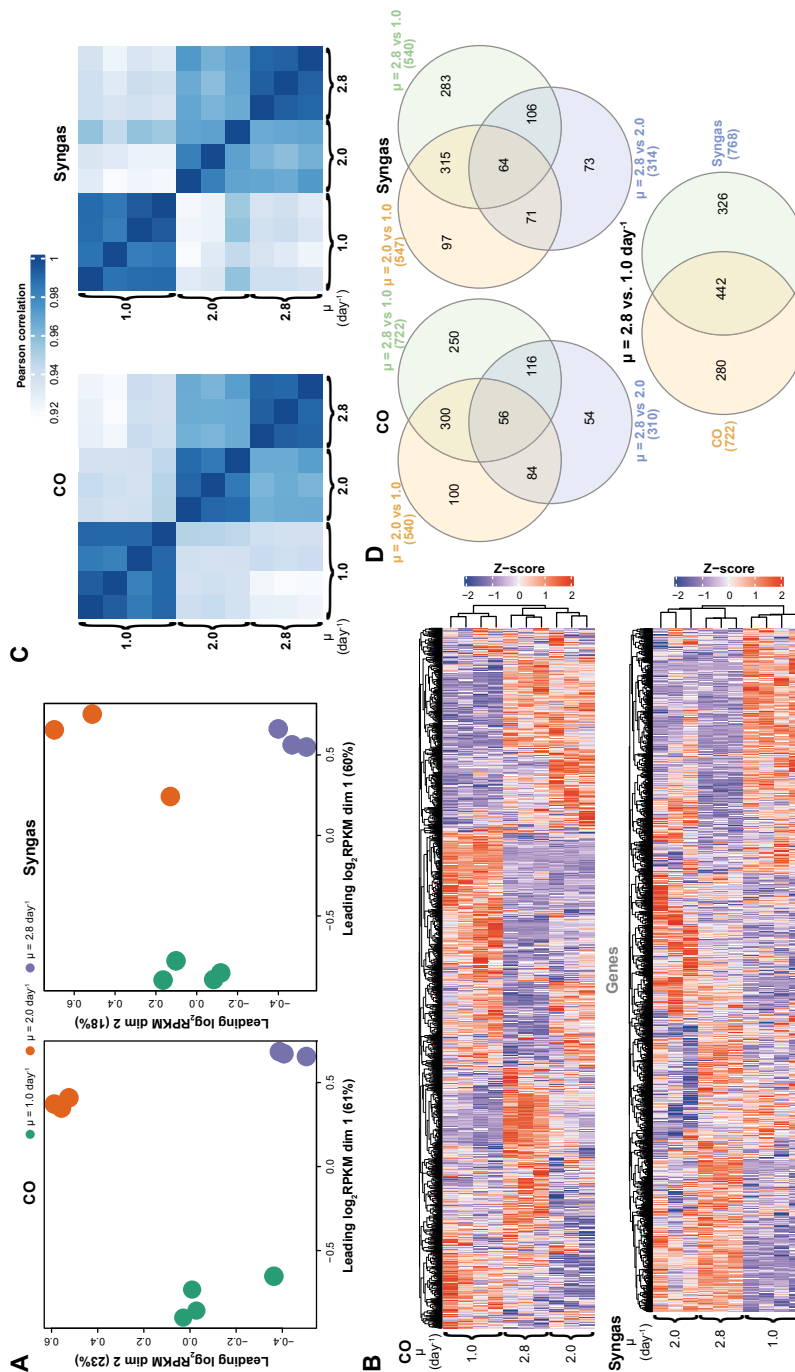


Figure 5. Specific growth rate-dependent transcriptome characteristics in CO- and syngas-grown *C. autoethanogenum* chemostats. **(A)** Multidimensional scaling (MDS) of bioreplicate transcript abundances (RPKM). **(B)** Hierarchical clustering of bioreplicate transcript abundances (Z-scores based on RPKMs). **(C)** Correlation of bioreplicate transcript abundances (RPKM) **(D)** Venn diagrams showing overlap of DEGs (number of DEGs for each comparison in brackets). *RPKM*, reads per kilobase of transcript per million mapped reads; *DEG*, differentially expressed gene (fold-change > 1.5 with q -value < 0.05); μ , specific growth rate. See **Tables S2, S3** and **S4, S5** in Publication 1 for RPKM and DEG data, respectively.

In order to gain additional insight into the global transcriptome changes that facilitate faster growth, an assessment of DEGs through functional gene classifications was carried out. The analysis of DEGs of $\mu = 2.8$ vs. 1.0 day^{-1} using the Cluster of Orthologous Groups (COG) classification (Tatusov et al., 2003) revealed that the majority of the identified DEGs were classified as “Function unknown” category (COG “S”; Figure 6A and Supplementary Table S6 in Publication 1). The latter highlights the necessity of genotype-phenotype mapping and the enhancement of gene annotations for acetogens. Additionally, Gene Ontology (GO) terms (Ashburner et al., 2000) were employed to achieve greater resolution behind global gene expression trends, resulting in clustering of the 1337 DEGs into six significant expression clusters (Figure 5B in Publication 1). These included, for example, terms related to translation and metabolism. Finally, we aimed to identify genes and GO terms that whose expression was tightly regulated in response to changes in μ , as evidenced by a consistent expression pattern across both gas mixtures. A total of 245 genes were identified that exhibited either a continuous increase or decrease in DEGs with increasing μ on both gases, while having a similar magnitude of expression change between the CO and syngas (Figure 5C in Publication 1). Again, translation-related transcripts were enriched and the analysis also identified enrichment of the GO term “mono-carboxylic acid metabolic process”.

Individual gene expression changes linked to metabolic rearrangements

As the results of previous transcriptomics and proteomics studies have indicated that the autotrophic metabolism of acetogens is not subject to hierarchical regulation of gene expression (Al-bassam et al., 2018; Mahamkali et al., 2020; Richter et al., 2016; Valgepea et al., 2018, 2017), an analysis of the DEGs with a particular focus on those that are associated with activities specifically relevant to acetogens, or with high expression changes was carried out. Numerous DEGs were identified in central metabolism, some of which can explain the metabolic rearrangements described above (Figure 6; Supplementary Figures S1 and S2 in Publication 1).

As a first example, an increase in carbon flux to 2,3-BDO accompanied by accelerated growth on syngas (Figure 3) was supported by 18.5-fold ($q < 0.01$) upregulation of the 2,3-BDO dehydrogenase (CAETHG_RS01830; BDH) and by 2.3-fold ($q < 0.01$) up-regulation of the pyruvate:Fd (flavodoxin) oxidoreductase (CAETHG_RS14890; PFOR) (all comparisons $\mu = 2.8$ vs. 1.0 day^{-1} unless otherwise noted; Figure 6). Despite the near-identical 2,3-BDO production pathway genes between the two species (Brown et al., 2014), it was demonstrated in *C. ljungdahlii* that the expression of all 2,3-BDO production pathway genes increased prior to 2,3-BDO production in autotrophic batch cultures (Köpke et al., 2011). This indicates that there is no transcriptional constraints in directing carbon from pyruvate to acetoin highlighting BDH as a key protein and target for metabolic engineering to enhance 2,3-BDO production in *C. autoethanogenum*.

Concurrently, the elevated production of ethanol (Figure 2 and 3) may be associated with increased expression (2.1- to 2.8-fold, $q < 0.01$) of multiple alcohol dehydrogenases (RS02620 and RS02630; Adh and Adh3), including the most abundant – Adh4 (RS08920), in *C. autoethanogenum* (Valgepea et al., 2022, 2017) on syngas comparison (Figure 6). The supply of acetaldehyde for the alcohol dehydrogenases was unlikely to have been direct from acetyl-CoA, as evidenced by the strong downregulation of two putative acetaldehyde dehydrogenases (RS08810 and RS08865; 11.5- and 17.0-fold, $q < 0.01$). Although there was a 4.8-fold ($q < 0.01$) upregulation of a bifunctional aldehyde/alcohol dehydrogenase (RS18395; AdhE1) that catalyses ethanol production directly from acetyl-coA, it is likely that the majority of ethanol was produced via acetate, utilising the AOR activity – AOR1 transcripts were approximately 160-fold more abundant than that of AdhE1 (Supplementary Tables S2 and S3 in Publication 1) across all experiments and AOR2 (RS00490) was up-regulated 2.5-fold ($q < 0.01$) (Supplementary Tables S4, S5 in Publication 1). This is in line with results from metabolic flux analysis (Figure 4) and of previous omics and knockout experiments in acetogens (Heffernan et al., 2020; Liew et al., 2017; Marcellin et al., 2016; Richter et al., 2016; Valgepea et al., 2018).

Faster growth demands more energy and this was evidenced by the elevated expression levels (1.5- to 2.2-fold, $q < 0.01$) of genes of the multi-subunit Fd-NAD⁺ oxidoreductase Rnf complex (RS15845-65) through the studied μ range (Figure 6). The Rnf complex is responsible for generating the proton motive force that drives the ATPase in *C. autoethanogenum* (Hess et al., 2016; Tremblay et al., 2012). Additionally, upregulation of NAD(P)- (RS02275) and FAD-dependent (RS04920) oxidoreductases was also detected (7.9- to 32.9-fold and 2.0- to 3.4-fold, respectively, $q < 0.01$) on both gases, which may play a role in the maintenance of redox homeostasis (Supplementary Figure S2 in Publication 1). It is noteworthy that, despite the increased H₂ uptake observed with faster growth on syngas (Figure 2C), there was a ~1.5-fold repression ($q < 0.01$) of the primary H₂ oxidiser (Mock et al., 2015; Valgepea et al., 2022; Wang et al., 2013), the HytA-E complex (RS13745–70) components (Figure 6). Moreover, comparable down-regulation was observed in CO cultures, indicating that μ -dependent regulation of the HytA-E complex may exist. This finding aligns with proteomics data indicating no alteration in the expression of hydrogenase proteins, even when the cells elevate its H₂ uptake from a near-zero to approximately 30 mmol/gDCW/h (Valgepea et al., 2018).

Importantly, a similar pattern was observed also for higher CO fixation – despite the accelerated growth, we could not detect up-regulation of any WLP genes (nor DEGs) while having substantially increased flux through the pathway (Figure 4). These observations indicate that *C. autoethanogenum* has evolved to prioritise pathways that maintain a sufficient baseline enzymatic capacity for core metabolism, thereby enabling increased flux throughput through key catabolic pathways for CO and H₂ metabolism when needed. This is consistent with the results of an integrative analysis in *C. autoethanogenum* (Valgepea et al., 2022) and various other microbes (Adamberg et al., 2012; Daran-Lapujade et al., 2007;

Valgepea et al., 2013). This adaptation could provide acetogens with an advantage in their natural habitats, as cells can respond more rapidly to the availability of CO and H₂, thereby enabling them to compete for resources with other organisms more successfully.

II. Adaptive laboratory evolution of wild-type *Clostridium autoethanogenum* JA1-1 (Publication 2)

Following a comprehensive characterisation of the metabolism of *Clostridium autoethanogenum* in chemostat on two different gases with an industrial strain, I then turned my focus on studying genotype-phenotype relationships in acetogens. The COVID-19 pandemic, however, forced us to change our approach towards this aim of my thesis. The initial plan was to explore genotype-phenotype relationships using a gene knockout collection, but our industrial collaborator LanzaTech was unable to deliver the strain collection on time due to the pandemic. We thus focused on exploring genotype-phenotype relationships through performing and coupling ALE, systems biology, and reverse engineering of the publicly accessible wild-type *C. autoethanogenum* JA1-1 strain. That strain was first isolated, characterised, and deposited in a strain collection in 1994 (Abrini et al., 1994) and became the type strain due to being the first isolate of the species (named *Clostridium autoethanogenum* by Abrini and co-workers). I used ALE, as it generates genotypic diversity, for studying genotype-phenotype relationships that can be explored through WGS and reverse genetic engineering. Furthermore, ALE also has the potential to tackle multiple limitations with public acetogen strains: as noted in the literature review, they often grow slowly and require complex media components, making research tedious and strains less efficient for industrial applications. Additionally, faster growth would speed up phenotyping and a more robust strain in continuous bioreactor cultures (e.g. low sensitivity to gas ramping, high biomass and dilution rates) would ease generating high-quality steady-state data. Moreover, using YE in the media also excludes metabolic modelling. Therefore, my aim became two-fold: studying genotype-phenotype relationships and develop a superior publicly available *C. autoethanogenum* strain.

a) An overview of ALE strategies across three laboratories

We decided to collaborate with another gas fermentation lab (University of Queensland) and the biotech company LanzaTech to take a more comprehensive approach of ALE. We used three different and independent ALE strategies across three facilities and continents to improve the phenotype of *C. autoethanogenum* (Figure 7) and obtain strains that can grow faster, without YE on minimal medium, and expectantly, are robust in operating continuous bioreactor cultures. I was responsible for the ALE experiment at the University of Tartu (UT ALE) which consisted of serial propagation of batch bottle cultures and was carried out for 142 generations with gradual/stepwise removal of YE at the start of the

experiment. My results will be presented in detail in this thesis while full description of the two other ALEs can be found in Publication 2. Briefly, at the University of Queensland (UQ ALE), batch cultures were propagated in serum bottles for 107 generations with gradual YE removal, then transferred to a continuous bioreactor culture for another 109 generations without YE. At LanzaTech (LT ALE), serial propagation in batch bottle cultures was omitted and instead cells from a bottle culture were exposed to the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG), then transferred to a bioreactor and grown without YE in chemostat conditions for about 50 days. The UT ALE experiment used CO, while the LT and UQ ALE experiments used syngas (CO, CO₂, H₂).

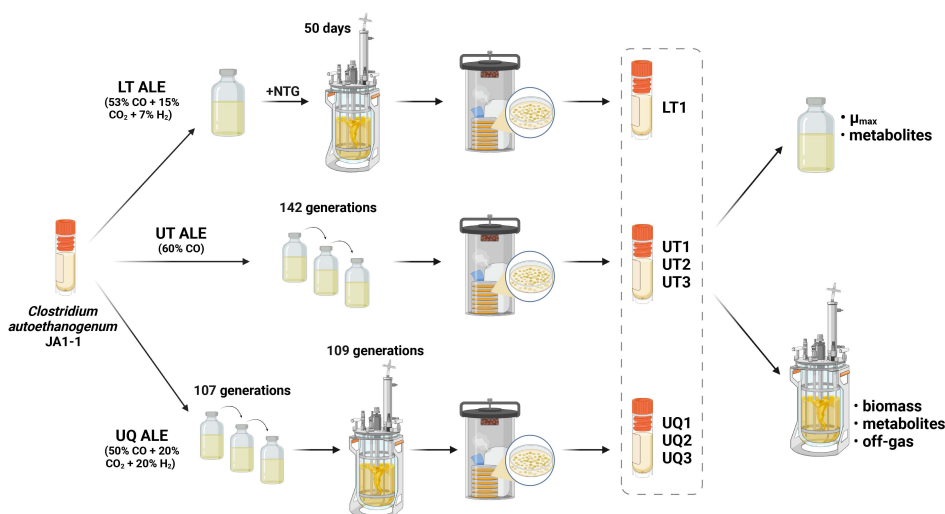


Figure 7. Overview of the three different autotrophic adaptive laboratory evolution (ALE) strategies and subsequent characterisation of ALE isolates in this work. Solid arrows show the ALE workflow and characterisation of isolates (LT1, UT1–3, and UQ1–3; grey dashed box). Created with BioRender.com. UT ALE, ALE executed at the University of Tartu; UQ ALE, ALE executed at The University of Queensland; LT ALE, ALE executed at LanzaTech.; μ_{max} , maximum specific growth rate.

b) Autotrophic ALE yields populations growing faster and without the need for complex nutrients

In the ALE conducted in Tartu (UT ALE), serial propagation of CO bottle cultures permitted the gradual removal of YE from the media by the fourth subculture. It is noteworthy that a sudden 2-fold increase in μ_{max} was observed during YE removal for the third sub-culture (first bottle with reduced YE, 0.5 g/L). Notable reductions in lag phase duration were also observed in the initial YE-free bottles (Figure 8A), indicating that substantial adaptations occurred during early stages of growth in response to new conditions, as previously demonstrated (Barrick et al., 2009). Also, a significant reduction in the duration of batch cultures was

noted through the UT ALE (Figure 8B), attributable to both a decrease in the lag phase and an increase in the $\mu_{\max}$.

