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Evaluation of Yeast Alcohol Acetyltransferases for Ethyl Acetate Production in *Clostridium ljungdahlii*

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ABSTRACT

Sustainable chemical production from C_1 gaseous substrates, such as syngas or CO_2/H_2, can be achieved through gas fermentation. In gas fermentation, acetogenic bacteria are able to utilize oxidized inorganic carbon sources as the sole carbon source and electron acceptor, while reduced inorganic species are used as the electron donor. *Clostridium ljungdahlii*, a model acetogen, is only capable of reducing CO_2 to acetate and ethanol, with H_2 as electron donor. In order to expand the product profile of this bacterium, five alcohol acetyltransferases (AATs) from yeast were heterologously expressed in *C. ljungdahlii* to evaluate its potential to produce ethyl acetate. When growing on CO_2 and H_2, up to 7.38 ± 0.43 mg/L of ethyl acetate were produced. Using fructose as the main carbon and energy source, up to 23.15 ± 1.28 mg/L of ethyl acetate were produced. Ethanol and fumarate supplementation were able to boost ethyl acetate titers (up to 37.51 ± 9.44 mg/L). Hence, ethyl acetate production was enabled in *C. ljungdahlii* at low titers, which could be explained by the high energetic cost of operation of AATs, and their shown promiscuity. However, we also show that this opens the door to more complex esterification reactions of higher added value biomolecules.

1 | Introduction

The production of chemicals from synthesis gas, or syngas, is one of the many efforts to promote the replacement of fossil fuels in chemical production by renewable means. Syngas, which here refers to a mixture of carbon monoxide (CO) and hydrogen (H_2), is mainly produced from natural gas and other fossil hydrocarbons. The most widespread technologies for syngas production are steam methane reforming and partial oxidation [1]. Other approaches, such as dry reforming, have the advantage of also consuming carbon dioxide (CO_2), which reduces associated emissions [2, 3]. Syngas is also produced as exhaust gas by the steel industry. Renewable syngas production technologies, like electrochemical syngas generation [4, 5], biomass gasification [6], or biogas reforming [7] still lack competitiveness. But with the rapid development of the field and renewed interest from the industry, these technologies will likely displace conventional fossil fuel-dependent syngas production technologies in the near future. Syngas can be chemically converted into H_2, ammonia, methanol, and more complex hydrocarbons [8]. Additionally, syngas can be biologically converted to other substances of interest reducing costs and expanding the options of (bio)chemical synthesis. Syngas fermentation is performed by autotrophic acetogenic bacteria, such as *Clostridium* spp., *Moorella thermoacetica*, or

Abbreviations: AAT, alcohol acetyltransferase; ANOVA, analysis of variance; ATP, adenosine triphosphate; CoA, coenzyme A; OD_{600}, optical density at 600 nm; PLS-DA, partial least squares discriminant analysis.

Eubacterium limosum [9], which mainly operate the Wood-Ljungdahl pathway to fix carbon (in the form of CO_2 or CO), using electrons derived from CO itself, hydrogen (H_2) or a negatively poised cathode. In the Wood-Ljungdahl pathway, 2 moles of CO_2-equivalents are reduced to 1 mol of acetyl-CoA using 4 moles of H_2-equivalents (8 electrons) and 1 mol of ATP. Acetyl-CoA is the central metabolite in the autotrophic metabolism of acetogens. To compensate for the ATP expenditure in the Wood-Ljungdahl pathway, most of the acetyl-CoA must be converted to acetate, recovering ATP. Therefore, more reducing power (as H_2-equivalents) is needed to produce a proton gradient, which subsequently generates a proton motive force that leads to ATP production. As a consequence of this, syngas fermentation is energetically more favorable than CO_2 and H_2 fermentation since CO, unlike CO_2, is already partially reduced [10]. Not surprisingly, when growing on CO_2 and H_2, acetogens are considered to live on the thermodynamic limit of life [11]. Nevertheless, CO_2 and H_2 fermentation achieves the direct transformation of CO_2 to substances of interest avoiding intermediate steps and, due to the toxic nature of CO for higher organisms, facilitates the overall process by avoiding its use. Both substrates, syngas, and CO_2—H_2, are sometimes referred to as "syngas" and are treated similarly, but in reality, the use of each substrate often results in a very different product portfolio in gas fermentation due to the different redox balances that bacteria must maintain to achieve homeostasis [12].

