Enhanced Medium-Chain Fatty Acid Production in *Megasphaera elsdenii* via Adaptive Laboratory Evolution and Genomic Insights

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Abbreviations

VFA - Volatile Fatty Acids

SCFA - Short-Chain Fatty Acids

MCFA - Medium-Chain Fatty Acids

ALE - Adaptive Laboratory Evolution

M. elsdenii - *Megasphaera elsdenii*

BA - Butyric Acid

HA - Hexanoic Acid

OD - Optical Density

FID - Flame Ionization Detector

GC - Gas Chromatography

DoE - Design of Experiment

HCl - Hydrochloric Acid

OD600 - Optical Density at 600 nm

F - Fructose

A - Acetate

A/F - Acetate/Fructose ratio

WT - Wild Type

Abstract

The transition to a sustainable bioeconomy has intensified research into microbial production of medium-chain fatty acids (MCFAs) such as butyric acid (BA) and hexanoic acid (HA), which are high-value precursors for biofuels and specialty chemicals. This study investigates the metabolic and genomic differences between *Megasphaera elsdenii* strains, JCM 1772 (ATCC 25940) and JCM 35779, focusing on their capacity for MCFA production. Preliminary analyses of JCM 1772 and JCM 35779, conducted using adaptive laboratory evolution (ALE) with ethyl methanesulfonate (EMS) mutagenesis, revealed notable differences between the two strains. JCM 1772 showed a capacity for adaptive evolution, leading to the emergence of mutants with increased HA production. In contrast, EMS was found to be toxic to JCM 35779 and was less effective in yielding mutants. Notwithstanding, JCM 35779 demonstrates a significant enhancement over JCM 1772 and its mutants, achieving up to 4.7 to 2.8-fold improvement in HA production while generally exhibiting lower BA production. Genomic analysis of these strains revealed the absence of a BA-specific CoA transferase in JCM 35779, which may explain its metabolic preference for HA over BA synthesis. This hypothesis was further supported by perturbation experiments involving acetate supplementation. These findings highlight the connections between genomic variation, metabolic pathways, and environmental perturbations in shaping MCFA output. By integrating genomic and metabolic insights, this work identifies targets for strain engineering to enhance MCFA production, advancing the potential of *M. elsdenii* as a sustainable bioproduction platform.

Keywords

Medium-chain fatty acids (MCFAs); Adaptive laboratory evolution (ALE); *Megasphaera elsdenii*; Fermentation optimization; ATCC 25940; JCM 1772; JCM 35779

1. Introduction

In the transition toward a sustainable bioeconomy, volatile fatty acids (VFAs) are increasingly recognized as versatile platform chemicals for the production of biofuels and biochemicals. As key intermediates in biorefinery processes, VFAs derived from lignocellulosic sugars represent an attractive route to reduce reliance on fossil-based resources (Kobayashi and Fukuoka, 2013). While short-chain fatty acids (SCFAs) are already well-established in industry, medium-chain fatty acids (MCFAs), such as butyric acid (BA) and hexanoic acid (HA), are gaining attention for their higher energy density and potential as precursors for high-value specialty chemicals and next-generation biofuels (Stamatopoulou et al., 2020; Wu et al., 2021). Furthermore, their hydrophobicity facilitates more cost-effective downstream recovery (Ho Ahn et al., 2023), making them particularly attractive for large-scale industrial production.

Among microbial platforms for MCFA production, anaerobic chain elongation via the reverse β-oxidation pathway (r-BOX) has recently emerged as a key strategy (Jeon et al., 2016; Liu et al., 2024). Within this context, *Megasphaera elsdenii* has garnered attention for its ability to utilize diverse carbon sources, such as glucose, lactate, and fermentation intermediates (Jeon et al., 2016; Choi et al., 2013; Sträuber et al., 2018). For example, *M. elsdenii* has been shown to produce up to 4.7 g/L of HA from sucrose (Choi et al., 2013). In comparison, *Megasphaera hexanoica*, a closely related species, has demonstrated promising but more substrate-specific MCFA production, reaching 8.9 g/L HA from lactate (Kang et al., 2022). While *M. hexanoica* shows strong titers, *M. elsdenii* stands out with its ability to produce a broader spectrum of MCFAs, particularly BA and HA, using both glucose and lactate as feedstock (Gildemyn et al., 2017); albeit with variations in MCFA production profiles across strains. For example, *M. elsdenii* ATCC 25940 produced 2.6 g/L HA from 40 g/L glucose (Roddick and Britz, 1997), whereas NCIMB 702261

and NCIMB 702410 yielded 2.2 g/L and 3.3 g/L HA, respectively, from 20 g/L sucrose (Choi et al., 2013). In comparison, *Megasphaera* sp. MH achieved up to 4.4 g/L HA under high selective pressure (100 mM acetate) when grown on fructose (Jeon et al., 2016). Although these studies have provided valuable insights, they have primarily focused on static culture conditions and a narrow set of well-characterized strains. Little attention has been paid to variations at strain-specific level, substrate utilisation and metabolic efficiencies observed across *M. elsdenii* isolates.