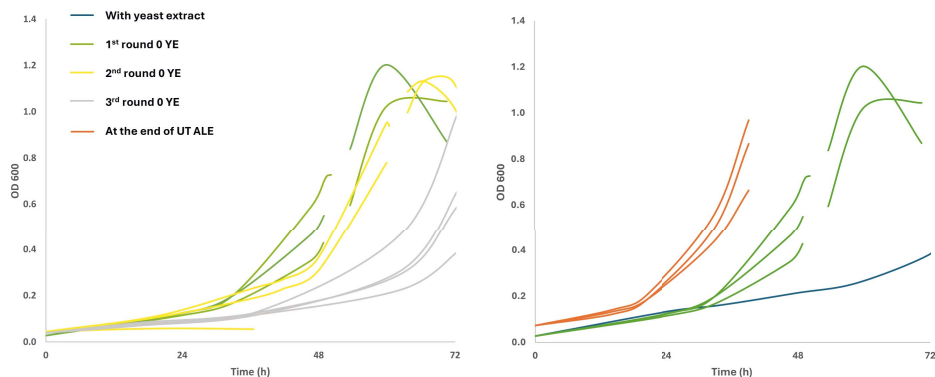


Figure 8. Growth curves of selected batch bottles from UT ALE. **A (Left panel)** – first three rounds of YE-free cultivations. **B (Right panel)** – overlay of growth curves from the beginning and the end of UT ALE; blue – with YE; grey – 1st YE-free round; yellow – 2nd YE-free round; green – 3rd YE-free round; orange – at the end of UT ALE. YE, yeast extract.

By the ~93rd generation, the population had reached its peak $\mu_{\max}$ of 0.10 h^{-1} (~4.6-fold higher than the initial $\mu_{\max}$ (Figure 9A). Subsequently, a decline in $\mu_{\max}$ was observed (Figure 9A) in the last 1/3 of the study, a phenomenon that has been previously observed when carrying out ALE under stable conditions (LaCroix et al., 2015; Wiser et al., 2013). This suggests that evolutionary forces other than those driving faster growth were involved in this ALE approach, most likely related to batch culture propagation details.

Additionally, we tested if higher $\mu_{\max}$ values could be attained by swapping the feedgas in bottles between CO and syngas, but this did not occur (Figure 10A). It could be that the population may have already undergone a metabolic streamlining process to focus on growth on CO and therefore adding CO₂ and H₂ from the ninth round had a negligible effect. Interestingly, the combined data from both UT ALE bottles revealed a declining trend of the acetate/ethanol ratio with increased $\mu_{\max}$ (Figure 9B). In contrast, some isolates of the ALE populations exhibited accelerated growth in parallel with elevated acetate production (Figure 10B; see details below). These findings at the isolate and population levels indicate that cells may employ diverse metabolic routes to achieve enhanced growth, which is in agreement with previous observations (Barrick et al., 2009; Fong et al., 2005b). We postulate that cross-feeding between subpopulations occurred in a manner analogous to that observed in previous bacterial ALEs (Good et al., 2017; Helling et al., 1987; Rozen and Lenski, 2000; Treves et al., 1998). Future co-culturing experiments of isolates with different phenotypes (e.g. high and low acetate producers) may provide insight into these possibilities.

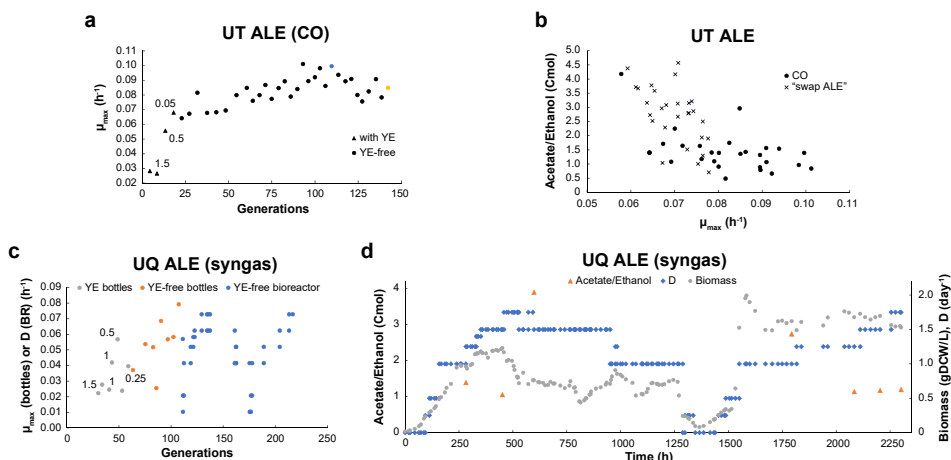


Figure 9. Autotrophic ALE of *Clostridium autoethanogenum* JA1-1 for improved growth phenotypes executed at the UT and UQ ALE. See **Figure 7** and text for more details on ALE strategies. **(a)** Change of maximum specific growth rate ($\mu_{\max}$) with batch culture generations during UT ALE on CO. Blue and yellow circles denote cultures used for isolating single colonies (see text for details). For cultures with generations ~43, ~48, ~96, and ~105, $\mu_{\max}$ was calculated using two data points from the exponential phase instead of using 3–5 data points as for the rest. **(b)** Change of acetate/ethanol (Cmol) with $\mu_{\max}$ in combined batch data from UT (UT ALE and UT “swap ALE”; see text for details). **(c)** Change of $\mu_{\max}$ in batch or dilution rate (D) in bioreactor continuous cultures with culture generations during UQ ALE on syngas. **(d)** Change of acetate/ethanol, biomass concentration (biomass), and D over fermentation time during the continuous culture of UQ ALE on syngas. Numbers next to triangles (panel a) or circles (panel c) denote YE concentrations. gDCW, gram of dry cell weight.

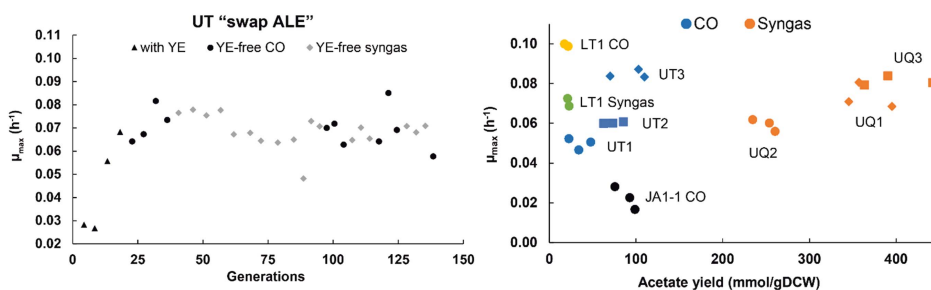


Figure 10. Dynamics of ALE approaches. **A (left panel):** Change of maximum specific growth rate ($\mu_{\max}$) with batch culture generations during UT “swap ALE”. YE, yeast extract. **B (right panel):** Correlation between maximum specific growth rate ($\mu_{\max}$) and acetate production yield (mmol/gDCW) in autotrophic batch cultures of selected isolates from UT, UQ, and LT ALE, and the wild-type *C. autoethanogenum* starting strain of ALE (JA1-1). Data for UT, UQ, and LT isolates are from yeast extract (YE)-free medium, JA1-1 data are from medium with YE. gDCW, gram of dry cell weight.

For UQ ALE, the experiment commenced with a fructose batch culture after which the culture was transferred to syngas bottles with a stepwise removal of YE (Figure 9C). The increase in $\mu_{\max}$ during YE removal was less pronounced than that observed in UT ALE but the increasing trend was clear and the final batch bottle culture exhibited a $\mu_{\max}$ of 0.08 h^{-1} . The evolved population was subsequently transferred to a chemostat bioreactor system, where additional selective pressure was applied by modifying the D (i.e. μ in steady-state conditions) over the course of approximately 109 generations (Figure 9D). Interestingly, J. K. Hefernan (UQ ALE operator) reported that during the bioreactor phase of the UQ ALE, the continuous culture started to crash. Following a period of batch cultivation and subsequent chemostat restart, the culture returned to a stable state. It has previously been proposed that oscillating selection pressures may sometimes facilitate plateaued ALE experiments to escape from the so-called “fitness valley” (Carpenter et al., 2023). A stable culture was obtained at high biomass concentrations and at $D = 1.8 \text{ day}^{-1}$ ($\mu = 0.08 \text{ h}^{-1}$). In the third ALE, which was conducted at LanzaTech (LT ALE), *C. autoethanogenum* was initially treated with the chemical mutagen NTG during batch growth with YE. Thereafter, the culture was used to inoculate a continuous bioreactor culture (with YE-free medium), which reached a final dilution of 1 day^{-1} , and was cultivated for approximately 50 days (Figure 7). The ALE experiments were terminated when significant changes in the evolving population phenotypes were no longer observed, for instance in terms of $\mu_{\max}$ and maximum biomass in bottle cultures or gas data in continuous cultures.

c) ALE isolates exhibit enhanced growth characteristics and metabolite production patterns

To isolate and conduct a comprehensive characterisation and evaluation of the level of phenotypic heterogeneity in single clonal isolates, evolved mixed populations were plated out on PETC-MES agar plates. Preferably, single clones from autotrophic ALE populations should be also isolated from autotrophic growth. For this, I developed an autotrophic agar bottle setup. A series of trials eventually resulted in a stable setup and workflow, which allowed the agar within pressure-resistant Schott bottles to be plated with cells and pressurised with CO-containing gas (Figure 11).

For UT ALE, clones were isolated from both the ALE culture glycerol stock at peak $\mu_{\max}$ of ~ 0.10 (blue marker in Figure 9A) and from the last ALE bottle with lower $\mu_{\max}$ (yellow marker in Figure 9A). From UT and UQ ALE, 10 colonies were randomly selected for a preliminary comparison of growth and by-product patterns in batch cultures using the same gas and media conditions as in ALEs (CO for UT isolates, syngas for UQ isolates respectively). It is notable that all isolates were capable of growth in the absence of YE, indicating that the ability to grow without YE may have been acquired by the majority of evolving populations. For UT ALE, it was hypothesised that isolates from the peak $\mu_{\max}$ population would demonstrate faster growth compared to isolates from the last,

slower-growing population. Nevertheless, this phenomenon was not observed (Supplementary Figure 4A in Publication 2). Variability in $\mu_{\max}$ of UQ ALE isolate batch cultures was also observed (Supplementary Figure 4B in Publication 2). Similarly, phenotypic heterogeneity was observed in terms of by-product patterns within isolates of both UT and UQ ALE (Supplementary Figure 4C–D in Publication 2). For instance, some isolates from UT ALE showed lower acetate/ethanol ratios, whereas three isolates from UQ ALE were detected with high ethanol production. As a result of the preliminary growth tests, three isolates, designated UT1, UT2, and UT3 were selected for further detailed characterisation on the basis of $\mu_{\max}$ and interesting metabolite production patterns observed. From our collaborators, three isolates from UQ ALE (UQ1, UQ2 and UQ3) and one isolate from LT ALE (LT1) were carried forward for further investigation.



Figure 11. Agar system developed in GasFermTEC for isolation of ALE isolates in autotrophic growth conditions.

The seven selected isolates were cultivated in autotrophic batch bottle cultures without YE with frequent sampling to acquire a more precise comparison of growth and by-product patterns. The UT and UQ ALE isolates were cultivated on the gas they were evolved on, namely CO and syngas. The isolate obtained through chemical mutagenesis, LT1, was cultivated on both gas mixtures. To establish a baseline for comparison, the starting wild-type strain for all ALE strategies, *C. autoethanogenum* JA1-1, was also cultured on both gases with YE supplementation. YE was added for JA1-1 because we could not observe growth on batch bottle cultures without YE (CO as a carbon source) after 520 hours. I was responsible for the CO dataset and JA1-1 growth experiments. On CO, UT3 exhibited the highest $\mu_{\max}$ (0.08 h^{-1}) among UT ALE isolates, while LT1 demonstrated the fastest growth on CO with a $\mu_{\max}$ of 0.10 h^{-1} (Figure 12A). In comparison, the starting ALE strain JA1-1 showed a $\mu_{\max}$ of 0.02 h^{-1} (with YE) that was 2- to 4-fold lower than observed for all the evolved isolates tested on CO. With regard to the growth tests conducted on syngas, UQ1, UQ3, and LT1 exhibited comparable growth ($\mu_{\max} \sim 0.08 \text{ h}^{-1}$), which was approximately 1.3- and 3-fold higher than for UQ2 and JA1-1 (with YE), respectively (Figure 12B). All

seven evolved isolates demonstrated higher $\mu_{\max}$ than the starting strain, thereby achieving one of the aims of our ALE approach: to acquire faster-growing strains.

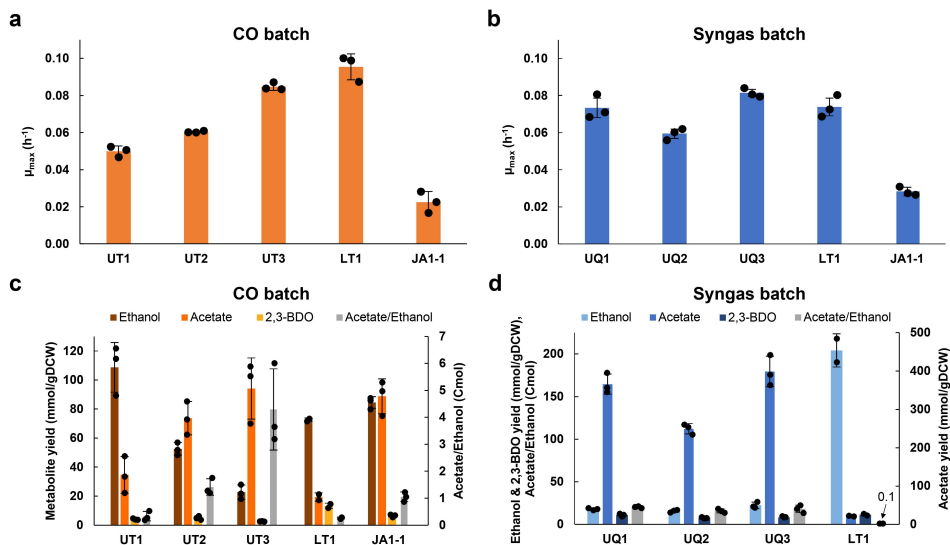


Figure 12. Autotrophic characterisation of selected isolates from UT, UQ, and LT ALE, and the wild-type *C. autoethanogenum* starting strain of ALE (JA1-1) in batch cultures. **(a)** Maximum specific growth rate ($\mu_{\max}$) of UT and LT ALE isolates and JA1-1 in CO batch cultures. **(b)** $\mu_{\max}$ of UQ and LT ALE isolate LT1 and JA1-1 in syngas batch cultures. **(c)** Production yields (mmol/gDCW) of growth by-products and acetate/ethanol (Cmol) of UT and LT ALE isolate LT1 and JA1-1 in CO batch cultures. **(d)** Production yields (mmol/gDCW) of growth by-products and acetate/ethanol of UQ and LT ALE isolate LT1 in syngas batch cultures. All data for UT, UQ, and LT1 are from yeast extract (YE)-free medium, JA1-1 data are from medium with YE. Bars show average $\pm$ standard deviation between bioreplicates, except for LT1 production yields (two bioreplicates). gDCW, gram of dry cell weight; 2,3-BDO, 2,3-butanediol.