Acetate is the main substance produced by acetogens during gas fermentation. However, these bacteria can generate an entire range of chemicals and biomolecules, including acids, alcohols, ketones, aromatic compounds, (poly)esters, and terpenoids [13, 14]. Furthermore, acetogens are also considered promising hosts for biomass production [15]. Ethanol was produced commercially from syngas by *Clostridium autoethanogenum* for the first time in 2018 by LanzaTech (Skokie, USA), and the same company has a broad portfolio of substances ready for production on an industrial scale [16], highlighting the relevance of the technology. Although *C. autoethanogenum* has been the workhorse for gas fermentation so far, its close relative *Clostridium ljungdahlii*, shows a promising but industrially less explored capacity for the production of substances of interest by gas fermentation. *C. ljungdahlii* is natively capable of producing acetate, ethanol, lactate, formate and 2,3-butanediol, although only acetate and ethanol are produced in the gram per liter range. Currently, the heterologous production of different substances in *C. ljungdahlii* is limited in the titers that can be achieved, due to the enormous energy limitation to which *C. ljungdahlii* is subjected when growing autotrophically. Nevertheless, in some cases, *C. ljungdahlii* has demonstrated the ability to overcome those limits. Thus, isopropanol was produced at 13.4 g/L as a result of the rational construction of a synthetic isopropanol pathway. In the same study, up to 3 g/L of 3-hydroxybutyrate were also obtained [17]. Butyrate production reached titers of 1.5 g/L by expressing butyrate biosynthetic genes from *Clostridium acetobutylicum* as well as by redirecting carbon flux from acetate to butyrate via critical knock-outs [18]. The inducible production of acetone up to 0.8 g/L was enabled expressing a synthetic operon derived from *C. acetobutylicum* [19]. The simultaneous integration of two synthetic clusters containing genes from *C. acetobutylicum*, *Clostridium kluyveri*, and *Clostridium carboxidivorans* led to the production of 0.4 g/L of hexanol [20]. Butanol was also produced at 0.2 g/L from butyrate using a *C. ljungdahlii* strain with integrated genes for butyrate production from *C. acetobutylicum* [21]. The production of mevalonate and isoprene at 68 mg/L and 2 µg/L, respectively, was reported due to the expression of a synthetic terpenoid pathway [22]. Isoamylalcohol and isobutanol production was also reported up to 35 and 30 mg/L, respectively [23].

With the aim of further contributing to the expansion of the product portfolio of *C. ljungdahlii*, here, ethyl acetate production was enabled during CO_2 and H_2 gas fermentation. The heterologous expression of alcohol acetyltransferases (AATs) from yeast, which catalyze the production of acetyl esters from the corresponding alcohol and acetyl-CoA, led to the production of ethyl acetate in a mineral salt medium with CO_2 and H_2 as the sole source of carbon and energy, respectively. While AATs have been explored previously in other *Clostridium* spp. [24, 25], this study focuses on their application in *C. ljungdahlii*. Five different AATs were evaluated, and different cultivation parameters were tested with the resulting strains to evaluate the factors influencing the production of ethyl acetate in heterotrophic and autotrophic conditions.