Previous comparative studies suggest that significant metabolic variability exists among *M. elsdenii* strains, likely reflecting differences in genetic background and ecological origin (Cabral and Weimer, 2024; Lee et al., 2020; Nelson et al., 2017). This variability presents an opportunity to explore evolutionary adaptability and strain-specific metabolic potential in two distinct *M. elsdenii* strains exhibiting contrasting fatty acid profiles. The first species, identified as JCM 1772, originates from sheep rumen and is also annotated as ATCC 29540 (Elsden et al., 1956). The second less studied species is JCM 35779, which was recently isolated from pig feces (Yoshikawa et al., 2024, M215). Here, comparative genomics and chemical profiling are performed to analyse the metabolic and evolutionary divergence between *M. elsdenii* strains JCM 1772 and JCM 35779, with a focus on their capacity for MCFA production.

Given the challenges in available genetic manipulation tools and the limited metabolic insights into these anaerobic organisms, adaptive laboratory evolution (ALE) emerges as a highly effective and versatile strategy for engineering microbial cell factories tailored for bioproduction (Hirasawa and Maeda, 2023). This method not only circumvents the limitations of traditional genetic engineering but has also proven successful in other anaerobic bacteria such as *Clostridium* spp., leading to increased metabolite yields and stress tolerance (Hirasawa and Maeda, 2023; Ingelman et al., 2024). To date, little effort has been made to exploit the evolutionary adaptability

of *M. elsdenii* under relevant selective pressures, which could improve MCFA yield. In addition to characterization of these strains, this study will also explore their evolutionary adaptability and strain-specific metabolic potential for the production of MCFA.

Overall, this study explores both production and genomic profiles, seeking to uncover the cellular responses and mechanisms that drive the production of MCFA, particularly on understanding the biosynthesis of BA and HA. Together, these findings open up new opportunities for optimizing MCFA production, with significant potential in engineering various physiological functions and impact downstream applications in biotechnology.

2. Materials and methods

2.1 Microorganism and medium preparation

Megasphaera elsdenii strains, JCM 35779 and JCM 1772, were provided by the RIKEN BRC through the National BioResource Project of the MEXT, Japan. Both strains were cultivated in mYPF medium, a medium designed to support the growth of *M. elsdenii* and facilitate the production of VFAs under anaerobic conditions. The mYPF medium was prepared with the following components per liter of distilled water: 10 g of yeast extract, 5 g of tryptone, 5 g of peptone, 5 g of beef extract, 10 g of fructose, 2 g of K_2HPO_4, 0.5 g of cysteine $HCl \cdot H_2O$, 1 mg of resazurin sodium salt, and 40 mL of a salt solution. The pH was adjusted to 6.8–7.0. The salt solution included: 0.25 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of K_2HPO_4, 1 g of KH_2PO_4, 10 g of $NaHCO_3$, and 2 g of NaCl. Yeast extract, beef extract, and calcium chloride dihydrate were purchased from Bio Basic Asia Pacific (Singapore), Difco (USA), and Kanto Chemicals (Japan), respectively. All other chemicals were sourced from Sigma-Aldrich (USA). For routine cultivation,

a Thermo Scientific™ Varioskan™ LUX plate reader (USA) was employed to monitor optical density and assess cell growth.

Genomic DNA was extracted from cell pellets using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany). The 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) (see **Table S1** for primers) and the resulting amplicons were subjected to Sanger sequencing for strain identification and confirmation.

2.2 Chemical mutagenesis

The wild-type (WT) strains *M. elsdenii* JCM 35779 and JCM 1772 were subjected to chemical mutagenesis using ethyl methanesulfonate (EMS). A range of EMS concentrations were tested to determine the optimal conditions for mutagenesis: 0.1% (1.24 g/L), 0.2% (2.48 g/L), 0.4% (4.97 g/L), 0.6% (7.45 g/L), 0.8% (9.93 g/L), 1.0% (12.4 g/L), and 1.2% (14.9 g/L). EMS was introduced into the cultures during the exponential growth phase. Following EMS addition, the cultures were incubated for one hour at 37 °C under continuous agitation. After the incubation period, the cultures were transferred to fresh mYPF medium and allowed to recover overnight at 37 °C. Growth rates of the mutagenized populations were monitored by measuring optical density at 600 nm (OD 600) every 30 minutes.

2.3 Adaptive lab evolution

For the adaptive laboratory evolution (ALE) of *M. elsdenii* strains, cultures were exposed to 0.1% EMS during the exponential growth phase for 1 hour to induce genetic diversity. After mutagenesis, the populations were grown in a formulated media system composed of 1 part of

mYPF medium and 8 parts of minimal medium (MM) , 200 µL 96-well clear flat bottom plate, over a period of 16-18 hours. The minimal medium (MM) contained the following components per liter of distilled water: 1 g NH_4Cl, 0.1 g NaCl, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.3 g K_2HPO_4, 0.5 g L-cysteine hydrochloride, 3.28 g sodium acetate, 7.4 g ethanol, 1 mg resazurin sodium salt, 10 µL of Vitamin Supplement (ATCC MD-VS™, USA), and 1 mL of Trace Mineral Supplement (ATCC MD-TMS™, USA). The pH of the medium was adjusted to 7.0. During this period, the optical density at OD 600 was measured every 30 minutes to monitor growth. Finally, cultures were recovered in rich mYPF media and incubated overnight at 37 °C. The recovered populations were then plated on mYPF agar plates and incubated at 37 °C to isolate individual colonies. Colonies that exhibited improved growth compared to the WT strain were selected for further characterisation for cell growth and acid production.