In addition to the accurate measurement of $\mu_{\max}$, the implementation of a frequent sampling enabled the precise determination of production yields (mmol/gDCW) for the detected growth by-products, namely acetate, ethanol, and 2,3-BDO. Notably, the lowest acetate/ethanol ratios were observed on CO for isolates LT1 (0.27) and UT1 (0.32), which were approximately ~4- and ~3-fold lower what was observed in JA1-1 (1.05) (Figure 12C). While UT1 demonstrated the highest ethanol production yield with the lowest $\mu_{\max}$ of the evolved strains, the fastest growing UT ALE isolate, UT3, exhibited the highest acetate production yield and the highest acetate/ethanol ratio of 4.14 (Figure 12A and C). The highest production yield of 2,3-BDO was observed for LT1, making the strain overall the most favourable on CO in terms of reduced by-products (Figure 12C). Furthermore, LT1 exhibited enhanced metabolic activity during growth on syngas in comparison with UQ isolates (Figure 12D). This strain demonstrated a >6-fold higher ethanol production yield while having the lowest acetate production yield, which resulted in a significantly low acetate/ethanol ratio of 0.10. The greatest yields of

2,3-BDO in syngas bottles were seen in strains UQ1 and LT1. Interestingly, higher acetate production yields observed in UT and UQ isolates are positively correlated with higher $\mu_{\max}$. Additionally, this might imply that there seems to be a trade-off between faster growth and ethanol production in isolates. In stark contrast, LT1 exhibits fast growth with significantly lower acetate production (Supplementary Figure 5 in Publication 2). The extracellular metabolomic data presented here provides additional evidence that evolving acetogens in the presence of C1 substrates triggers rearrangements in central carbon metabolism, as previously seen (Kwon et al., 2022; Moon et al., 2024; Pregnon et al., 2022; Tremblay et al., 2015).

d) Bioreactor continuous gas fermentation of ALE isolates

Once the bottle batch studies had been completed, we proceeded to test the performance and robustness of all UT ALE isolates and LT1 ($D = 1$ and 2 day^{-1} datapoints) in the industrially relevant condition (Köpke and Simpson, 2020): continuous culture gas fermentation. Additionally, UQ1 and LT1 (at $D = 0.5 \text{ day}^{-1}$) experiments were conducted in an identical bioreactor setup in the University of Queensland. As previously outlined in the thesis introduction, steady-state continuous cultures also facilitate the acquisition of data essential for characterising the cell's physiological state (e.g. μ , gas uptake rates, by-products profile and carbon distribution) (Adamberg et al., 2015) in comparison to the data obtained from bottle cultures. A total of 25 biologically independent steady-states were achieved across 8 different experiments for strains grown on CO or syngas at D ranging from approximately 0.5 to 2 day^{-1} (Table 2).

Table 2. Summary of 25 steady state chemostat cultures of UT, LT1, and UQ1 isolates.

Strain	Feed gas	Gas flow rate (mL/min)	Agitation (RPM)	D (day^{-1})	Biomass concentration (gDCW/L)	Number of replicates
UT2	CO	35	690	1.00 ± 0.00	0.52 ± 0.03	3
UT3*	CO	28	570	0.49 ± 0.01	1.03 ± 0.15	3
UT1	CO	28	570	0.53 ± 0.00	1.13 ± 0.06	3
UT2	CO	24	570	0.52 ± 0.01	0.88 ± 0.09	4
LT1	Syngas	50	675	0.99 ± 0.04	1.41 ± 0.09	4
LT1	Syngas	72	800	1.97 ± 0.11	1.50 ± 0.07	4
LT1	Syngas	35	525	0.50 ± 0.06	0.42 ± 0.05	2
UQ1	Syngas	35	525	0.50 ± 0.06	0.47 ± 0.05	2

D , dilution rate; gDCW, gram of dry cell weight. Data are average $\pm$ standard deviation between bioreplicates. Syngas composition – 50% CO, 20% CO₂, 20% H₂, 10% Ar. All steady-states are biologically independent. Asterisk denotes that steady-state were reached only for gas uptake and production rates.

The initial objective was to run all strains at $D = 1 \text{ day}^{-1}$. However, despite multiple attempts (including with various different mass-transfer ramping strategies), it was not possible to achieve a steady state at $D = 1 \text{ day}^{-1}$ on CO for UT2 isolate at COUR of approximately 1200 mmol/L/day and biomass concentrations of $\sim 1.5 \text{ gDCW/L}$. A comparable challenge was documented in the UQ1 isolate experiment, in which attempts were made to reach a steady state at $D = 1 \text{ day}^{-1}$. We did obtain steady-state at $D = 1 \text{ day}^{-1}$ for UT2 at COUR $\sim 400 \text{ mmol/L/day}$, while interestingly, CO uptake could only be increased after a significant reduction of CO% in the bioreactor headspace by abruptly changing the gas-liquid mass transfer (Figure 13 between ~ 380 to $\sim 440 \text{ h}$). However, reaching a stable high-mass steady state was not possible. We hypothesise that UT2 might have encountered difficulties due to CO toxicity, which might have been caused from too rapid mass-transfer ramping during the initial phases of the experiment. Interestingly, in one test experiment conducted with strain DSM 23693 at a very high D , we also observed that a reduction in gas-liquid mass transfer increased gas uptake rates. Moreover, later in my doctoral studies, a comparable phenomenon was observed on few occasions with strain RE3 (see Chapter III), at higher D conditions (data not shown). Taken together, these findings may suggest that CO toxicity is particularly problematic and pronounced when operating near the growth limits of a particular strain.

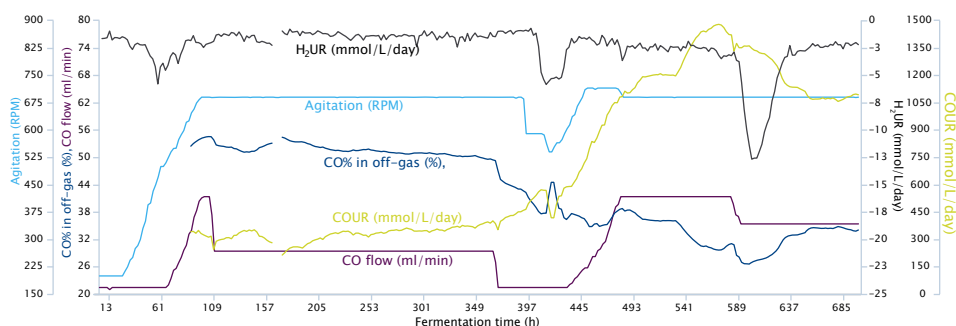


Figure 13. Chemostat fermentation profile of UT2 at a dilution rate of 1 day^{-1} on CO. Data until $\sim 380 \text{ h}$ is of the “CO toxicity” steady-state (in **Table 2**) and from $\sim 500 \text{ h}$ of the non-stable “non-CO toxicity” state (see text for details). Note the increase of the CO uptake rate (COUR; mmol/L/day) after gas-liquid mass transfer adjustment between ~ 380 to $\sim 440 \text{ h}$. Off-gas analysis was unavailable between ~ 162 to $\sim 175 \text{ h}$ due to technical problems. RPM, rounds per minute; COUR, CO uptake rate (mmol/L/day); H_2UR , H_2 uptake rate (mmol/L/day).

To further investigate this effect, we ran a transcriptome analysis on this steady-state (UT2 at $D = 1 \text{ day}^{-1}$; up to $\sim 380 \text{ h}$ on Figure 13) and compared it with other isolates. Transcriptome analysis revealed a significantly divergent transcriptional profile when compared to all other cultures (Supplementary Figure 6 in Publication 2). Furthermore, the transcriptional profile was also markedly distinct from the unstable “non-CO toxicity” phase of the same culture operated at the same D (from $\sim 480 \text{ h}$ Figure 13). For instance, our analysis of UT2 in this “CO

toxicity” state revealed a notable up-regulation of numerous hypothetical, putative membrane, and sporulation-related proteins components (Supplementary Table 2 in Publication 2). Intriguingly, these proteins may serve as promising candidates for potential overexpression, whereby they could play a role in mitigating CO toxicity. Furthermore, we observed a 2-fold down-regulation of components of the HytA-E/formate dehydrogenase (FdhA) complex (Wang et al., 2013). The latter plays a pivotal role in the reduction of CO₂ to formate in *C. autoethanogenum*, utilising NADPH and reduced ferredoxin (Fd) or H₂ (Mock et al., 2015; Valgepea et al., 2017a; Wang et al., 2013). The authors who purified the complex postulate that it may also serve to protect cells from the over-reduction of NADP and Fd pools during growth on CO (Wang et al., 2013). Intriguingly, we also detected significant HytA-E/formate dehydrogenase (FdhA) complex downregulation with strain DSM 23693 with faster growth (Figure 6). This might indicate that such downregulation might be a general mechanism in response to increased CO exposure of cells, leading to overfeeding conditions. Furthermore, Wang and co-workers propose that a reduction in CO oxidation increases the CO concentration in cells, which in turn inhibits CO₂ reduction to formate by the complex that lowers re-oxidation of Fd. To circumvent this over-reduction state, NADPH and Fd has to be deoxidised by HytA-E through proton reduction to H₂. Our results are in accordance with the hypothesis proposed, as evidenced by the detection of H₂ production when attempting to improve COUR of the UT2 “CO toxicity” culture by decreasing gas-liquid mass transfer, i.e. CO oxidation (Figure 13). Furthermore, we have detected H₂ production in CO-chemostats with multiple other strains in similar situations (data not shown). We hypothesise that such regulation may be linked to the down-regulation of HytA-E/FdhA complex component genes. The metabolism of *C. autoethanogenum* thus demonstrates reactional flexibility in selecting electron sinks to drive CO₂ reduction, as proposed previously (Lemaire et al., 2020; Wang et al., 2013) and such flexibility could facilitate growth advantages when faced with changing environmental conditions.

In light of the challenges encountered with the UT2 and UQ1 cultures at $D = 1 \text{ day}^{-1}$, we decided to use $D = 0.5 \text{ day}^{-1}$ to further compare different strains and gas conditions. In the case of UT3, however, only data regarding gas uptake and production at $D = 0.5 \text{ day}^{-1}$ could be obtained, as acetate and ethanol production were found to be highly sensitive to any operational activities conducted on the bioreactor cultures. For example, halting agitation for a volume check or sampling of 1% of the culture volume often had a significant impact on the bioreactor stability. Furthermore, adjustment of gas-liquid mass transfer was needed even after reaching seemingly robust (high biomass) conditions (Figure 14). In stark contrast, operating LT1 cultures was a relatively straightforward process, and we were able to achieve steady states at $D = 1$ and 2 day^{-1} at high COUR and biomass concentrations (Figure 14). To illustrate, the COUR for LT1 growing on syngas at $D = 1 \text{ day}^{-1}$ was triple the steady-state value of UT2 on CO (Supplementary Figure 7 in Publication 2). Furthermore, in consideration of the time-scales involved, faster gas-liquid mass transfer ramping and elevated Ds also

resulted in the achievement of two steady states for LT1 with half the time it took to achieve one steady-state for UT3 (Figure 14). In terms of time-scales involved, this could translate to significant savings, for example, when testing lots of strains or conditions at multiple D values. Additionally, since higher D values reach steady state in less time in continuous cultures, the time-savings are far from linear, as Figure 14 illustrates. The robustness of LT1 is highly relevant for the acetogen gas fermentation field as it makes the operation of continuous gas fermentation cultures for the acquisition of high-quality data considerably less challenging. Therefore, a robust strain for bioreactor continuous gas fermentation was obtained, which fulfils the third objective of the ALE project.

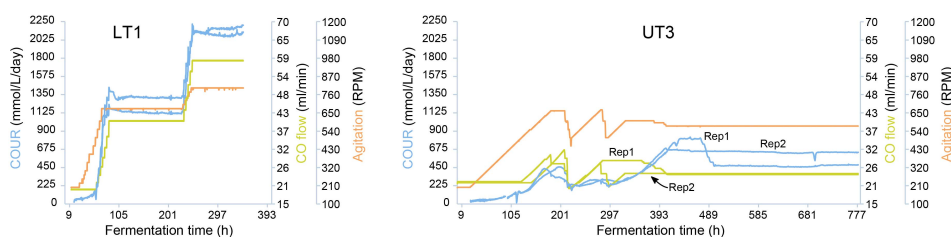


Figure 14. Robustness of strain LT1 in terms of operation of bioreactor continuous gas fermentation cultures. LT1 data are for two of the four chemostats that achieved steady-states at D of 1 and 2 day⁻¹; UT3 data are for two (Rep1 and Rep2) of the three chemostats that achieved steady-states at D = 0.5 day⁻¹. See **Table 2** for more details on LT1 and UT3 chemostats. COUR, CO uptake rate (mmol/L/day); RPM, revolutions per minute.

A detailed examination of the steady-state data from the bioreactor runs reveals that a number of metabolic rearrangements occurred during the three ALE strategies. In contrast to the batch data (Figure 12), the UT strains showed nearly identical growth characteristics in CO chemostats, namely q_{EtOH} and acetate/ethanol ratios (Figure 15). Although the acetate concentration for UT2 was lower, this was likely due to the lower biomass concentration (Figure 15A). However, the specific by-product production rates (Figure 15B) and specific gas rates (Figure 15C) exhibited comparable values with the exception of the specific production rate of 2,3-BDO ($q_{2,3\text{-BDO}}$; mmol/gDCW/day).

Furthermore, the chemostat data diverged from the batch data when comparing UQ1 and LT1 in syngas chemostats at D = 0.5 day⁻¹, as both strains exhibited comparable performance in terms of by-product production and gas uptake and production (Figure 15). The data suggests that batch bottle cultures may not be a reliable predictor of performance in continuous cultures. To illustrate, only LT1 demonstrated the capacity to perform above D = 0.5 day⁻¹, whereas the majority of evolved strains exhibited comparable μ_{max} in batch cultures, which was > 0.5 day⁻¹. For example, UQ3 strain displayed one of the highest μ_{max} values in batch dataset but D = 1 day⁻¹ was unobtainable. Moreover, attempts to operate UT2 chemostats at elevated D and gas-liquid mass transfer rates appeared to be compromised by CO toxicity (see above), which was absent in batch cultures with lower gas-liquid mass transfer. These findings underscore the importance of

evaluating novel strains (e.g., acquired through ALE or metabolic engineering) in industrially relevant continuous fermentation conditions (Köpke and Simpson, 2020).

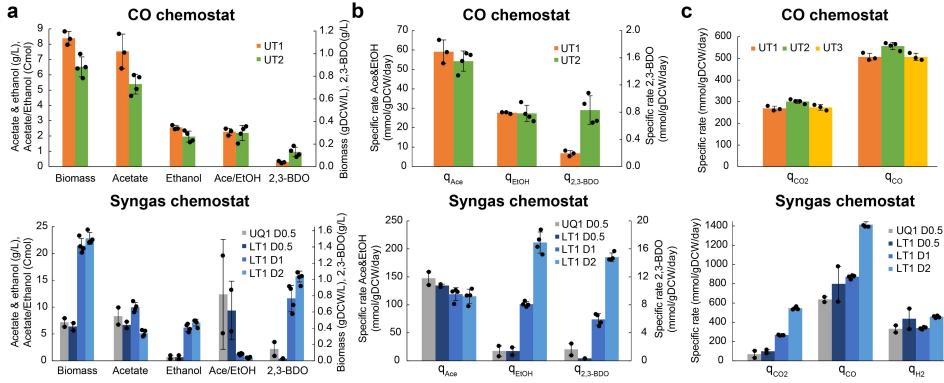


Figure 15. Characterisation of selected isolates from UT, UQ, and LT ALE in steady-state autotrophic chemostat cultures. **(a)** Biomass and by-product concentrations and acetate/ethanol (Ace/EtOH). **(b)** Specific by-product production rates (mmol/gDCW/day). **(c)** Specific gas uptake (CO , H_2) and production (CO_2) rates. CO chemostat data for UT1 and UT2 are from dilution rate (D) 0.5 day^{-1} ; for syngas chemostat data, the number following D denotes D value in day^{-1} . UT3 reached steady-state only regarding gas uptake and production, thus only these data are shown. Bars show average $\pm$ standard deviation between four (UT2, LT1 D1, LT1 D2), three (UT1), or two (UQ1 D0.5, LT1 D0.5) bioreplicates. See **Table 2** for details. gDCW, gram of dry cell weight; 2,3-BDO, 2,3-butanediol; q , specific rate.