2 | Materials and Methods

2.1 | Bacterial Strains and Culture Conditions

All chemicals were purchased from Thermo Fisher Scientific (Waltham, USA), Merck (Darmstadt, Germany), or Carl Roth (Karlsruhe, Germany). The strains *C. ljungdahlii* DSM 13528, *Saccharomyces cerevisiae* DSM 70449 and *Kluyveromyces marxianus* DSM 5418 were purchased from the DSMZ (Braunschweig, Germany). *C. ljungdahlii* was cultured in the minimal medium: modified PETC (20 g/L MES, 2 g/L NH_4Cl, 0.2 g/L KCl, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L NaCl, 0.2 g/L KH_2PO_4, 80 mg/L $CaCl_2 \cdot 2H_2O$, 2 mg/L $CoCl_2 \cdot 6H_2O$, 8 mg/L $(NH_4)_2Fe(SO_4)_2$, 10 mg/L $MnSO_4$, 20 mg/L nitrilotriacetic acid, 2 mg/L Na_2MoO_4, 0.2 mg/L Na_2SeO_4, 0.2 mg/L Na_2WO_4, 2 mg/L $ZnSO_4$, 0.02 mg/L biotin, 0.05 mg/L pantothenic acid, 0.05 mg/L thiamine·HCl, 0.2 g/L L-cysteine·HCl, 1 mg/L resazurin) adjusted to pH 5.7. The medium was supplemented with thiamphenicol (5 µg/mL), fructose (7.2 g/L), folic acid (1 mg/L), formate (0.1 g/L), ethanol (0.4 g/L), or fumarate (5.8 g/L), when needed. Anaerobic cultures of *C. ljungdahlii* were performed in 250 mL serum bottles with a filling volume of 30 mL, at 37°C and with minimal shaking (50 rpm). A fresh culture growing on fructose was used as the inoculum, at a starting OD_{600} of 0.1, unless indicated otherwise. The headspace of the serum bottles was filled with nitrogen (1 bar) in heterotrophic cultures or CO_2 and H_2 (20:80 v/v; 2.5 bar) in autotrophic or mixotrophic cultures. All used gases were sterile and of high purity (Air Liquide, Paris, France). All the handling of *C. ljungdahlii* was performed under strict sterile and anaerobic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, USA). *S. cerevisiae* and *K. marxianus* were cultured in YPD medium (20 g/L peptone, 20 g/L glucose, 10 g/L yeast extract) at 30°C. *Escherichia coli* DH5α (New England BioLabs, Ipswich, USA) and *E. coli* pANA1 [26] was cultured in LB Broth and LB Agar at 37°C. The media were supplemented with chloramphenicol (25 µg/mL), when needed. *E. coli* DH5α was used for plasmid construction

TABLE 1 | Plasmids used in this study.

Name	Resource	Origin
pMTL83151	*E. coli—Clostridium* shuttle plasmid. Backbone for expression plasmids	[27]
pMTL82152	Source of *thl* promoter (P_{thl})	
pMTL_ATF1_Sc	Expression plasmid for ATF1 from *S. cerevisiae*	This study
pMTL_ATF2_Sc	Expression plasmid for ATF2 from *S. cerevisiae*	
pMTL_ATF1_Km	Expression plasmid for ATF1 from *K. marxianus*	
pMTL_EAT1_Km	Expression plasmid for EAT1 from *K. marxianus*	
pMTL_trEAT1_Km	Expression plasmid for truncated EAT1 from *K. marxianus*	

TABLE 2 | Oligonucleotides used for plasmid construction in this study. Overhangs are underlined. Used restriction sites are bold.

Name	Resource	Sequence (5′–3′)
thl_fw_BglII	*thl* promoter (P_{thl}) amplification	ccccc**agatct**TTTTTAACAAAATATATTGATAAAAATAATAATAG
thl_rv_AatII		ccccc**gacgtc**AACTAACCTCCTAAATTTTG
ATF1_Sc_fw_AatII	ATF1 gene amplification (*S. cerevisiae*)	ccccc**gacgtc**ATGAATGAAATCGATGAGAAAAATC
ATF1_Sc_rv_NotI		ccccc**gcggccgc**CTAAGGGCCTAAAAGGAGAG
ATF2_Sc_fw_AatII	ATF2 gene amplification (*S. cerevisiae*)	ccccc**gacgtc**ATGGAAGATATAGAAGGATAC
ATF2_Sc_rv_NotI		ccccc**gcggccgc**TTAAAGCGACGCAAATTC
ATF1_Km_fw_AatII	ATF1 gene amplification (*K. marxianus*)	CCCCCC**GACGTC**ATGATGAAATTACAGAAAATGTC
ATF1_Km_rv_NotI		CCCCCC**GCGGCCGC**TTATAATGTAGTCAAGTTGTTTTC
EAT1_Km_fw_AatII	EAT1 gene amplification (*K. marxianus*)	CCCCCC**GACGTC**ATGCTTCTCGCTTACACCGTC
EAT1_Km_rv_NotI		CCCCCC**GCGGCCGC**TTAATCTCTAGCACTTTTGAGAGATTC
trEAT1_Km_fw_AatII	Truncated EAT1 gene amplification (*K. marxianus*)	CCCCCC**GACGTC**ATGTCTGCTACTGCCAGAGCCTTC