2.4 High throughput BA and HA screening

Mutants obtained through ALE were plated on mYPF agar and incubated overnight at 37 °C. Single colonies were isolated from each mutant and cultured overnight in liquid mYPF medium. These cultures were subsequently used to inoculate the formulated medium described above in 2 mL deep-well plates for high-throughput screening of BA and HA. The deep-well plates were incubated at 37 °C for four days without agitation. Mutants exhibiting increased BA and HA production were selected for further analysis. A time-course study was conducted over a period of seven days in 20 mL serum bottle fermentations using mYPF medium at 37 °C and 200 rpm agitation (**Fig. S1**).

2.5 Fermentation

M. elsdenii was cultivated overnight in an anaerobic environment at 37 °C with shaking at 200 rpm for 16-18 hours. The overnight culture was then used to inoculate serum bottles (50 mL, Wheaton) containing 20 mL of media, with the specific media composition adjusted depending on the requirements of the experiment. Fermentation was carried out in batch mode under anaerobic conditions, with serum bottles purged with nitrogen gas after each sample collection. Samples were routinely collected at 12-hour intervals throughout the fermentation process to measure biomass growth (via OD 600), monitor acetate consumption, and analyse the production of MCFAs, i.e., BA and HA.

2.6 Analytical quantification of Acetate utilisation

Acetate consumption during fermentation was analyzed using a gas chromatography (GC) system (Agilent 6890 N, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and a DB-WAX fused silica capillary column (60 m length x 0.53 mm diameter x 1.00 µm film thickness). Helium was used as the carrier gas. The GC oven was initially set to 35°C with a 4-minute hold, followed by a temperature ramp of 10°C/min to a final temperature of 220°C, which was maintained for 6 minutes. Both the injector and detector temperatures were set to 300°C. Prior to GC analysis, fermentation samples were centrifuged at 10,000 rpm for 10 minutes to remove cells and particulate matter. A 0.25 mL aliquot of the supernatant was then acidified by adding 11.25 µL of 6 M HCl. The acidified sample was centrifuged at maximum speed for 5 minutes before being injected into the GC for analysis. Calibrated standard curve (**Fig. S2**) was used to calculate the acetate concentration.

2.7 Analytical quantification of Butyric acid (BA) and Hexanoic acid (HA)

To quantify BA and HA, 0.4 mL of supernatant was acidified by adding 15 μL of 6 M HCl, followed by extraction with 0.1 mL of hexane. A 1 μL aliquot of the extracted hexane was then injected into a gas chromatograph (GC, Agilent 7890A, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and a fused silica capillary column (HP-5, 30 m × 0.320 mm diameter × 0.5 μm film thickness). Helium was used as the carrier gas. The GC oven was initially set to 40°C with a 3-minute hold, followed by a temperature ramp to 130°C at a rate of 30°C/min, and a subsequent ramp to 300°C at 20°C/min. Concentrations of BA and HA were determined by comparing the peak areas to calibrated standard curves (**Fig. S3**).

2.8 Read Processing and Alignment

Raw paired-end Illumina reads were quality-filtered and adapter-trimmed using FastQC v12.1 and fastp v1.0.1 (Andrew, 2010; Chen et al., 2018). Trimmed reads were aligned to the *M. elsdenii* reference genome with BWA-MEM v0.7.19 (default parameters) (Li and Durbin, 2009). Resulting SAM files were converted to BAM, sorted, and indexed using SAMtools v1.22 (Li et al., 2009).

To evaluate gene presence and absence with nucleotide-level resolution, we leveraged annotated coding sequences (CDS) from the *M. elsdenii* reference GenBank file (Accession No: NZ_CP027570.1), extracting CDS features, including genomic coordinates, locus tags, gene names and corresponding translated protein sequences. Per-base coverage depth was calculated with SAMtools, and CDS-level proportional coverage was utilized for further gap identification and downstream gene-absence analysis.

3. Results and discussions

3.1 Chemical mutagenesis mediated adaptive lab evolution (ALE)

This study first investigates the evolution capabilities of two phylogenetically distinct strains of *M. elsdenii* towards BA and HA production; a well-characterized *M. elsdenii* (JCM 1772, also classified as ATCC 25940), isolated from sheep rumen, and a newly isolated strain (JCM 35779), sourced from pig feces. Notably, JCM 1772 been widely studied in the literature (Hatmaker et al., 2019), and serves as a valuable reference for comparative evaluation of strain performance.