It is noteworthy that LT1 demonstrated superior performance in comparison to all other strains with regard to acetate/ethanol ratios, exhibiting values less than 1 at $D = 1$ and 2 day^{-1} (Figure 15A). The ability to achieve higher biomass concentrations for LT1 $D = 1$ and 2 day^{-1} cultures resulted in the highest ethanol and 2,3-BDO levels (Figure 15A), and, due to faster growth, also led to elevated specific production rates (q_{EtOH} and $q_{2,3\text{-BDO}}$) (Figure 15B). Furthermore, LT1 cultures at $D = 1$ and 2 day^{-1} demonstrate the highest carbon flux to reduced products (ethanol and 2,3-BDO) across the studied strains and steady-states (Figure 16). It is noteworthy that the q_{H_2} for LT1 did not increase in proportion to D (Figure 15C), as observed in the initial chapter of the thesis with strain DSM 23693. This finding indicates that this phenomenon may be more widespread within *C. autoethanogenum* and may present an alternative avenue to enhance the performance of *C. autoethanogenum* through ALE, i.e. improving H_2 uptake.

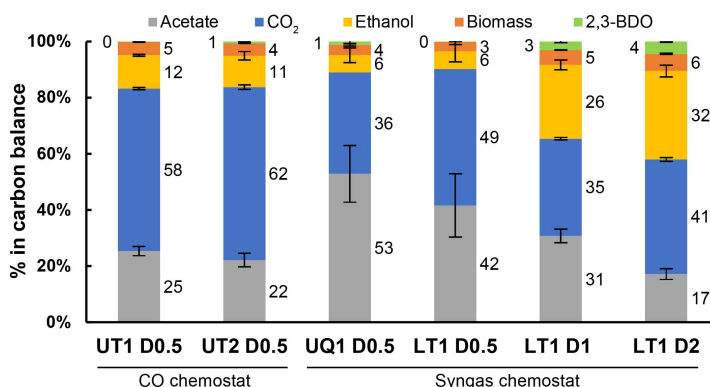


Figure 16. Carbon balances of selected isolates from UT, UQ, and LT ALE in autotrophic chemostat cultures. The number following D (dilution rate) denotes D value in day⁻¹. Carbon flow into CO₂ for UQ1 D0.5 and LT1 D0.5 were calculated by subtracting measured carbon flows into other products and biomass from 100%. Carbon recoveries for UT strains and LT D1 and D2 were normalised to 100% to have a fair comparison of carbon distributions between different conditions. Bars show average $\pm$ standard deviation between four (UT2, LT1 D1, LT1 D2), three (UT1), or two (UQ1 D0.5, LT1 D0.5) bioreplicates. See **Table 2** for details. 2,3-BDO, 2,3-butanediol.

It is evident that the isolation of LT1 from a chemostat culture following NTG mutagenesis may have contributed to the enhanced performance of LT1 in our bioreactor continuous cultures. Upon comparing our various ALE approaches, it is evident that the combination of NTG with selective pressure during continuous cultivation in LT ALE proved to be the most successful strategy – it may be surmised that NTG treatment introduced high genetic diversity, thus paving the way for subsequent selection under operational conditions. These results support the effectiveness of NTG-induced mutagenesis in combination with selective pressure in microbial organisms, as previously demonstrated (Bennett et al., 2021; Lee et al., 2011), including in acetogens (Otte et al., 2009). Furthermore, they indicate that isolating evolved strains from conditions similar to those where the strain would eventually be used for scientific investigation or industrial exploitation could be of significant value.

In conclusion, the bottle batch and bioreactor data analysis demonstrated that the evolved strain LT1 met all the objectives of our ALE work to create an enhanced model acetogen strain accessible to the public. It is capable of growth without complex nutrients on minimal medium, exhibits high gas uptake enabling accelerated growth, and is robust in terms of operating both continuous bioreactor gas fermentation cultures and bottle batch experiments. Furthermore, the metabolic feature of high carbon flux into reduced growth by-products makes LT1 an attractive chassis for metabolic engineering. Concurrently, this strain, designated LAbriini (i.e., Laboratory Adapted Abriini), has been deposited at the DSMZ strain collection (DSM 115981) for public use. It is the aspiration of all the authors of the ALE study (including the thesis author) that it will become a model acetogen strain, given the promising potential of Labriini's enhanced characteristics for accelerating the phenotyping and engineering of acetogens.

e) Genotypes of ALE isolates

Subsequently, WGS was employed to identify genetic mutations that may have resulted in the improved phenotypes observed in isolates derived from the three ALE strategies. Furthermore, WGS data facilitates the comparison of genetic variability and the observation of sample subpopulations of evolved strains. The starting strain, JA1-1, and seven key evolved strains (UT1, UT2, UT3, UQ1, UQ2, UQ3, and LT1) were sequenced, and a mutation analysis was performed using breseq against the NCBI reference genome assembly ASM148472v1 (GenBank CP012395.1) of *C. autoethanogenum* JA1-1 (Humphreys et al., 2015). The starting strain for our three ALE strategies was sequenced to ensure reliable detection of genetic mutations that appeared only during ALEs, as opposed to relying exclusively on the published genome sequences for *C. autoethanogenum* JA1-1 (Brown et al., 2014; Humphreys et al., 2015). The latter enabled the exclusion of two false positive mutations from the mutation results (see Materials and Methods in Publication 2 for more info). Furthermore, within this work, the Illumina data was employed to refine the PacBio genome assembly of LT1 using the Pilon v1.24 tool (Walker et al., 2014), and subsequently this assembly was submitted to the NCBI genome submission portal (Genbank CP110420) for annotation with the NCBI Prokaryotic Genome Annotation Pipeline. See Supplementary Table 1 in Publication 2 for a comparison of LT1 strain gene identifiers with previously published complete assemblies of *C. autoethanogenum* JA1-1 genome sequences (Brown et al., 2014; Humphreys et al., 2015). A comparison of the genome assemblies of JA1-1 and LT1 reveals a change in total length from 4,352,446 to 4,349,910 nucleotides. Additionally, the number of coding sequences (CDSs) differs, with 3964 and 3942 CDSs observed for JA1-1 and LT1, respectively. It is notable that the GC content remains consistent at 31.1%.

A total of 25 clean mutations (occurring at nearly 100% in the isolate sequencing samples) were identified in the evolved strains. Of those mutations, 21 were found to affect protein-coding sequences (Figure 17 and Supplementary Table 6 in Publication 2). Of these 25 mutations identified, 17 were SNP-s, eight were deletions, while four being ≥ 168 bp in length (Figure 17B). The analysis revealed the presence of hotspots that accumulated mutations across multiple ALE strategies (Figure 17A). Of particular note are two genes that exhibited a number of mutations: mutations #4–5 in the sporulation transcriptional activator Spo0A (CLAU_3129) and mutations #19–23 CLAU_0471. These two genes are examples of convergent evolution in autotrophic selection pressure conditions across three distinct ALE strategies. When also including sub-population results (occurrence $< 95\%$), then UQ1 and UQ2 samples displayed a potential third hotspot – genes CLAU_0418, CLAU_0419, CLAU_0420, and CLAU_0424 acquired 10 SNP-s in a ~ 7000 bp area, with some affecting 30% of the population (average frequency 12.5% for 10 SNP-s).

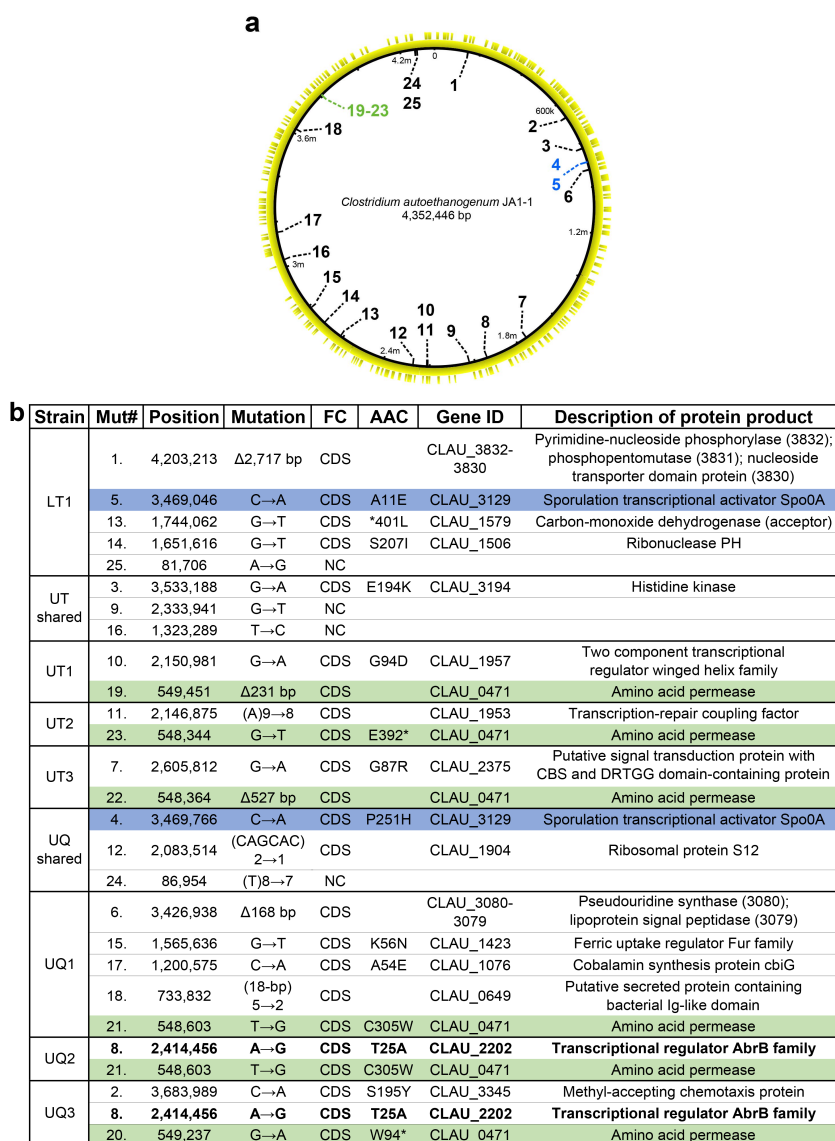


Figure 17. Whole-genome sequencing of the selected isolates from UT, UQ, and LT ALE and identified mutations relative to the wild-type *C. autoethanogenum* starting strain of ALE (JA1-1). **(a)** Distribution of the identified 25 mutations across the circular genome of JA1-1. The number denotes mutation shown in panel b (column Mut#) and colour matches the shading of mutation in panel b (exceptions are black numbers with white shading in panel b). Yellow layers are protein-coding sequences (CDS) on forward (inner) and reverse (outer) strands. **(b)** Details of the 25 mutations identified in the seven ALE strains. Mutations with colour shading appeared in the same genes across multiple ALE strategies, i.e. convergent mutations, while the non-convergent mutation in bold font (#8) appeared in two isolates within one ALE strategy (details shown only for the first occurrence); shading colours match colour of numbers (Mut#) on panel a. The bases within the parentheses in column “Mutation” denote the repeat sequence that decreased from the number of repeats indicated on the left of the arrow (→) to the number indicated on the right. See Supplementary Table 6 in Publication 2 for additional details on mutations. Mut#, mutation number; FC, feature class; AAC, amino acid change; Gene ID, gene identifier; SNP, single nucleotide polymorphism; Δ, deletion; NC, non-coding; *, stop codon.

One of the genes under higher selection in our ALE experiments is a proposed amino acid permease (CLAU_0471; function predicted based on conserved domain PotE) that was affected by two deletions and three different SNPs. One 231 bp deletion was observed in UT1, and one 527 bp deletion was observed in UT3. Two hits were identified in UQ isolates and one in UT2 (mutations #19–23 in Figure 17B and Supplementary Table 6 in Publication 2). It is noteworthy that the nucleotides at these three SNP positions of CLAU_0471 in the ALE starting strain JA1–1 are fully conserved across homologs of *Clostridia* species (Supplementary Figure 9 in Publication 2) and two of those SNPs resulted in stop codons. While clean mutations were observed exclusively in ALE approaches where serial propagation in bottle cultures and stepwise removal of YE were employed (UT and UQ ALE), mutations were also detected in CLAU_0471 at LT1 sequencing sample. LT1 reads exhibited a single stop codon at the 72nd amino acid (24% of the reads) and a 32-basepair deletion spanning 415–446 nt (9% of the reads). It is currently unclear why a proposed amino acid permease is undergoing such strong selective pressure, however, our findings suggest that it is subjected to elevated selective pressure in YE-free autotrophic ALE conditions.

The second gene, transcription factor *spo0A* (CLAU_3129), was found to be affected by a G-to-T SNPs at two distinct locations within the UQ and LT ALE (mutations #4–5 in Figure 17B and Supplementary Table 6 in Publication 2). Similarly to the mutations observed in the CLAU_0471 gene (Supplementary Figure 9 in Publication 2), the corresponding nucleotides in the starting strain JA1–1 at both positions of *spo0A* are highly conserved across homologues of *Clostridia* species (Supplementary Figure 10 in Publication 2). This gene is regarded as a master regulator of sporulation and solventogenesis in *Clostridia* (Dürre, 2016; Kolek et al., 2017; Paredes et al., 2005; Steiner et al., 2011). The down-regulation or inactivation of *spo0A* in *C. acetobutylicum* has been observed to result in a reduction in sporulation and an increase in solvent production (Harris et al., 2002; Li et al., 2016). However, in other *Clostridia*, the role of Spo0A appears to be more complex, with studies suggesting that it may improve fitness (Humphreys et al., 2023) and regulate metabolic and virulence factors that extend beyond the sporulation process (Talukdar et al., 2015). Given that mutations in *spo0A* occurred exclusively in ALE strategies where syngas (i.e., a mixture of CO₂ and H₂ in addition to CO) and chemostat conditions were present, we postulate that *spo0A* may be subjected to heightened selective pressure when these conditions are present. A batch serial passage ALE at syngas conditions may prove an effective method of excluding either of the aforementioned variables.

It is noteworthy that mutations #10 in a predicted phosphorelay regulator (CLAU_1957) and mutation #3 (present in all UT isolates) in a proposed histidine kinase (CLAU_3194) may be associated with the regulation of sporulation in UT1. The latter genes are involved in phosphorylation circuits involving histidine kinases (Loomis et al., 1997) and similar kinases have been shown to regulate sporulation, metabolism and solventogenesis in *Clostridium beijerinckii* and *C. acetobutylicum* (Xin et al., 2020; Xu et al., 2015). Additionally, both histidine kinases (Paredes et al., 2005; Steiner et al., 2011) and CLAU_1957 (Fujita and

Losick, 2003) could affect Spo0A directly. Furthermore, we note that CLAU_1957 had an additional, unique SNP in UT3 sequencing sample (12% of the population according to breseq pipeline). The occurrence of mutations in two-component sensor systems composed of histidine kinases and response regulators in ALE experiments with other bacteria has led to the hypothesis that these mutations are associated with the general adaptation of the organism to its growth environment (Lim et al., 2021). It is also possible that sporulation networks were affected in UQ1 through mutation (#6) that was identified to cause a 168 bp deletion across genes encoding for a lipoprotein signal peptidase (CLAU_3079) and a pseudo-uridine synthase (CLAU_3080), that are involved in sporulation (Fimlaid et al., 2015) and pyrimidine metabolism processes, respectively (Figure 17B and Supplementary Table 6 in Publication 2). It is noteworthy that the latter gene exhibited increased expression in a *spo0A* overexpression mutant strain of *Clostridium acetobutylicum* subjected to butanol stress (Alsaker et al., 2004).