and propagation following manufacturer's instructions, and *E. coli* pANA1 was used for plasmid methylation as previously described [26].

2.2 | Plasmid Construction and Electroporation

Phusion high-fidelity DNA polymerase, genomic DNA purification, plasmid DNA extraction, and DNA gel extraction kits were purchased from New England BioLabs (Ipswich, USA) and used following the manufacturer's instructions. FastDigest restriction enzymes, FastAP phosphatase and T4 DNA ligase were purchased from Thermo Fisher Scientific (Waltham, USA) and used following the manufacturer's instructions. Plasmids used in this study are shown in Table 1.

Oligonucleotides were purchased from Eurofins Genomics (Ebersberg, Germany) and are shown in Table 2.

The shuttle plasmid pMTL83151 [27] was the backbone for all constructed expression plasmids. The constitutive promoter P_{thl} was chosen to assure constant and strong expression. P_{thl} was amplified from the plasmid pMTL82152 [27] using the primers: thl_fw_BglII and thl_rv_AatII. Subsequently, the amplicon was digested and ligated between the BglII and AatII restriction sites of the backbone. The genes ATF1 (YOR377W) and ATF2 (YGR177C) from *S. cerevisiae* were amplified from genomic DNA using the primer pairs: ATF1_Sc_fw_AatII-ATF1_Sc_rv_NotI and ATF2_Sc_fw_AatII-ATF2_Sc_rv_NotI, respectively. The genes ATF1 (KLMA_30203), EAT1 (KLMA_10805) and the truncated EAT1 (KLMA_10805: p. Leu2_Tyr19del) from *K. marxianus* were amplified from genomic DNA using the primer pairs: ATF1_Km_fw_AatII-ATF1_Km_rv_NotI, EAT1_Km_fw_AatII-EAT1_Km_rv_NotI and trEAT1_Km_fw_AatII-EAT1_Km_rv_NotI, respectively. The amplicons were inserted between the AatII and NotI restriction sites, resulting in the expression plasmids listed in Table 1. The plasmid map of pMTL_ATF1_Sc is shown in Figure S1 as an example. The identification of the mitochondrial transit peptide of EAT1 was based on the structural model obtained from AlphaFoldDB (Uniprot ID: W0T4A7).

E. coli pANA1 methylated plasmids were used for electroporation in *C. ljungdahlii*, following a method modified from [26]. Briefly, the electroporation was performed in 2 mm electroporation cuvettes (Bio-Rad Laboratories, Hercules, USA) using the following parameters: 2500 V, 10 μF, 600 Ω (Electroporator 2510; Eppendorf, Hamburg, Germany).

2.3 | Fermentation Broth Analysis

The concentrations of organic acids, sugars, and alcohols were determined using an HPLC system (Jasco, Tokio, Japan) equipped with PDA, RI, and UV (210 nm) detectors. The column Aminex HPX-87H, 300 mm × 7.8 mm, 9 µm (Bio-Rad Laboratories, Hercules, USA), protected with the precolumn Kromasil 100 C18, 40 mm × 4 mm, 5 µm (Dr. Maisch, Ammerbuch, Germany), was used at 50°C with an isocratic mobile phase flow rate of 0.5 mL/min. The samples were diluted 1:10 with the mobile phase (0.005 M H_2SO_4). Appropriate standards were prepared accordingly. The injection volume was 50 µL.