Strain identities of the strains were confirmed by 16S rRNA phenotyping (**Tables S1** and **S2**). Alignments of 16S showed that the strains were matched to their respective *M. elsdenii* strains; JCM 1772 with DSM 20460 (ATCC 25940) and JCM 35779 with strain M215. Both strains exhibited optimal growth at 37 °C in mYPF medium and primarily produced BA and HA, consistent with previously reported metabolic capabilities of *M. elsdenii* strains (Choi et al., 2013, Nelson et al., 2017).

To enhance fatty acid production, ethyl methanesulfonate (EMS), a potent chemical mutagen was used to generate genetically diverse mutant libraries. EMS acts by alkylating guanine bases in DNA, inducing point mutations and enabling exploration of beneficial genotypes through selective evolution. It has been previously used to boost metabolite production, including a 3.9-fold increase in BA yield in *Clostridium carboxidivorans* (Lakhssassi et al., 2020). In this study, a range of EMS concentrations (0.1-1.2%) was tested. As shown in **Fig. 1A**, 0.2% EMS was identified as the maximum tolerated concentration that still permitted growth in JCM 1772.

For strain JCM 1772, a two-step strategy was employed, combining EMS-induced mutagenesis with ALE to further drive metabolic improvements. EMS mutagenesis was performed

prior to each ALE cycle (**Fig. 1B**), following a serial transfer method (Hirasawa and Maeda, 2023) (**Fig. S1**). ALE has previously been shown to increase resistance to various environmental stressors, including chemicals (Imamovic et al., 2018; Lázár et al., 2014; Maeda et al., 2020). ALE was carried out using minimal media supplemented with acetate, where acetate is proposed to be both an electron sink and a growth inhibitor (Hino et al., 1991). Growth curves of evolved mutant populations (M1, M2) after the 8th ALE round showed improved growth kinetics compared to the WT strain in acetate-based medium (**Fig. 1B**). This indicates successful adaptation and enhanced fitness under acetate stress. Fermentation trials comparing WT and selected mutant strains further revealed significant differences in metabolic output (**Fig. 1C, D**). For JCM 1772, evolved strains (E1, E5, F4) showed a 1.2- to 2.2-fold increase in BA (0.2 g/L) and HA (0.5 g/L) production relative to WT. No significant difference in growth rate was observed between mutants and wild type under rich media.

In contrast, JCM 35779 demonstrated a lower tolerance to EMS, where growth was nearly halved that of WT, even at the lowest concentration 0.1 % of EMS (**Fig. 1F**). Despite this, growth was still possible at a higher EMS concentration of up to 1.2%, albeit at a reduced rate. However, a second round of EMS exposure proved to be toxic, where no growth was observed. Thus, mutants were only selected after one round of EMS. The selected best mutants (H1, H5, G2) obtained from these experiments have demonstrated improvements in fatty acid production, mainly in the first 24 hours, compared to WT (**Fig. 1G, H,** 1.1-1.7-fold change over 24 hours). Notably, strain JCM 35779 and its mutants exhibited a substantially higher HA to BA ratio (8.9 to 22.9) compared to JCM 1772 and its mutants (1.0 to 2.8). In addition, JCM 35779 WT has a much higher HA yield of 1.4 g/L at 48 hours, compared to JCM 1772-E1 (0.5 g/L) and JCM 1772 WT (0.3 g/L).