Furthermore, it is likely that pyrimidine metabolism could have played a role in the improvement of phenotypes in our ALE approaches, including LT1: the large 2717 bp deletion in LT1 (mutation #1) resulted in the deletion of two genes (CLAU_3830 and 3831) and truncation of one gene (CLAU_3832), which are all associated with pyrimidine metabolism (Figure 17B and Supplementary Table 6 in Publication 2). This could potentially facilitate a more rapid supply of RNA monomers, thereby enabling higher translation rates and accelerated growth. Among the other mutations identified in the LT1 genome, the mutation that is potentially the most relevant to the improved phenotype of LT1 is a SNP (mutation #13) in the carbon monoxide dehydrogenase gene *AcsA* (CLAU_1579). This mutation may be of particular significance with regard to enhanced autotrophic growth, as the *AcsA* gene encodes the CODH subunit of the CODH/ACS protein complex (Figure 17B and Supplementary Table 6 in Publication 2). The CODH/ACS complex plays a crucial role in the oxidation of CO, reduction of CO₂ and synthesis of acetyl-CoA (Lemaire and Wagner, 2021), and therefore, its activity is indispensable for the autotrophic growth of *C. autoethanogenum* (Liew et al., 2016a). The removal of the stop codon resulting from this mutation gives rise to a putative translational fusion product. It is noteworthy that the *acsA* (CLAU_1579) stop codon removal in strain LT1 has been documented in an independent study that observed translational read-through at the protein level (Liew et al., 2016a). Interestingly, mutations in the CODH/ACS complex have been observed in previous CO ALEs in acetogens (Kang et al., 2020; Moon et al., 2024), postulated to be associated with adaptations against increased CO toxicity (Kang et al., 2020).

It is notable that the application of NTG treatment during LT ALE did not result in a significant increase in the number of mutations observed in LT1 when compared to those identified during UT and UQ ALE sequenced isolates. Strikingly, when the subpopulation mutations were also taken into account, LT1 had mutations in both genes (*spo0A* and CLAU_0471) that demonstrated convergent evolution in this dataset. Therefore, the enhanced performance of LT1 in continuous bioreactor cultures and its capacity to grow in the absence of YE can be attributed to its genotype and the selection strategy employed during its development. This

further showcases the benefits of combining mutagen treatment to generate a population with high genetic diversity followed by selection for the most fit organisms under intended isolate operational conditions (e.g. autotrophic YE-free continuous bioreactor cultures). Further studies are required to establish a link between the identified mutations and the corresponding phenotype, as well as to gain a deeper understanding of the structural and functional attributes of the CODH/ACS enzyme complex and the regulatory mechanisms involved.

Among other detected mutations, an additional SNP was observed in a typically highly conserved genomic region within the Clostridia species (Supplementary Figure 11 in Publication 2) – mutation #8 in an AbrB family transcriptional regulator (CLAU_2202) seen in UQ ALE (Figure 17B and Supplementary Table 6 in Publication 2). It is noteworthy that CLAU_2202 is among the top 100 abundant proteins in *C. autoethanogenum* (Valgepea et al., 2022). Other potentially relevant mutations in CDSs were identified in the ribosomal protein S12 (mutation #12) and transcription-associated genes (#11, and #15). Notably, a homolog of the Ferric uptake regulator of the Fur family (CLAU_1423), which acquired mutation #15 in our study – the putative transcriptional regulator PerR – has acquired numerous mutations in the ALE experiments compiled in the ALEdb database (Phaneuf et al., 2019). Interestingly, mutation #9 (present in all UT isolates) – although intergenic, is positioned next to TetR family transcriptional regulator (–157 bp downstream of CLAU_2136 start) and, additionally, a separate SNP in CLAU_2136 was detected in UQ1 subpopulation (7%). These results indicate that transcriptional regulators might be under strong selection in the studied ALE conditions.

The analysis of WGS data of the seven selected ALE isolates confirmed the findings of previous studies that different ALE approaches frequently result in similarly improved phenotypes, albeit through disparate underlying genetic alterations (Barrick et al., 2009; Fong et al., 2005b; Good et al., 2017; Helling et al., 1987; Treves et al., 1998). As briefly discussed before, we observed increased selection pressure for CLAU_0471 in batch bottle ALE conditions (with stepwise removal of YE) and for *spo0A* – in conditions where syngas and chemostat selection was used. Further ALE experiments are needed to test whether there was causality between these observed patterns. Importantly, recurrent mutations in independent replicate lines strongly imply a causal relationship (Conrad et al., 2009). A deeper understanding of the direct causal relationships between the aforementioned genetic changes and their physiological consequences can be gained through reverse genetic engineering these mutations into the starting strain of ALE (e.g. wild-type). This would also mitigate the potential confounding and epistatic effects that ALE isolates may harbour. Therefore, we next reverse engineered some of the uncovered mutations into the starting strain of ALE (JA1-1) and comprehensively phenotyped the acquired strains.

III. ALE-guided reverse engineering and characterisation of *Clostridium autoethanogenum* strains (Publication 2 and 3)

a) Construction of the ALE guided reverse-engineered strains of *Clostridium autoethanogenum*

The targets for reverse genetic engineering were chosen based on WGS results in the isolates from Publication 2 by my colleague postdoc Kurshedaktar Majibullah Shaikh, my supervisor Kaspar Valgepea, and by myself. Firstly, we only focused on mutations in CDS areas, as we expect these to have the most significant effects on the phenotypes. Secondly, we reasoned that the occurrence of convergent evolution indicated significant links between selection conditions (presence of CO), realised phenotypes (faster, YE-free growth), and genomic changes.

Therefore appearance of mutations in *spo0A* during both LT and UQ ALE (Figure 17B) was likely significant, potentially due to the role of *spo0A* as a master regulator of sporulation and solventogenesis in Clostridia (Dürre, 2016; Kolek et al., 2017; Paredes et al., 2005; Steiner et al., 2011). Additionally, mutation #10 observed in UT1 isolate occurred in a two-component transcriptional regulator CLAU_1957 (based on GenBank CP012395.1 annotation; (Humphreys et al., 2015) that could also be involved in sporulation processes (e.g. phosphorelay, sporulation activator activity (Hoch, 1993)) based on predictions using protein sequence or structure homology by DeepFRI (Gligorijević et al., 2021), ProteInfer (Sanderson et al., 2023), and UNIPROT database (Bateman et al., 2023) (Supplementary Table 7 in Publication 2; UniProtKB U5RXC5). Specifically, mutation #10 occurred in a predicted response regulator receiver domain (one of the two predicted domains). Therefore, we targeted to modify *spo0A* and CLAU_1957 in wild-type strain JA1-1 to investigate whether alterations to the proposed sporulation processes could, at least partially, replicate the improved phenotypes obtained during our ALE. For the third reverse engineering target, we chose gene CLAU_0471 (annotated as an amino acid permease) as the latter acquired genetic mutations across all the sequenced isolates when also including subpopulation sequencing results. The mutations varied considerably in type and size – including two partial gene deletions and two premature stop codons, signifying the level of selection pressure applied on gene CLAU_0471 under three ALE conditions. We thus reasoned to delete the gene CLAU_0471 in wild-type strain JA1-1, to evaluate the effects of mutations, and subsequently, characterise the effect on the strain phenotype. The strains were genetically engineered by Kurshedaktar Majibullah Shaikh. Briefly, the *C. autoethanogenum* strain JA1-1 (Abrini et al., 1994) (deposited as DSM10061 in the German Collection of Microorganisms and Cell Cultures) was used as the base strain for reverse genetic engineering of the three following strains shown in Table 3.

Table 3. Summary of the reverse-engineered strains

Strain	Genotype	Gene description
RE1	deletion of CLAU_3129	Sporulation transcriptional activator Spo0A
RE2	modified CLAU_1957	Two component transcriptional regulator winged helix family, G-to-A nucleotide change resulting in glycine to aspartate change in 94 th amino acid
RE3	deletion of CLAU_0471	Proposed amino acid permease

b) Performance of reverse-engineered strains growth in autotrophic bottle batch cultures

First, we studied the reverse-engineered strains RE1, RE2, and RE3 in autotrophic batch bottle cultures (Figure 18). LAbriini (LT1 in Publication 2) strain data from Publication 2 are incorporated into this chapter for comparative purposes. Experiments conducted without YE (Figure 18A) demonstrated that all three strains are capable of growth in the absence of YE (in this media, the wild-type strain JA1-1 is unable to grow without it). It is noteworthy that, RE1 autotrophic batch bottle cultures displayed a $\mu_{\max}$ of approximately 0.1 on both gases, which is comparable to LAbriini results on CO and is >4-fold faster growth than JA1-1 with YE. Strains RE2 and RE3 show lower values than RE1, with both demonstrating marginally elevated $\mu_{\max}$ on CO condition compared to syngas, a trend also seen with LAbriini. A comparison of the $\mu_{\max}$ value from the CO condition of RE2 and its respective mutation source strain (UT1; an ALE strain with the same SNP as RE2) shows that RE2 has a $\mu_{\max}$ value comparable to that of UT1 while being significantly faster (>3-fold) than JA1-1 on YE (Figure 18B).

The analysis of growth by-products for the YE-free media bottle batch experiments (Figure 18C) demonstrated a markedly elevated production of 2,3-BDO for all three reverse-engineered strains when cultivated on syngas in comparison to CO (p-values = 0.001–0.02). Conversely, no notable distinction has been observed for LAbriini (Nwaokorie et al., 2023 and Publication 2). Ethanol production was observed to be higher on CO for RE1 (p-value = 0.01), while acetate production was found to be higher on syngas for RE2 (p-value = 0.001). Additionally, acetate/ethanol ratios (yield/yield) were noted to be higher for both strains on syngas (p-value of 0.02 for RE1 and 0.01 for RE2) (Figure 18C). This is unexpected, given that the H₂ in syngas is expected to support synthesis of reduced products (see discussion below). A comparison between reverse-engineered strains reveals that RE2 produces less 2,3-BDO than RE3 and RE1. Additionally, RE3 appears to potentially produce more 2,3-BDO than RE1, similar to observations made in chemostat cultures (see Figure 19 and 20) although the difference in batch is not statistically significant (p-value = 0.06 for syngas comparison). With regard to ethanol yields, production was indifferent between RE3 and RE1 (p-values of 0.99 and 0.85) while both RE3 and RE1 demonstrated a greater ethanol production on CO than RE2 (p-values = 0.01).

It is noteworthy that RE2 produced significantly more acetate on syngas compared to RE3 and RE1 (p-values of 0.01 and 0.05, respectively). When comparing yields to LAbriini, RE1 and RE3 exhibit higher ethanol yields on CO (p-values of 0.01 and 0.03), while all three engineered strains demonstrate lower 2,3-BDO yields on CO (p-values = 0.001–0.004) (Figure 18C). Both RE1 and RE2 produced more acetate than LAbriini on both gases (p-values = 0.002–0.005) which also results in acetate/ethanol ratios being 2–4 times higher (p-values = 0.01–0.02) (Figure 18C).

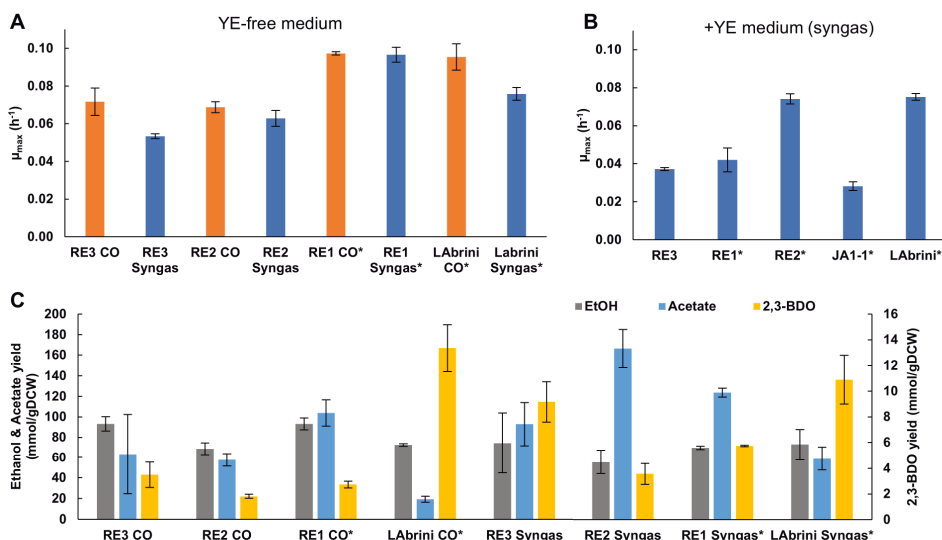


Figure 18. Autotrophic batch bottle characterisation of three reverse-engineered strains. **(A)** Comparison of maximum specific growth rate (μ_{max}) of studied strains on YE-free medium. Orange bar denotes CO feed gas, blue syngas. The wild-type strain JA1-1 does not grow in YE-free medium, therefore it was not included in this comparison. Bars show average $\pm$ standard deviation between three bioreplicates, except two bioreplicates for LAbriini syngas and RE1 syngas (YE-free medium). **(B)** μ_{max} of studied strains on medium with YE on syngas. Bars show average $\pm$ standard deviation between three bioreplicates. **(C)** Production yields (mmol/gDCW) of growth by-products of studied strains. Bars show average $\pm$ standard deviation between three bioreplicates, except two bioreplicates for LAbriini CO and RE1 syngas. Asterisk behind strain name denotes previously shown data from Publication 2 and Nwaokorie et al., 2023. gDCW, gram of dry cell weight; 2,3-BDO, 2,3-butanediol.

Since the wild-type strain JA1-1 is unable to grow in a YE-free medium, we also cultivated the strains RE1-3 in PETC-MES+YE media to obtain a fair μ_{max} comparison with the starting strain JA1-1. Strikingly, only RE2 exhibited a significantly higher μ_{max} than JA1-1, and strains RE1 and RE3 grew notably slower with YE than without it (Figure 18). It is notable that another partially reverse-engineered strain (containing partial deletion of gene CLAU_3194 – a proposed histidine kinase; containing PocR ligand-binding domain) demonstrated a similarly observed phenomenon in multiple media switching (with and without YE)

attempts (data not shown). Therefore, it can be proposed that the presence of YE in the growth medium seems to have a trade-off on $\mu_{\max}$ in YE-free media for several reverse engineered strains. A similar trade-off was observed in several ALE isolates. For instance, carbon-limitation adapted yeast isolates exhibited a fitness cost in resource abundant conditions (Wenger et al., 2011). A recent investigation involving *E. coli* isolates from glutarate tolerance ALE demonstrated a reduced growth rate on M9+glucose minimal media (i.e. the base medium prior to starting tolerance ALE) (Lennen et al., 2023), indicating that growth rate of a given ALE isolate in non-stress conditions is not always predictive of the evolved trait. It has been demonstrated that this phenomenon may be attributed to a “fear-greed” trade-off in a resource-limited environments (Anand et al., 2019; Sastry et al., 2019), illustrating the ever-present resource allocation trade-off between growth and stressor tolerance in microbes. Regarding YE-free growth, all three reverse-engineered strains (RE1, RE2 and RE3) demonstrated the capacity to grow in autotrophic batch cultures in the absence of YE, which is required for wild-type strain JA1-1. Previously *C. ljungdahlii* and *C. autoethanogenum* have been adapted to enable growth without YE (Martin et al., 2015; Schulz et al., 2023). These results demonstrate that YE-free growth can be achieved through single-gene targeted modifications. Furthermore, all the reverse-engineered strains grew significantly faster without YE compared to the wild-type strain in the presence of YE. This provides further evidence in support of the benefits of reverse-engineering mutations identified in ALE studies (Dragosits and Mattanovich, 2013; Ingelman et al., 2024).

c) Reverse-engineered strains exhibit enhanced solvent production and performance in autotrophic continuous bioreactor cultures

In addition to a comparison of strains in bottle batch cultures, we tested reverse-engineered strains RE1, RE2, and RE3 in chemostat cultures in order to obtain bioreactor steady-state data on minimal medium (Table 4).

Table 4. Summary of chemostat cultivations with key parameters in this chapter.

Strain	Gas flow rate (mL/min)	Agitation (RPM)	D (day ⁻¹)	Biomass concentration (gDCW/L)	Number of replicates	Feed gas
RE1	45	680	0.96 ± 0.01	1.50 ± 0.04	4	Syngas
RE1	72	810	1.99 ± 0.06	1.58 ± 0.04	4	Syngas
RE3	25	580	0.53 ± 0.01	1.09 ± 0.15	4	Syngas
RE3	45	680	1.00 ± 0.04	1.57 ± 0.05	3	Syngas
RE2	21	570	0.49 ± 0.02	0.97 ± 0.12	4	CO
RE2	45	680	0.96 ± 0.01	1.66 ± 0.07	4	CO

Syngas composition – 50% CO, 20% CO₂, 20% H₂, 10% Ar.; CO gas composition – 60%, 40% Ar. All reported steady-states are biologically independent. D, dilution rate; gDCW, gram of dry cell weight. Data are average ± standard deviation between bioreplicates.