The concentrations of ethyl acetate were determined using a GC system (Shimazdu, Kyoto, Japan) equipped with an FID (flame ionization) detector. The capillary column VF-5 ms, 34 m × 0.25 mm × 0.25 µm + 5m EZ-Guard (Agilent Technologies, Santa Clara, USA) was used. The oven was kept at 50°C. A temperature ramp of 20°C/min up to 250°C was programmed, starting 3 min after injection, and held for 4 min at 250°C. The injection was performed with a split ratio of 10.0 at 250°C. Nitrogen was used as carrier gas with a flow rate of 1 mL/min. The FID detector was kept at 300°C and constant flows of fuel-H_2 (32 mL/min), air (200 mL/min) and make up gas-nitrogen (24 mL/min) were used. Samples were extracted with dichloromethane at a volumetric rate 1:1, for 30 min at 37°C. Appropriate standards were prepared accordingly and extracted in the same way. The injection volume was 1 µL.

Optical density at 600 nm (OD_{600}) was measured to follow the growth of *C. ljungdahlii* with a BioPhotometer (Eppendorf, Hamburg, Germany). The pH was measured with a pH meter for small volumes (Mettler Toledo, Columbus, USA).

2.4 | Permanent Gas Determination

The concentration of CO_2 and H_2 in the headspace of the serum bottles were determined using a GC system (Shimazdu, Kyoto, Japan) equipped with a BID (barrier discharge ionization) detector. The capillary column Supelco Carboxen 1010 PLOT, 30 m × 0.32 mm × 15 µm (Merck, Darmstadt, Germany) was used. The oven was kept at 32°C for 10 min, followed by a temperature ramp of 30°C/min up to 250°C. The injection was performed with a split ratio of 50.0 at 230°C. Helium was used as carrier gas with an initial flow rate of 0.6 mL/min for 10 min, followed by a flow ramp of 0.1 up to 1 mL/min, and held at 1 mL/min for 3 min. The BID detector was kept at 300°C with a constant flow discharge gas-helium (50 mL/min). Samples were injected using an automatic injection valve with a 200 µL sample loop.

Gas pressure in the headspace of the serum bottles was measured with a digital pressure gauge DPG110 (Omega Engineering, Norwalk, USA).

2.5 | Measurement of Volatiles

Volatile substances in the cultures were directly measured by GC-MS using static headspace sampling of 10 mL *C. ljungdahlii* cultures growing in 20 mL headspace vials. A GC-MS Triple-Quadrupole 7000A (Agilent Technologies, Santa Clara, USA) with an MPS Robotic autosampler (Gerstel, Mülheim an der Ruhr, Germany), equipped with a capillary column VF5-ms (30 m × 0.25 mm × 0.25 µm) + 10 m EZ-guard (Agilent Technologies, Santa Clara, USA) was used. The oven was kept at 38°C. A temperature ramp of 3°C/min up to 50°C was programmed, starting 1 min after injection, followed by another temperature ramp of 20°C/min up to 250°C, after 1 min at 50°C, and kept 5 min at 250°C. The headspace vials were incubated at 80°C–250 rpm for 60 min before injection. A total of 250 µL were used for injection, which was performed with a split ratio of 10.0 at 150°C. Nitrogen was used as carrier gas with a flow rate of 1.2 mL/min. The MS interface was set to 280°C, with an ESI source temperature of 250°C and a mass scan range from 38 to 900 m/z. Peak detection was carried out using the MassHunter software (Agilent Technologies, Santa Clara, USA), and the compound class was induced from the comparison of the MS spectra against the NIST MS Search 3.0 database.

2.6 | Statistical Analysis

The nonparametric Kruskal–Wallis rank sum test was performed to determine the statistical significance of observed differences. Post hoc analysis was performed when necessary, using Dunn's test. p values indicated in the text always refer to the adjusted p values (using the Benjamini–Hochberg method) of the aforementioned statistical tests, unless indicated otherwise. The statistical analysis of volatiles was performed using partial least squares discriminant analysis (PLS-DA) and analysis of variance (ANOVA) in log-normalized data. All calculations and plots were performed in R/RStudio [28].