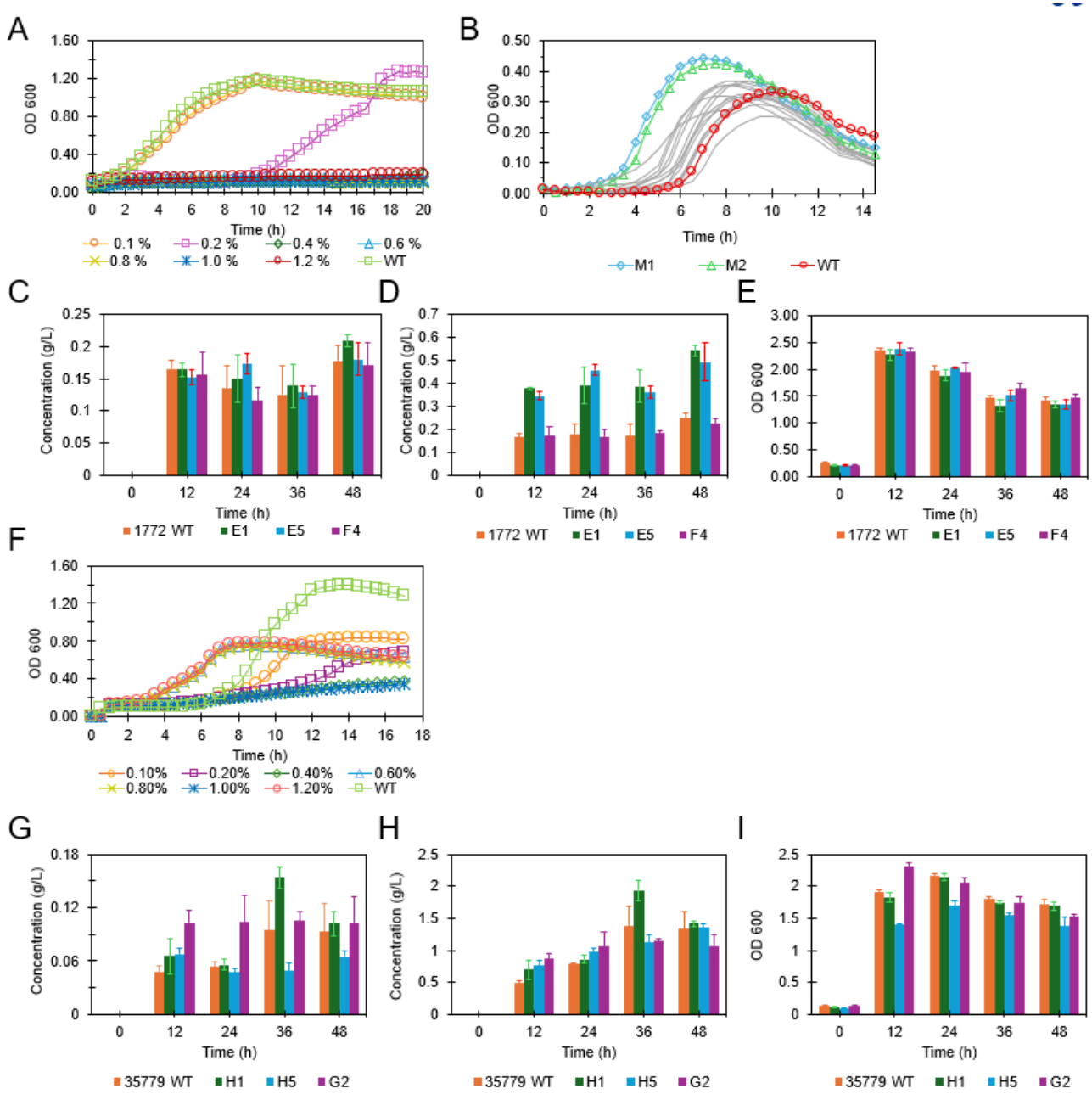


Fig. 1. Effects of EMS-induced mutagenesis and Adaptive Lab Evolution (ALE) on JCM 1772 and JCM 35779. Growth curves in the presence of varying concentrations of EMS (%) for **(A)** JCM 1772, and **(B)** Growth curves of JCM 1772 mixed mutant populations (representation populations

are labelled as M1, M2) in acetate-supplemented media over 14 hours after the 8th round of ALE. Fatty acids production **(C)** BA and **(D)** HA and **(E)** cell density between WT strains JCM 1772 and isolated mutants at various time points of fermentation in mYPF medium. Growth curves in the presence of varying concentrations of EMS (%) for **(F)** JCM 35779. Fatty acids production **(G)** BA and **(H)** HA, and **(I)** cell density between WT strains JCM 35779 and selected best mutants at various time points of fermentation in mYPF medium. Experiments were conducted in biological triplicates. **(A)**, **(B)**, **(F)** experiments were conducted in 96-well plates, whilst the rest were conducted in 20 mL serum bottles (more details in methods).

3.2 Reference-Based Coverage Analysis of Genomes

Although JCM 35779 has been classified as *M. elsdenii* based on 16S rRNA analysis from Yoshikawa et al. and our analyses (**Table S2**), its genome has not yet been sequenced. Conversely, JCM 1772 has been identified as *M. elsdenii* ATCC 25940. Despite genomic similarities based on species classification, JCM 35779 consistently outperformed JCM 1772 in HA production, where a 4.7 to 2.8-fold improvement was observed compared to JCM 1772 and its derivatives (**Fig. 1**). The production levels of HA and BA indicate that these strains, despite being classified as the same species, are distinct from one another. This disparity may stem from inherent differences in redox metabolism, carbon flux allocation, or CoA regeneration pathways between the strains (Jarboe et al., 2013; Jeon et al., 2016).

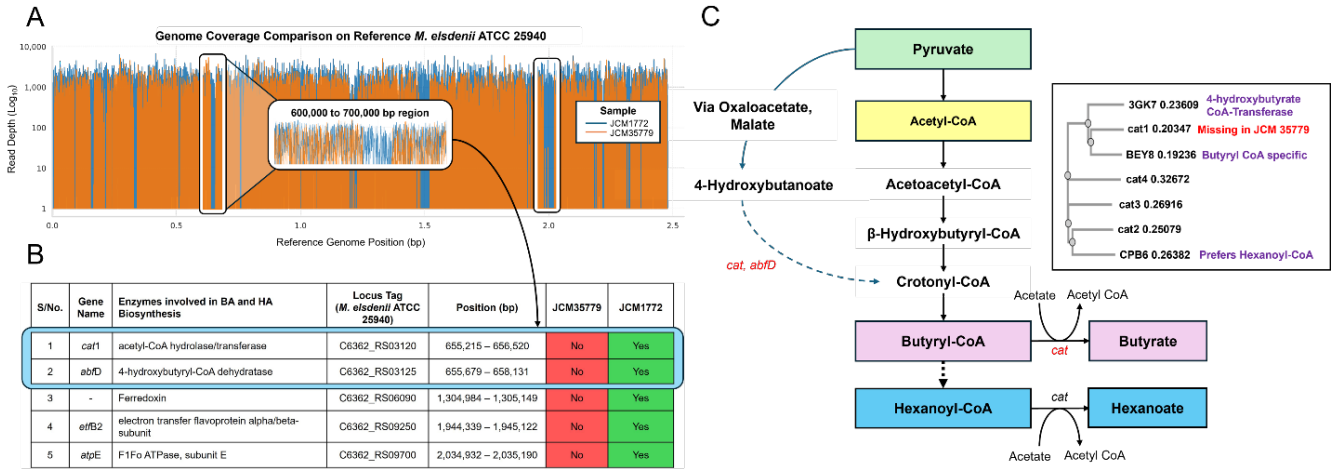
To systematically dissect the genetic and metabolic divergence between *M. elsdenii* strains JCM 1772 and JCM 35779, comparative genomics was employed, leveraging whole-genome resequencing and reference-based coverage analysis. Whole-genome resequencing was performed using Illumina HiSeq platform, generating paired-end reads with an average read length of 150 bp.