Syngas chemostat experiments

The following strains were cultivated on syngas – RE1 at $D = 1$ and 2 day^{-1} , and RE3 at $D = 0.5$ and 1 day^{-1} (see below for RE2 data on CO). The bioreactor performance of RE3 proved to be less robust than that of RE1 and ALE isolate LAbriini; thus, operating the culture at $D = 2 \text{ day}^{-1}$ was not attempted. As in autotrophic batch bottle data, LAbriini strain data from Chapter 2 (and Publication 2) are incorporated into this chapter for comparative purposes. To ensure a fair comparison between strains, cultures of different strains at the same D were operated at similar gas-liquid mass-transfer conditions (i.e., biomass concentrations) (see Materials and Methods).

When examining by-product production, it is notable that the q_{EtOH} for RE3 at $D = 1 \text{ day}^{-1}$ was observed to be higher than the q_{ace} . This pattern was not observed in either RE1 nor ALE isolate LAbriini (Figure 19A). In general, RE3 demonstrates an elevated $q_{2,3\text{-BDO}}$ ($p\text{-value} = 0.036$) and a reduced q_{ace} ($p\text{-value} = 0.003$) at $D = 1 \text{ day}^{-1}$ in comparison to RE1 (Figure 19A). We hypothesise that the shift from acetate towards ethanol production on syngas growth occurs at lower D in RE3 compared to other *C. autoethagenum* strains (e.g. see Chapter I of Results). At $D = 2 \text{ day}^{-1}$, the strain RE1 exhibited considerably reduced solvent production rates in comparison to ALE isolate LAbriini ($p\text{-values}$ of 0.006 and 0.002 for q_{EtOH} and $q_{2,3\text{-BDO}}$, respectively). This finding is in accordance with the observed differences in metabolite concentrations between RE1 and LAbriini (Supplementary Figure 2 in Publication 3). Interestingly, the increase in q_{ace} was not proportional to the increase of D for both reverse-engineered strains studied at syngas conditions (RE1 and RE3). Furthermore, a significant positive correlation between q_{EtOH} and $q_{2,3\text{-BDO}}$ was observed in all examined syngas-fermenting culture steady-states (Figure 19B). The elevated 2,3-BDO production in RE3 is further supported by its $D = 1 \text{ day}^{-1}$ datapoint clearly deviating from the general trend of increasing $q_{2,3\text{-BDO}}$ with increasing D while displaying lower q_{EtOH} values than RE1 and LAbriini at same D (Figure 19B). This implies that compared to $D = 0.5 \text{ day}^{-1}$ and other strains at $D = 1 \text{ day}^{-1}$, there is a potential carbon shift from ethanol to 2,3-BDO production in RE3 strain at $D = 1 \text{ day}^{-1}$.

With regard to the gas consumption and production, the performance of RE1 was very similar to LAbriini at both $D = 1$ and 2 day^{-1} (Figure 19C). As anticipated for CO-limited chemostats, the q_{CO} exhibited a nearly proportional increase with D (Figure 19C). However, the increase of the q_{H_2} displayed a non-proportional increase with D , as observed previously (Publication 1). At the same time, the correlation between the total specific electron consumption rate (sum of H_2 and CO) and the acetate/ethanol ratio (Supplementary Figure 3 in Publication 3) is consistent with the findings of the aforementioned study: cultures growing at higher D demonstrate a reduced carbon allocation towards acetate. For RE3, genome-scale metabolic flux simulations using FBA showed that all the H_2 was used for the reduction of CO_2 to formate in the WLP (Supplementary Figure 4; Supplementary Tables 3 and 4 in Publication 3). Notably, we observed a positive correlation (R^2 value 0.77) between acetate/ethanol (C-mol) and $q_{\text{H}_2}/q_{\text{CO}}$ ratios (Figure 19D), while a strong negative correlation was evident between $q_{2,3\text{-BDO}}$ and $q_{\text{H}_2}/q_{\text{CO}}$ (Figure 19E) (R^2 value 0.88). This suggests that the production of

reduced products (i.e. ethanol and 2,3-butanediol) is lower when a higher supply of carbon-free redox (i.e. hydrogen) is available, at least when comparing syngas with CO. It is notable that this result is inconsistent with previously published data for *C. autoethanogenum* grown on syngas (50% CO, 20% CO₂ and 20% H₂) and CO+H₂ (15% CO, 45% H₂) (Valgepea et al., 2018), wherein the researchers demonstrated that providing additional carbon-free redox (as H₂) directs more carbon into reduced metabolites, including ethanol. Regarding specific electron consumption rates within this dataset, a strong correlation (R^2 value of 0.97) was identified with q_{EtOH} (Figure 19F), which is in accordance with previous findings (Allaart et al., 2023). It is notable that the flux simulations indicated a reduction in both maintenance energy and the proportion of the latter from total ATP production in RE3 (Supplementary Figure 4; Supplementary Tables 3 and 4 in Publication 3).

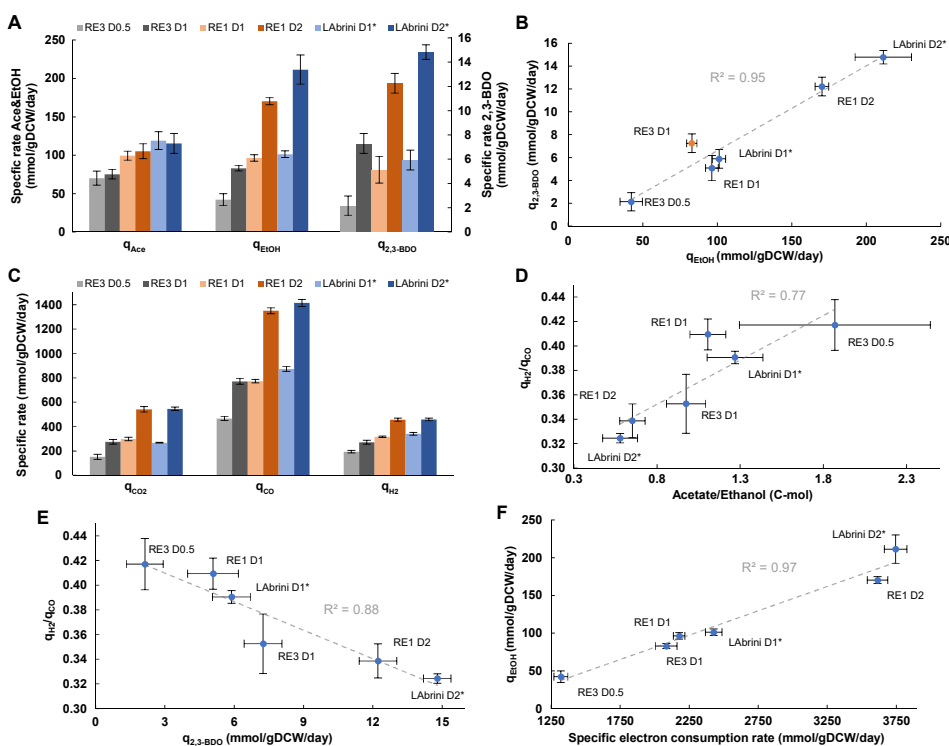


Figure 19. Performance of RE3, RE1, and LAbriini in syngas-fermenting chemostat cultures. **(A)** Specific by-product production rates. **(B)** Correlation between specific 2,3-BDO production rate and specific ethanol production rate across the whole dataset. RE3 D = 1 day⁻¹ is denoted with orange colour. **(C)** Specific gas uptake and production rates. **(D)** Correlation between $q_{\text{H}_2}/q_{\text{CO}}$ and acetate/ethanol (C-mol) ratios. **(E)** Correlation between $q_{\text{H}_2}/q_{\text{CO}}$ ratio and specific 2,3-BDO production rate. **(F)** Correlation between q_{EtOH} and specific electron consumption rate ((q_{H_2} and q_{CO} summed) $\times 2$). The number following D (dilution rate) denotes D value in day⁻¹. Bars show average $\pm$ standard deviation between bioreplicates (see Table 4 and Publication 3 Methods for additional details). Asterisk behind name denotes previously shown data from Publication 2. EtOH, ethanol; 2,3-BDO, 2,3-butanediol; Ace, acetate; q, specific production rate, except specific uptake for CO and H₂.

Carbon balance analysis indicates that RE1 exhibits a high degree of similarity to LAbriini, while displaying a slightly reduced ethanol production and increased carbon flow into CO₂ (Figure 20). At D = 1 day⁻¹, the values are almost identical, while RE1 showed slightly lower carbon flow into acetate and more into CO₂; at D = 2 day⁻¹, RE3 produces slightly more CO₂ and less ethanol. A comparison of RE3 with RE1 and LAbriini at D = 1 day⁻¹ reveals that RE3 also displays a lower acetate share. However, a greater proportion of carbon is allocated to CO₂ and 2,3-BDO in comparison to LAbriini and RE1. Taken together, both reverse-engineered strains nearly recover the 2,3-BDO and ethanol carbon shares of LAbriini, at D = 1 day⁻¹. The effect of increasing D on the carbon balance is comparable across the three strains. For instance, increasing D diverted more carbon into solvents and CO₂, which aligns with the observation that the acetate/ethanol ratio decreases (Supplementary Figure 2 in Publication 3) at higher D.

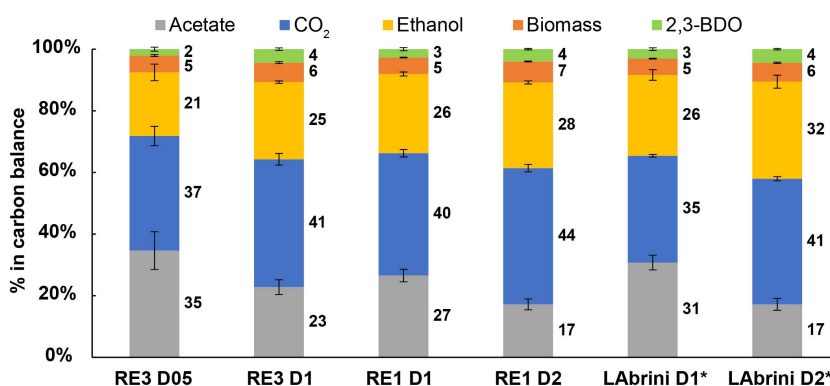


Figure 20. Carbon balances of RE3, RE1 and LAbriini syngas-fermenting chemostat cultures. The number following D (dilution rate) denotes D value in day⁻¹. Carbon recoveries were normalised to 100% to have a fair comparison of carbon distributions between strains and conditions. Bars show average $\pm$ standard deviation between bio-replicates (see **Table 4** and Publication 3 Methods for additional details). Asterisk behind name denotes previously shown data from Publication 2. 2,3-BDO, 2,3-butanediol.

CO chemostat experiments

In addition to the syngas continuous culture experiments presented in the preceding pages, the reverse-engineered strain RE2 was cultivated on CO at D = 0.5 and D = 1 day⁻¹ to test its performance. For this strain, CO was selected as the feed gas since the mutation from which RE2 gene modification originated was evolved on CO (UT1). The respective LAbriini data from D = 1 day⁻¹ acquired by my colleagues are incorporated into this chapter for comparative purposes. See Table 4 for exact gas-liquid mass-transfer conditions and other experimental details.

Given the considerable challenges encountered in cultivating UT strains to high biomass concentrations on CO (see ALE chapter of the thesis), expectations regarding the performance of RE2 were understandably limited. However, we

were unexpectedly able to efficiently operate the culture at $D = 0.5 \text{ day}^{-1}$ and furthermore, achieved steady-state at high gas uptake rates and biomass concentrations at $D = 1 \text{ day}^{-1}$ (double of the UT and UQ strains; Figure 21). The robustness of RE2 in continuous cultures was unexpected, given that only a single SNP was introduced into the wild-type JA1-1 background. Despite the considerable disparity in bioreactor operation between UT1 and RE2 cultures, the two strains exhibited comparable growth characteristics (e.g. biomass concentration, specific by-product production rates, specific gas rates) at $D = 0.5 \text{ day}^{-1}$. With one exception – for 2,3-BDO production RE2 strain demonstrated significantly higher values, for example $q_{2,3\text{BDO}}$ rate was 4.7-fold higher than UT1 at $D = 0.5 \text{ day}^{-1}$ (Figure 21B). When looking at $D = 1 \text{ day}^{-1}$ data on CO, it becomes evident that a higher D resulted in comparable effects for RE2 and LAbriini. The metabolite values and specific production rates were found to be largely identical between the two strains, with the exception of 2,3-BDO production and q_{CO} . The results of the carbon balance analysis are strikingly similar, indicating that RE2 recovers the carbon balance of LAbriini on CO-chemostats at $D = 1 \text{ day}^{-1}$ (Figure 21D).

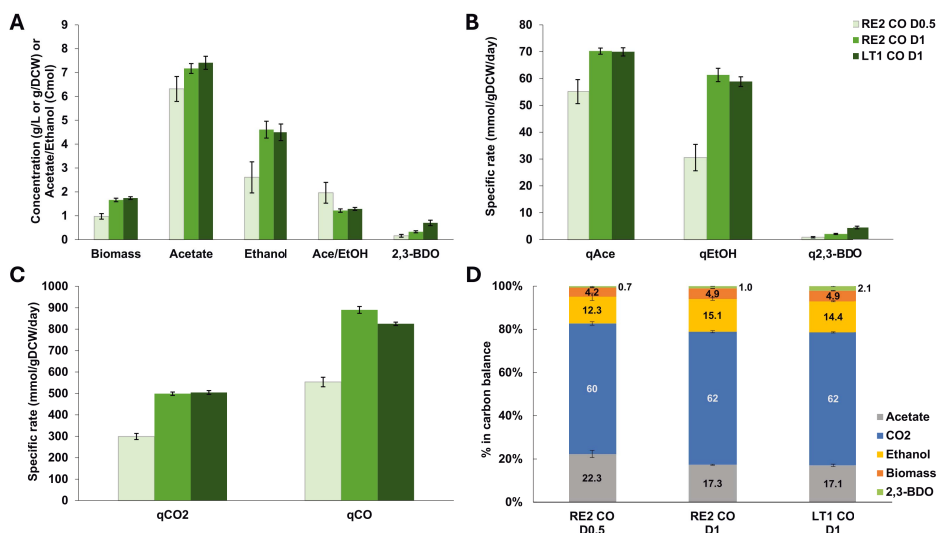


Figure 21. Performance of RE2 and LAbriini (LT1) in CO-fermenting chemostat cultures. (A) Biomass and byproduct concentrations. (B) Specific by-product production rates. (C) Specific gas uptake and production rates. (D) Carbon balances. Bars show average $\pm$ standard deviation between bioreplicates (see **Table 4** and Publication 3 Methods for additional details). The number following D (dilution rate) denotes D value in day^{-1} . gDCW, gram of dry cell weight; Ace, acetate; EtOH, ethanol; 2,3-BDO, 2,3-butanediol; q, specific production rate, except specific uptake for CO.

The performance of reverse-engineered strains in batch and continuous cultures demonstrate that genes under higher selection pressure in ALE may represent promising targets for targeted genetic engineering (Dragosits and Mattanovich, 2013; Wu et al., 2022). Importantly, the reverse-engineered strains display enhanced robustness compared to the majority of isolates from ALE that harboured multiple mutations (Chapter II of the thesis Results). For example, we previously encountered recurring challenges in achieving stability of the bioreactor continuous cultures for the majority of ALE isolates. Conversely, as an example, achieving a steady state for RE1 was a relatively straightforward even at $D = 2 \text{ day}^{-1}$. These observations may indicate that we have avoided the epistatic effects commonly seen in ALE-derived strains (Carpenter et al., 2023; Chou et al., 2011; Khan et al., 2011) by reverse-engineering the mutations identified in ALE into strains RE1, RE2, and RE3. Consequently, the superior characteristics identified in the ALE study isolates – namely, faster growth, growth without complex nutrients, and robustness in continuous culture operation – were also recovered.

d) Similarities in proteome responses of reverse-engineered strains

As part of a reverse-engineered strain characterisation study, we investigated proteome expression patterns for the three reverse-engineered strains as functional genomics has been informative for elucidating genotype-phenotype links in engineered strains (Wang et al., 2021). Furthermore, it has been shown that mutations in sporulation-related genes have led to significant effects on Clostridial phenotypes other than sporulation (Diallo et al., 2021).