3 | Results

3.1 | Evaluation of Alcohol Acetyltransferases for Ethyl Acetate Production

Four different AATs were initially selected for constitutive expression in *C. ljungdahlii* to produce ethyl acetate. The enzymes ATF1 and ATF2 from *S. cerevisiae* (hereinafter referred to as ATF1 Sc and ATF2 Sc, respectively), and ATF1 and EAT1 from *K. marxianus* (hereinafter referred to as ATF1 Km and EAT1 Km, respectively) were selected due to their well-known activities for the production of esters [29–32], in their respective hosts. The engineered *C. ljungdahlii* strains expressing the corresponding AAT were initially monitored for 1 week in heterotrophic conditions, with fructose as the sole carbon and electron source (Figure 1A). Additional incubation (up to three weeks) was used to ensure product stability and verify that all reactions had completed. *C. ljungdahlii* ATF1 Sc produced 22.98 ± 0.91 mg/L of ethyl acetate after 1 week of cultivation, being the only strain that was able to produce ethyl acetate (limit of detection: 0.1 mg/L, among the evaluated strains). Ethyl acetate titers did not change significantly ($p > 0.1$) over the rest of the experiment, reaching 23.15 ± 1.28 mg/L after 3 weeks of cultivation. Acetate and ethanol titers reached 2.01 ± 0.14 and 1.90 ± 0.25 g/L after 1 week, respectively. During the remaining experiment, likely corresponding to stationary phase, only an increase in acetate titers up to 3.49 ± 0.18 g/L was observed, while ethanol titers and pH decreased,