These reads were first quality-checked and adapter-trimmed using FastQC and fastp, respectively. Subsequently, the reads were aligned to the *M. elsdenii* ATCC 25940 reference genome using the Burrows-Wheeler Aligner (BWA-MEM, Li and Durbin, 2009). To quantify genomic variation, per-base coverage was computed using SAMtools. This generated a comprehensive map of nucleotide coverage across the reference genome, enabling the identification of regions with significantly reduced or absent coverage in the target strains. Discrete intervals of zero coverage were flagged as putative deletions or strain-specific absent loci, potentially representing structural variations such as insertions, deletions, or gene loss (**Fig. 2, Fig. S4**).

Per-base coverage analysis further revealed that JCM1772 and JCM35779 have varying alignment profiles with the reference genome ATCC 25940, with an alignment of 99.5 % for JCM 1772, highlighting a near identical genomic profile between JCM 1772 and the reference genome (**Table S3**). This observation confirms that JCM 1772 is correctly identified as ATCC 25940. However, only 83.4 % of sequences from JCM 35779 that could be mapped onto the reference genome of ATCC 25940. The coverage plot (**Fig. 2A**) revealed discrete regions of zero coverage, indicating putative deletions or strain-specific absent loci in JCM 35779.

To identify strain-specific gene deletions or truncations, coordinates of no-coverage intervals were intersected with the annotated gene set of the reference genome (NCBI GenBank, *M. elsdenii* ATCC 25940, NZ_CP027570.1). Here, we identified several missing genes in JCM 35779, including those involved in BA and HA biosynthesis (**Fig. 2B, C**). Notably, the missing genes include the beta-subunit of the electron-bifurcating flavoprotein complex, which is postulated to facilitate crotonyl-CoA conversion to butyryl-CoA (etfB2). Additionally, there are two missing genes involved in succinate reduction to crotonyl-CoA: acetyl-CoA hydrolase/transferase (cat1) and 4-hydroxybutyryl-CoA dehydratase (abfD) (**Fig. 2, Table S4**).

Although four CoA transferase (cat) enzymes are proposed in JCM 1772, sequence alignment with known BA/HA-specific CoA transferases (Yang et al., 2021) suggests that cat1 functions as a BA-specific CoA transferase (Figure 2B inset). cat2 is hypothesized to be similar to CPB06, which reacts with both butyryl CoA and hexanoyl CoA, with preference for hexanoyl CoA. Removing cat1 is hypothesized to not only limit crotonyl-CoA formation from 4-hydroxybutanoate but also decrease BA production, as the remaining CoA transferases show a stronger preference for hexanoyl CoA. This observation correlates with the HA/BA profiles observed in JCM 1772 and JCM 35779.



S/No.	Gene Name	Enzymes involved in BA and HA Biosynthesis	Locus Tag (*M. elsdenii* ATCC 25940)	Position (bp)	JCM35779	JCM1772
1	*caf*1	acetyl-CoA hydrolase/transferase	C6362_RS03120	655,215 – 656,520	No	Yes
2	*abf*D	4-hydroxybutyryl-CoA dehydratase	C6362_RS03125	655,679 – 658,131	No	Yes
3	-	Ferredoxin	C6362_RS06090	1,304,984 – 1,305,149	No	Yes
4	*etf*B2	electron transfer flavoprotein alpha/beta-subunit	C6362_RS09250	1,944,339 – 1,945,122	No	Yes
5	*atp*E	F1Fo ATPase, subunit E	C6362_RS09700	2,034,932 – 2,035,190	No	Yes

Fig. 2. Comparative genome coverage of JCM 1772 and JCM 35779 aligned to *M. elsdenii* ATCC 25940 reveals putative deletions or strain-specific gene absences. **(A)** Read alignment coverage shows that JCM 1772 (blue) exhibits consistently high genome-wide coverage, whereas JCM 35779 (orange) displays several regions with markedly reduced or absent coverage. **(B)** There are two possible pathways to BA and HA in JCM 1772 and JCM 35779, with genes missing the route via oxaloacetate in JCM 35779 highlighted in red. An absent CoA transferase (cat) is proposed to responsible for activities of both 4-Hydroxybutyrate CoA-transferase and butyryl CoA transferase.