In the first subset (from Publication 2), RE1, RE2 and wild-type (JA1-1) were cultivated in syngas bottle cultures with YE. Rationale for adding YE was since JA1-1 does not grow without YE in these conditions. Furthermore, it is notable that these proteome expression patterns are most likely influenced by variable μ_{max} values (Figure 22) and potential variations in the consumption of YE components between the three strains. Our analysis identified a total of 284 differentially expressed proteins (DEP; fold-change > 2 and q-value < 0.05) between RE1 and JA1-1, and 514 between RE2 and JA1-1 (Supplementary Table 5 in Publication 2). The proteomic responses in RE1 and RE2 were similar in general (Figure 22B; Supplementary Figure 17 in Publication 2), with 205 DEP-s shared between the two. This similarity may be attributed to the 66-fold down-regulation of Spo0A in RE2 (Figure 22C; Supplementary Table 5 in Publication 2). Notably, peptides for five sporulation-related proteins that were shown to be highly expressed in JA1-1 (CLAU_1942, 1946, 3117, 3233, 3248) were not identified in either of the engineered strains. Similarly, the absence of Spo0A peptides in RE1 indicates the successful knockout in RE1. Concurrently, three histidine kinases and two phosphatases with potential links to Spo0A regulation were up-regulated in both RE1 and RE2 (Figure 22C). Of particular interest is CLAU_3194, since this gene was mutated in all UT isolates (mutation #3 in Figure 17B). The phosphate metabolism was additionally influenced by 12 differentially expressed kinases not shared between the two strains (Supplementary Table 5 in

Publication 2). Furthermore, we detected that CLAU_3080 (gene partially deleted in UQ1, see Chapter II.e for more details) was up-regulated 3-fold in the RE2 strain (Supplementary Table 5 in Publication 2). Both reverse-engineered mutations in strains RE1 and RE2 triggered widespread proteomic responses, demonstrating their role on cell metabolism well beyond the sporulation process. However, we note that the exact regulatory mechanisms behind the proteome changes that resulted in enhanced phenotypes for RE1 and RE2 remain to be elucidated.

With regard to the functionalities specifically relevant for acetogens, it was found that the electron-bifurcating hydrogenase HytA-E complex was down-regulated. This may have been compensated for by the induction of hydrogenase CLAU_1535 and its associated proteins in both strains (Figure 22C). Furthermore, a formate dehydrogenase (CLAU_2907) and the methylene-THF dehydrogenase (CLAU_1574) within the WLP displayed a reduction in expression levels in both strains. Additionally, the redox metabolism was significantly impacted in both RE1 and RE2, as evidenced by the differential expression of oxidoreductases (proteins involved in ethanol metabolism and electron transfer) in comparison to JA1-1 (Figure 22C). Lastly, we hypothesise that the necessary transcriptional changes needed enhance the phenotypes of RE1 and RE2 could have been achieved through the up-regulation of seven transcriptional regulators by a factor of 2 to 10 and/or the down-regulation of two transcriptional regulators by a factor of 2 to 6 (Supplementary Table 5 in Publication 2).

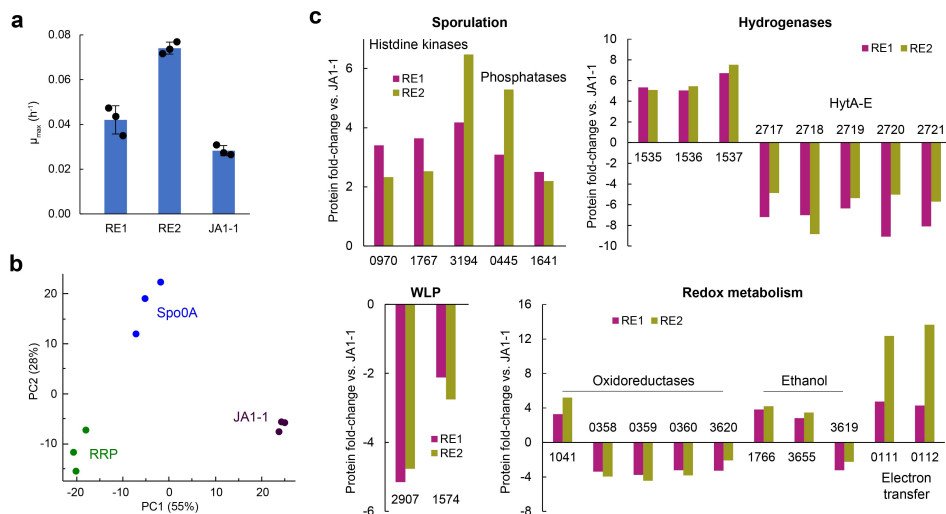


Figure 22. Proteomic responses of the wild-type *C. autoethanogenum* starting strain of ALE (JA1-1) to the reversed engineered mutations in syngas bottle cultures. **(a)** Maximum specific growth rate (μ_{max}) for reverse-engineered strains RE1, RE2, and JA1-1 (data from Figure 9C) with YE in medium. Bars show average $\pm$ standard deviation between three bioreplicates. **(b)** Principal component analysis of protein MS intensities for the three bioreplicate cultures of RE1, RE2, and JA1-1. PC, principal component. **(c)** Potential key proteins differentially expressed (fold-change > 2 and q -value < 0.05) for both RE1 vs JA1-1 and RE2 vs JA1-1 comparisons. Protein IDs are preceded with “CLAU”. See Supplementary Table 5 in Publication 2 for data.

In the second proteome expression study, a comparative analysis was conducted of chemostat-grown strains cultivated on syngas at $D = 1 \text{ day}^{-1}$. The strains RE1 and RE3 were compared to the ALE-derived strain LABrini. A total of 107 DEP-s (fold-change > 1.5 and $q < 0.05$) were identified between RE1 and LABrini, 46 between RE3 and LABrini, and 24 shared DEPs between RE1 and RE3 (Supplementary Table 5 in Publication 3). The number of DEPs was considerably higher in comparison to the batch dataset, indicating a higher degree of similarity between the RE strains and LABrini. In comparison to LABrini, CLAU_0471 (LABRINI_02360) and carbamate kinase (10405) were not identified in RE3, whereas a transcriptional regulator (05890) was not detected in RE1. Conversely, the expression of a transcriptional regulator (00325) and alcohol dehydrogenase (Adh) (05230) was observed in RE3, but not in LABrini.

In contrast to the numerous sporulation-related proteins that were found to be differentially expressed in RE1 and RE2 in comparison to JA1-1 in batch proteomics comparisons (see previous page), the expression of sporulation-related proteins was not different between RE1 and RE3 in comparison to LABrini in chemostats (Supplementary Table 5 in Publication 3). Interestingly, the histidine kinase CLAU_3194 (16380), which may be involved in sporulation networks (see Chapter II.e) displayed a 2-fold increase in expression in RE3 relative to LABrini (Supplementary Table 5 in Publication 3). However, it was not identified as a DEP in RE1. This protein was identified as having a shared mutation among all three sequenced UT isolates (see Chapter II.e for more details), and it is noteworthy that CLAU_3194 was identified as a DEP in both RE1 and RE2 in batch proteomics dataset (see previous page). A comparison of the proteome expression of RE3 and JA1-1 autotrophic batch cultures also identified CLAU_3194 as a DEP, among other numerous proposed histidine kinases (Supplementary Table 6 in Publication 3).

For both RE1 and RE3 chemostat data, we observed a 4-fold upregulation of the alcohol dehydrogenase (Adh4; 09125) with highest protein expression in *C. autoethanogenum* (Valgepea et al., 2022). Additionally, a 3-fold increase in another Adh (18005) was noted in RE3 (Table 5). Within the carbon-fixing WLP pathway, a 2-fold increase in the expression of formate-tetrahydrofolate ligase (Fhs; 08015) and cyclodeaminase/cyclohydrolase family protein (08010) was observed in RE3, while a formate dehydrogenase (13975) exhibited a 2-fold increase in RE1 (Table 5, Supplementary Table 5 in Publication 3). Concurrently, a formate dehydrogenase (00410), its preceding 4Fe-4S cluster protein (00405), and a carbon monoxide dehydrogenase (08025) exhibited a 2-fold reduction in expression in RE3. Furthermore, a number of oxidoreductases were observed to be differentially expressed in both strains (e.g. 04430, 00385, 05235; Supplementary Table 5 in Publication 3). However, only one exhibited a similar trend, namely the proposed FAD-dependent oxidoreductase (15005) displayed 6- or 7.5-fold down-regulation for RE1 or RE3, respectively. Lastly, in RE1, we observed a significant increase in the expression of gluconeogenesis enzymes (16255 and 16260; 10-fold), as well as a notable induction in a sigma54-dependent transcriptional regulator (05770, exhibiting a 4-fold increase). Furthermore, there was

a 4-fold decrease in the expression of aldehyde ferredoxin oxidoreductase AOR1 (00445) and a 3-fold decrease in the expression of carbon starvation protein A (07885) in RE1. The latter observations may indicate either changes in sensing carbon limitation or in sporulation processes (Kevorkian et al., 2016) in RE1. However, it should be noted that we failed to detect sporulation in JA1-1 and RE1, RE2, RE3 strains (see next subchapter).

Table 5. Notable differentially expressed proteins in RE1 and RE3 strains compared to LAbriini in syngas-fermenting chemostat cultures.

Protein ID	Description of protein product	RE1 vs. LAbriini			RE3 vs. LAbriini		
		q-value	Log ₂ FC	FC	q-value	Log ₂ FC	FC
<i>Ethanol production</i>							
LABRINI_18005	Iron-containing alcohol dehydrogenase				0.02	1.6	2.9
LABRINI_09125	Iron-containing alcohol dehydrogenase	0.01	1.9	3.6	0.02	2.0	4.0
LABRINI_00445	Aldehyde ferredoxin oxidoreductase	0.02	−1.1	2.1			
<i>CI-fixing WLP pathway</i>							
LABRINI_00405	4Fe-4S dicluster domain-containing protein	0.02	−0.7	1.6	0.04	−0.8	1.7
LABRINI_00410	Formate dehydrogenase subunit alpha				0.03	−0.8	1.8
LABRINI_08010	Cyclodeaminase/cyclohydr olase				0.01	0.9	1.8
LABRINI_08015	Formate–tetrahydrofolate ligase				0.03	0.9	1.9
LABRINI_08025	Carbon-monoxide dehydrogenase catalytic subunit				0.04	−0.7	1.6
LABRINI_13975	Formate dehydrogenase subunit alpha	0.01	1.1	2.1	0.05	1.1	2.2

ID, identifier; FC, fold-change. Positive FC means up-regulation, negative down-regulation. See Supplementary Table 5 in Publication 3 for all differentially expressed proteins.

e) Sporulation tests on reverse-engineered strains

As several mutations discovered in ALE isolates and reverse engineering targets were annotated as sporulation related, we reasoned to test whether the engineered mutations could have affected the sporulation ability of the reverse-engineered

strains. We cultured strains RE1, RE2, RE3, and JA1-1 both autotrophically and heterotrophically and used microscopy to detect potential differences in sporulation from stationary phase samples. In addition, we plated heterotrophic cultures of RE1, RE2 and JA1-1 before and after a heat-shock to observe potential heat-resistant spores. It was unexpectedly observed that neither microscopy (Figure 23) nor plating (Supplementary Figure 16 in Publication 2) identified the presence of any spores or cells undergoing sporulation for any of the strains (including JA1-1). While Abrini et al., who have isolated *C. autoethanogenum* JA1-1, report it as a spore-former (Abrini et al., 1994), a recent study has indicated that JA1-1 does not form spores (Piatek et al., 2022). The latter and our observations are corroborated by a review of Clostridial sporulation, which concludes that sporulation is rarely observed in acetogens (Diallo et al., 2021).

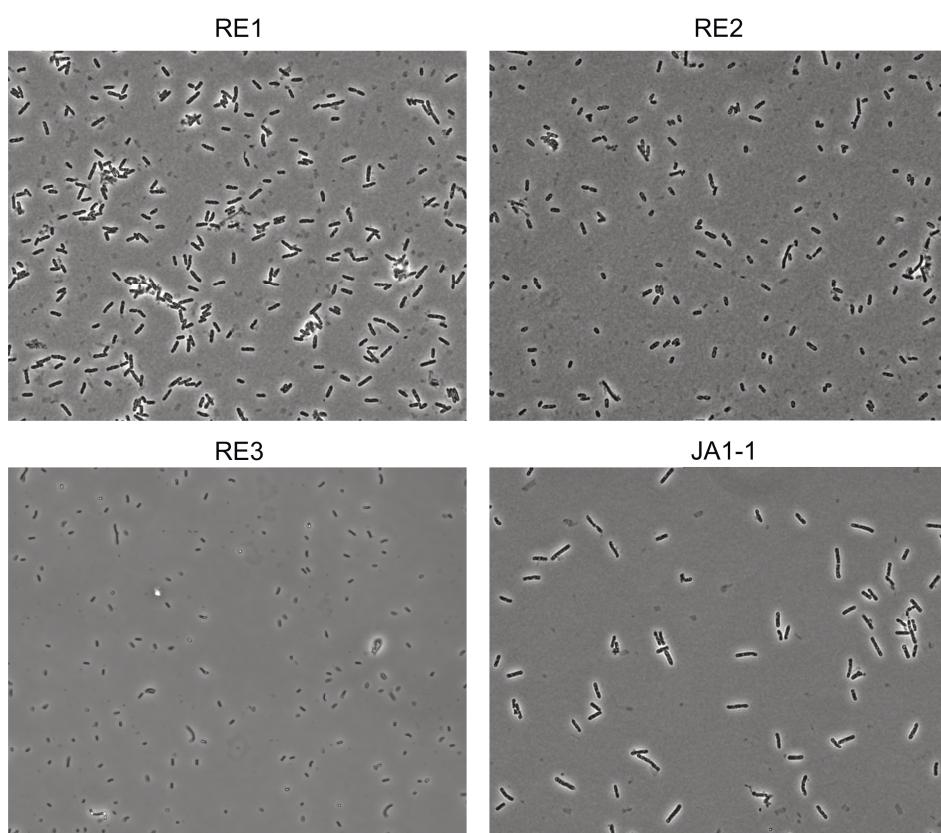


Figure 23. Micrographs showing lack of spores or sporulating cells in autotrophic cultures of JA1-1, RE1, RE2 and RE3 after lag phase. Micrographs taken regularly during the incubation period and from YTF cultures showed same results. Spores or endospores are expected to be white and we note that white specs on RE3 micrograph are technical artefacts from microscope (same white cells appear transparent in next micrographs).

f) Reverse-engineered genes may be involved in overlapping regulatory networks

The RE3 strain carrying CLAU_0471 deletion demonstrated significant phenotypes, such as reduction in $\mu_{\max}$ in YE-media compared to its absence and a high 2,3-BDO production in both bottle batch and continuous culture experiments. As noted above, it is unclear why an amino acid permease (as CLAU_0471 is annotated by gene sequence homology) would undergo multiple mutations, including extensive deletions, across multiple distinct ALE strategies during autotrophic growth on minimal media (see Results & Discussion in Chapter II.e for more details). Nevertheless, alternative functions for CLAU_0471 emerge from bioinformatic searches employing methodologies other than gene sequence homology. To illustrate, DeepFRI (Gligorićević et al., 2021) prediction based on protein structures expands the CLAU_0471 function to a transmembrane (ion) transporter. Regardless of the actual function of the predicted membrane protein, its abundance is observed to be very low according to previously published data (Valgepea et al., 2022), ~20–35x lower than for CODH. Thus, it seems implausible that evolutionary pressures during ALE selected against this protein to conserve energy or membrane surface area due to a lack of its synthesis. According to CDART (Geer et al., 2002), the PotE domain present in CLAU_0471 is also found in proteins with proposed histidine kinase and signal transduction histidine kinase functions in a number of other taxa, including some *Clostridium* species. This is notable as strains RE1 and RE2 were engineered with single mutations in putative sporulation-related genes, which resulted in the restoration of the superior phenotypes observed in many ALE isolates.

The transcription start site data of *C. autoethanogenum* (de Souza Pinto Lemburger et al., 2019) indicates that CLAU_0471 does not belong to an operon. In accordance with the STRING protein-protein interactions database (Szklarczyk et al., 2015), genes up- and down-stream of CLAU_0471 appear to possess a range of functions, which are either related to metabolism or cell signalling. For instance, two translated proteins situated downstream on the same strand are associated with energy and pyrimidine metabolism. The first is CLAU_0470, a SPFH domain-containing protein within local clusters labelled “ATP synthesis” and “Oxidative phosphorylation”. The second is CLAU_0469, a proposed sugar kinase within the local cluster labelled “Pyrimidine metabolism”. The gene upstream on the same strand (CLAU_0473), which encodes a proposed methyl-accepting chemotaxis protein, is associated with biological processes related to “Signal transduction” and “Chemotaxis” according to STRING database.

The two other reverse-engineered strains – RE2 and RE1, have genetic modifications in CLAU_1957 and Spo0A, respectively. According to STRING, both genes are predicted to interact with over ten other functional partners. Spo0A is associated with sporulation, while CLAU_1957 is implicated in the “Two-component regulatory system” and the “Phosphorelay sensor kinase activity” local network clusters. As noted in the proteome analysis subsection, we saw differing expression of various genes putatively related to sporulation processes in RE1 and RE2 strains. Additionally, changes in expression of genes in *Clostridium* sporulation networks have been shown to regulate gene expression

A comparison of the CLAU_1957 and Spo0A sequences against a reported list of conserved sporulation genes in all spore-forming bacilli and clostridia (Galperin et al., 2012) revealed that both proteins share significant similarity (CLAU_1957 – 31.7% identity with 49% query coverage; E value $3e^{-15}$; Spo0A – 57.6% identity with 98% query coverage; E value e^{-108}) with the *B. subtilis* Spo0A protein (NCBI reference number NP_390302.1). Intriguingly, comparison of the predicted structures of CLAU_1957 (Figure 25C) and Spo0A (Figure 25D) (Jumper et al., 2021) with the only fully experimentally determined structure of Spo0A – from *Geobacillus stearothermophilus* (Lewis et al., 2000), revealed that the N-terminal subdomain of all three proteins is highly conserved (Figure 25A). It is noteworthy that the introduced mutation in RE2 (Gly to Asp change in 94th position) is situated within the same subdomain, the phosphorelay signal receiver domain (Diallo et al., 2021). Furthermore, the structural comparison demonstrated that the C-terminal subdomain (the DNA-binding domain; (Brown et al., 1994)) of the proposed *C. autoethanogenum* Spo0A displays a high degree of similarity with the experimentally determined structures from *B. subtilis* and *G. stearothermophilus* (Figure 25B). Moreover, the N-terminal subdomain of CLAU_1957 protein is found to be conserved when compared to the closest analogue with a determined structure – “Sensory transduction protein regX3” from *Mycobacterium tuberculosis* (data not shown). The combined evidence indicates that both CLAU_1957 and Spo0A may have been relatively well-conserved components of sporulation systems in a common ancestor. To conclude, the results of this analysis indicate that the protein products of several mutated genes in the ALE experiments and mutations in strains RE1, RE2 and RE3 examined in this chapter may be part of overlapping regulatory networks or have carried signaling protein functions in evolutionary ancestors. In order to further test these hypotheses, additional studies employing a combination of multiple gene edits, including disruption and partial deletions, are required.

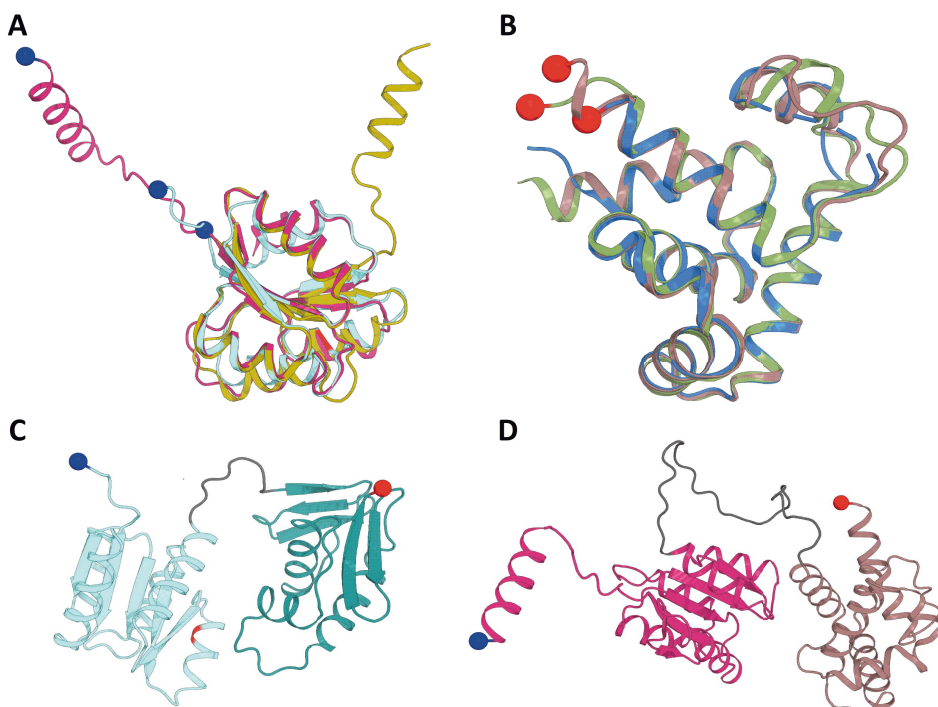


Figure 25. Comparison of AlphaFold 2 predicted domains of CLAU_1957 and Spo0A with their closest structural homologues. **(A)** Superposition of the N-terminal domains of *C. autoethanogenum* Spo0A AlphaFold 2 model (Uniprot no. U5RXI2; shown residues 1–144; pink), CLAU_1957 AlphaFold 2 model (Uniprot no. U5RXC5; shown residues 1–123; blue) and *Geobacillus stearothermophilus* Spo0A structure determined by X-ray diffraction (PDB no. 1DZ3, (Lewis et al., 2000a); shown residues 1–124; yellow). Superposition of U5RXC5 to U5RXI2 resulted in an RMSD of 0.84 Å for 92 Ca aligned; superposition of 1DZ3 to U5RXI2 resulted in an RMSD of 0.84 Å for 86 Ca aligned. **(B)** Superposition of C-terminal domains of *C. autoethanogenum* Spo0A AlphaFold 2 model (Uniprot no. U5RXI2; shown residues 174–291; brown) with *B. subtilis* Spo0A (PDB no. 1LQ1, (Zhao et al., 2002); shown residues 150–264; blue) and *Geobacillus stearothermophilus* Spo0A (PDB no. 1FC3, Lewis et al., 2002; shown residues 140–258; green) structures determined by X-ray diffraction. Superposition of 1LQ1 or 1FC3 on U5RXI2 resulted respectively in an RMSD of 0.38 Å for 92 Ca aligned or an RMSD of 0.38 Å for 94 Ca aligned. **(C)** AlphaFold 2 proposed model of *C. autoethanogenum* CLAU_1957 (Uniprot no. U5RXC5) with mutation in RE2 strain in phosphorelay signal receiver domain highlighted in red (in 94th amino acid, glycine exchanged for an asparagine). The N-terminal half is shown in cyan, the linker in grey and the C-terminal half in dark teal. **(D)** AlphaFold 2 proposed model of *C. autoethanogenum* Spo0A (Uniprot no. U5RXI2) with the N-terminal half colored in pink, the linker shown in grey and the C-terminal half in brown. The N and C termini are highlighted as blue and red spheres, respectively. Sequence alignment was performed using PyMOL version 2.2.0 (Schrödinger, LLC).

CONCLUSIONS

Chapter I provides important quantitative data and a comprehensive systems-level analysis of the impact of the key physiological parameter, μ , on acetogen metabolism and the gas fermentation bioprocess during steady-state cultures. Our results show that the bioprocess benefits from faster growth of *C. autoethanogenum*, which in turn leads to an increase in both product yield and productivity for industrially relevant compounds. Moreover, this research contributes to the advancement of understanding of transcriptional regulation in acetogens, and supports the concept that cells maintain sufficient “baseline” expression of key catabolic pathways for increasing flux throughput.

The comprehensive ALE approaches, outlined in Chapter II, demonstrate that ALE can be employed to obtain *C. autoethanogenum* strains that grow fast without complex nutrients and are robust in continuous gas fermentation cultures (e.g. display low sensitivity to gas ramping and are able to achieve high biomass and dilution rate conditions). Consequently, we conclude that these strain characteristics can be leveraged to accelerate the phenotyping and metabolic engineering of acetogen cell factories. Furthermore, Chapter II offers insights into convergent evolutionary pathways in acetogens. It concludes that convergent evolution across disparate ALE strategies has resulted in analogous phenotypes, albeit through disparate genetic pathways. Additionally, it identifies novel metabolic engineering targets for potentially enhanced phenotypes.

In Chapter III, I have covered the study that validated the effects of mutations in three genes observed in the previous ALE study by constructing and characterising the reverse-engineered strains RE1, RE2, and RE3. The various single-gene edits introduced were able to reproduce the superior phenotypes of ALE isolates in both bottle batch and bioreactor continuous cultures. Further studies are required to investigate the potential cumulative or epistatic effects resulting from the combination of the tested gene targets and the other mutations identified in ALE isolates covered in this thesis. This should involve reconstructing mutations of interest, both in combination and in isolation, and assaying their contribution to observed phenotypes.

Based on the results of three studies in this thesis, the following conclusions can be made:

- The gas fermentation bioprocess benefits from faster growth of *C. autoethanogenum*, which in turn leads to an increase in product yields and productivities for industrially relevant compounds
- ALE can be used to acquire *C. autoethanogenum* strains with significantly improved phenotypes which can be leveraged to accelerate the phenotyping and metabolic engineering of acetogen cell factories
- Studying ALE-inspired reverse-engineered acetogen strains using gas fermentation and -omics methods advanced the understanding of genotype-phenotype relationships in gas fermenting acetogen *C. autoethanogenum*

In conclusion, the global pandemic effectively acted as a driving force for the rapid and extensive adaptation of my study plans and actions, resulting in a significantly altered thesis and a markedly different journey than what was originally planned. This illustrates that as evolving cells in a population, adaptation is similarly crucial for graduate students. This thesis exemplifies that ALE is a powerful tool that, when used correctly, can facilitate the acquisition of significant amount of novel information. ALE helped to identify novel gene candidates for the engineering of microbial cell factories and advanced the understanding of genotype-phenotype relationships in the gas-fermenting acetogen *C. autoethanogenum*.

SUMMARY IN ESTONIAN

Clostridium autoethanogenum-i metabolismi süsteemibioloogiline kirjeldamine ja parendamine

Atsetogeensete bakterite uurimine on viimastel aastatel pälvinud kasvavat tähelepanu nende võime tõttu muundada kasvuhoonegaase ning vesinikku väärtuslikeks produktideks, sealhulgas etanooliks, atsetaadiks ja 2,3-butaandiooliks läbi gaasfermentatsiooni. Tehnoloogia võib tulevikus mängida olulist rolli kliimamuutuste leevendamisel ja jäätmekäitluse probleemide lahendamisel, kuid tehnoloogia globaalsel rakendusel seisavad ees mitmed väljakutsed. Esiteks on arusaam atsetogeenide metabolismist piiratud – atsetogeenide metabolismi mitmed nüansid, sh regulatiivsed mehhanismid ning genotüübi ja fenotüübi suhted on suures osas veel lõpuni mõistmata. Teiseks on atsetogeenide geneetilise manipuleerimise valdkond võrreldes mudelorganismidega veel üsnagi algusjärgus, seda peamiselt atsetogeenide range anaeroobsuse vajaduse ja aeglase kasvu tõttu. Kolmandaks on suureks väljakutseks sihipärasele ainevahetuse inseneerimisele asjaolu, et enamike atsetogeenide geenide funktsioonid on teadmata või ebaselged.

Käesolev doktoritöö keskendus nende väljakutsete lahendamisele, ühendades gaasfermentatsiooni süsteemibioloogia, adaptiivse laboratoorse evolutsiooni (ALE) ja geneetilise inseneerimisega. Doktoritöö eesmärk oli süvitsi uurida atsetogeenide kasvuerikiirusest sõltuvat metabolismi ning kasutada ALE-i, et luua *C. autoethanogenum* tüvesid, mis kasvaksid oluliselt kiiremini minimaalsöötmel ning oleksid paremini kohastunud läbivoolu kultiveerimiseks bioreaktories. Lisaks oli eesmärgiks parendada genotüüp-fenotüüp seoste mõistmist atsetogeenides, kasutades multidistsiplinaarset lähenemist, mis integreerib ALE ning erinevad süsteemi- ja sünteetilise bioloogia lähenemised.

Doktoritöö esimene peatükk sisaldab kvantitatiivseid andmeid kasvuerikiiruse mõjust atsetogeenide metabolismile ja gaasfermentatsiooni protsessile. Tulemused näitavad, et *C. autoethanogenumi* kiirendatud kasv suurendab tööstuslikult oluliste ühendite saagikust ja tootlikkust. Lisaks edendab see uurimus arusaamist atsetogeenide transkriptsioonilisest regulatsioonist.

Töö teises peatükis kirjeldatud ALE-lähenemised näitavad, et ALE abil on võimalik saada *C. autoethanogenumi* tüvesid, mis omavad oluliselt parenenud kasvuerikiirust, suudavad kasvada minimaalsöötmel ning näitavad head võimekust autotroofsetes gaasifermentatsiooni läbivoolukultuurides. Tänu ALE katsetest eraldatud isolaatide DNA analüüsile tuvastati läbiviidud ALE katsetes konvergentse evolutsiooni olemasolu ning mitmeid sihtmärke paremate omadustega atsetogeensete tüvede loomiseks läbi geneetilise inseneerimise.

Kolmandas peatükis uuriti kolme ALE-i tüvedes esinenud mutatsiooni mõju, konstrueerides metsiktüvel põhinevad tüved RE1, RE2 ja RE3. Eelmainitud tüvesid uuriti põhjalikult autotroofsetes pudeli- ning läbivoolukultuurides ning näidati, et ALE-st inspireeritud geneetilist inseneerimist kasutades on võimalik

saada oluliselt parendatud omadustega gaasfermenteerivad *C. autoethanogenum* tüved. Edasised uuringud peaksid keskenduma mutatsioonide võimalike kumulatiivsete või epistaatiliste mõjude uurimisele.

Kokkuvõttes illustreerib see töö, et ALE on võimas tööriist, mida saab edukalt kombineerida süsteemi- ja sünteetilise bioloogia meetoditega, et mõista paremini atsetogeeni *C. autoethanogenum* metabolismi. Töö tulemusena saadi uusi teadmisi atsetogeenide kasvuerikiirusest sõltuva metabolismi, genotüüp-fenotüüp seoste ning gaasfermentatsiooni valdkonna erinevate kultiveerimismeetodite kohta. Selle töö tulemused võiksid aidata kaasa efektiivsemate, tööstuslikult relevantsete atsetogeenitüvede väljatöötamisele.

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PUBLICATIONS

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Kaldalu, N., Jöers, A., Ingelman, H., Tenson, T., 2016. A General Method for Measuring Persister Levels in *Escherichia coli* Cultures. *Methods in Molecular Biology*, 1333, 29–42. DOI: 10.1007/978-1-4939-2854-5_3.
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