An inset displays a phylogenetic tree of CoA transferases, including the four cat enzymes in JCM 1772 (cat1-4). BEY8, CPB6 are described in Yang et al., 2021. A characterized 4-Hydroxybutyrate CoA-transferase is also annotated (PBD ID: 3GK7). More details on genes are given in Table S4-S6.

3.3 Effect of Acetate

Biosynthesis of BA is hypothesized to proceed through the condensation of two acetyl-CoA molecules, followed by the conversion of butyryl-CoA to BA via a key CoA transferase enzyme (Choi et al., 2013; Jeon et al., 2016; Kim et al., 2019; Detman et al., 2019). It was hypothesized that the absence of a key CoA transferase enzyme, involved in converting butyryl-CoA to BA, resulted in significantly lower BA levels in JCM 35779 compared to JCM 1772. To address this, acetate, which serves as both a CoA acceptor and substrate, was proposed to assess its effects on the production of both BA and HA in wild-type and evolved strains.

Following acetate supplementation, a significant increase in BA production was observed across all strains, with the response being particularly pronounced in JCM 35779 strain and its derived mutant H1 (**Fig. 3A, C**). Notably, this finding aligns with general trends (Choi et al., 2013; Jeon et al., 2016; Kim et al., 2019) that indicate an enhancement in BA production when acetate is present. JCM 35779 and its mutant H1 exhibited an eight-fold increase in BA production, reaching peak titers of 1.1 g/L at 36 hours in acetate supplemented medium, compared to 0.1 g/L in no acetate-supplemented controls. In contrast, JCM 1772 and its mutant E1 showed a more moderate two-fold increase, with maximum BA concentrations of 0.8 g/L under acetate supplementation (**Fig. 3C**). With acetate supplementation, acid yields shifted, with JCM 1772-E1 producing 1.2-fold more BA and 1.8-fold less HA than the WT, with total acid production decreased slightly to 1.4 g/L, suggesting possible inhibitory effects of acetate accumulation. These results support the

hypothesis that exogenous acetate supplementation can improve the availability of CoA acceptor and acetyl CoA, which in turn affects the production of BA, either as a substrate or to mediate the conversion of butyryl-CoA to BA. This effect is particularly pronounced in strains like JCM 35779 that have impaired butyryl-CoA to BA conversion.

Despite its clear benefit for BA biosynthesis, acetate supplementation did not enhance HA production in a comparable manner. Notably, for JCM 1772 and its mutant E1, HA production was inhibited by acetate, with yields reduced by approximately 50% relative to controls (**Fig. 3D**). In JCM 35779 strains, where HA titers generally also did not increase significantly despite the presence of acetate (**Fig. 3B**). These findings align with previous research (Hino et al., 1991), which suggests that acetate supplementation may create redox-limited conditions in particular scenarios. Such an environment would then favor the energetically less demanding biosynthesis of BA, ultimately leading to the observed trade-off between BA and HA production.

Overall, the results indicate that using a common metabolite such as acetate may be a limiting factor for the subsequent elongation to long chain fatty acids (HA). This is likely due to acetate multifaceted roles, including acting as a substrate in acetyl-CoA generation and as a Co-A acceptor for Co-A transferase reactions, yet it may also induce metabolic stress or redox imbalance (Jarboe et al., 2013; Kim et al., 2019). Additionally, elevated acetate levels can disrupt intracellular pH homeostasis and reduce the driving force for r-BOX, which may further impair the elongation pathway toward HA (Jeon et al., 2016). Therefore, although acetate plays a beneficial role in boosting BA, its complex metabolic influence may limit its effectiveness as a universal enhancer for long chain fatty acid production.

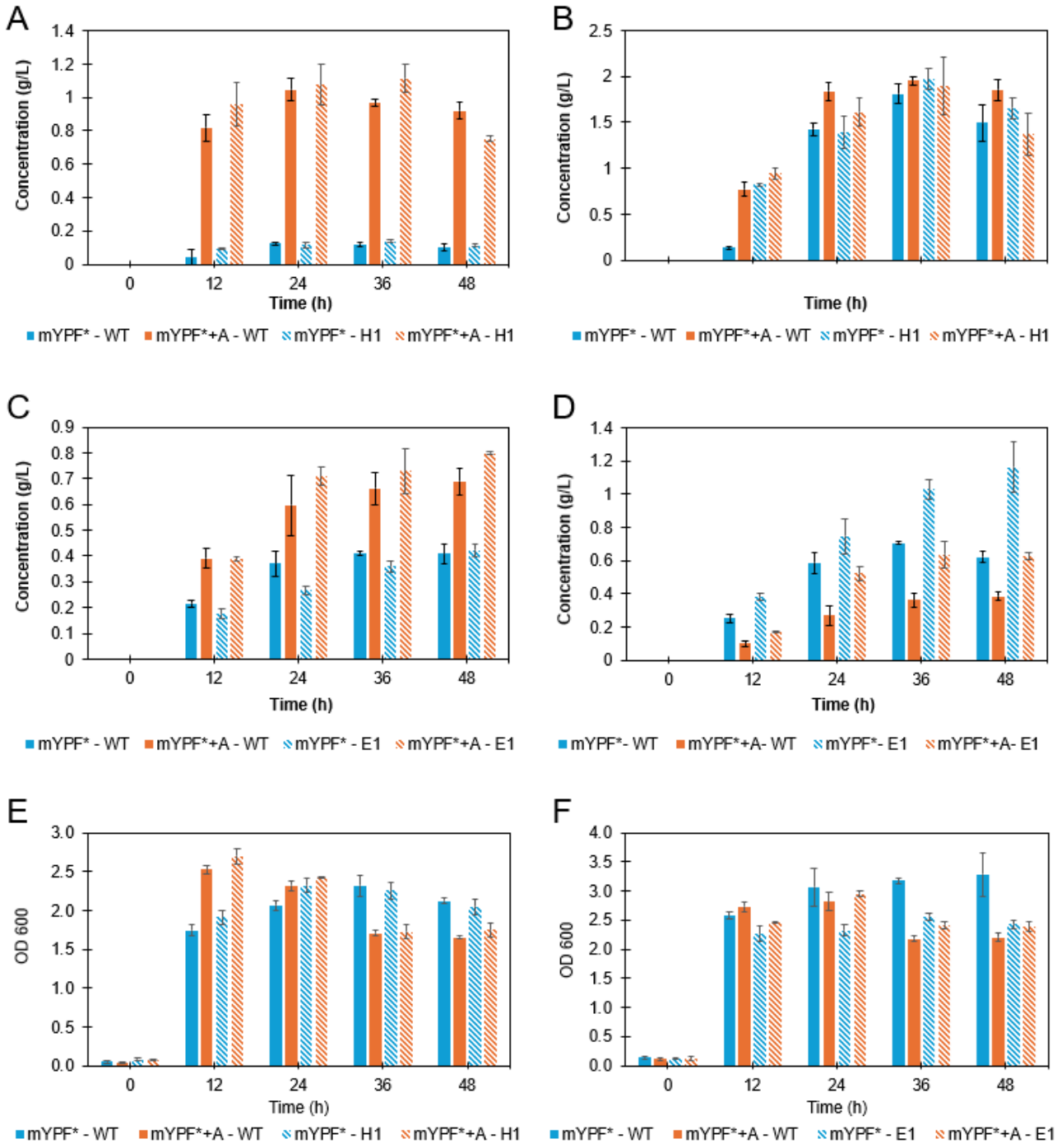


Fig. 3. Metabolite production and cell growth of JCM 35779 and JCM 1772 strains over 48 hours in mYPF* where fructose concentration is increased to 20 g/L and mYPF*+A, which is mYPF* supplemented with 100 mM sodium acetate. **(A)** BA and **(B)** HA production by strain JCM 35779 and 35779-H1. **(C)** BA and **(D)** HA production by strain JCM 1772 and 1772-E1. Cell density (OD

600) of strains **(E)** JCM 35779 and 35779-H1 and **(F)** JCM 1772 and 1772-E1 with and without acetate supplementation. Experiments were conducted in biological triplicates. Acetate utilization can be found in **Fig. S5**.

4. Conclusion

This study highlights the potential of *M. elsdenii* as a producer of BA and HA, through chemical mutagenesis and genomic profiling. Comparative analysis between *M. elsdenii* strains derived from sheep rumen (JCM 1772) and pig feces (JCM 35779) highlighted importance of strain screening for MCFAs production. To overcome genetic framework, combining mutagenesis with ALE offers a promising route to optimize MCFAs production and reveal underlying metabolic processes. Chemical mutagenesis accelerated ALE, generating mutants 1.2- to 2.2-fold higher BA and HA yields under HTP conditions. Genomic insights revealed the loss of butyryl CoA: CoA transferase and 4-hydroxybutyryl-CoA dehydratase in JCM 35779, which in turn may explain its elevated HA-to-BA production ratio, as compared to JCM 1772.

Future work should focus on optimizing process parameters such as electron donors, pH control (Fernández-Blanco et al., 2023), and in situ product removal (Lakhssassi et al., 2020; McDowall et al., 2022) which have been known to improve yield and support sustainable fatty acid biomanufacturing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors contribution

AC: Conceptualisation, methodology, validation, formal analysis, investigation, data curation, visualisation, writing (original draft). AY: Validation, investigation, writing (review & editing). D.T.: Validation, investigation, writing (review & editing). W.K.: Validation, investigation, supervision, writing (review & editing). YWT: Validation, investigation, formal analysis, visualisation, writing (review & editing). YHL: Conceptualisation, writing (original draft), resources, supervision, project administration, funding acquisition, writing (review & editing). FTW: Conceptualisation, writing (original draft), investigation, resources, formal analysis, supervision, project administration, funding acquisition, writing (review & editing).

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