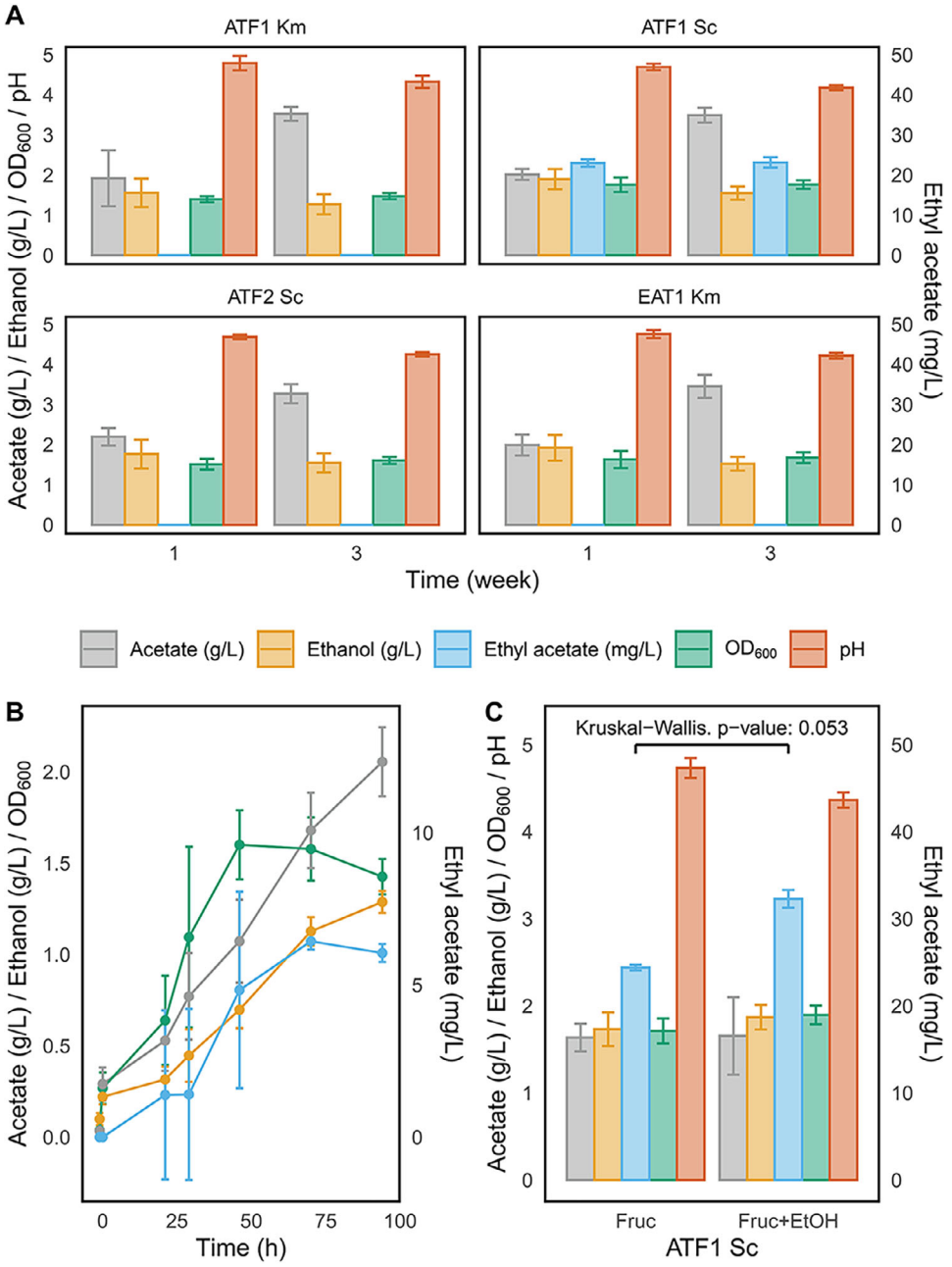

FIGURE 1 | Ethyl acetate production by *C. ljungdahlii* under heterotrophic conditions. (a) Heterotrophic growth and product formation on fructose of the four initially evaluated strains: *C. ljungdahlii* ATF1 Km, *C. ljungdahlii* ATF1 Sc, *C. ljungdahlii* ATF2 Sc, *C. ljungdahlii* EAT1 Km. Key performance parameters are reported for 1 and 3 weeks after the start of the cultivation. Only *C. ljungdahlii* ATF1 Sc was able to produce ethyl acetate. (b) Growth dynamics of *C. ljungdahlii* ATF1 Sc growing on fructose, inoculated at OD_{600}: 0.02. pH was omitted here for the sake of clarity. (c) Effect of ethanol supplementation (0.4 g/L) on ethyl acetate production by *C. ljungdahlii* ATF1 Sc growing on fructose (Fruc+EtOH), compared to unsupplemented cultures (Fruc). Key parameters are reported for 1 week after the start of the cultivation. The experiments were all conducted in triplicates ($n = 3$) and mean ± standard deviation is represented.

and OD_{600} remained stable. An independent repetition of the experiment was performed obtaining similar results after 1 week (Figure S2). The activity of the AATs requires the simultaneous occurrence of acetyl-CoA and ethanol inside the cell, and since *Clostridium* spp. fermentations often have two phases: acidogenesis and solventogenesis, ethanol availably in the early exponential phase would be crucial for ethyl acetate production. To assess this, *C. ljungdahlii* ATF1 Sc growth was followed in detail to determine the onset of ethyl acetate synthesis. An inoculation density of 0.02 (OD_{600}) was used here to slow down initial growth, which allowed a more resolved observation of growth phases. However, this resulted in lower ethyl acetate titers (6.44 ± 0.27 mg/L, Figure 1B) as compared to the usual inoculation density (OD_{600}: 0.1) used in experiments of Figure 1A. Ethyl acetate was mostly produced in late exponential phase and did not increase further when stationary phase was reached, unlike acetate and ethanol. This suggested that ethanol availability in early stages of growth is important for the production of ethyl acetate. But it is probably

not the biggest limitation, since further synthesis should have been possible in the stationary phase. Therefore, ethanol was supplemented to the growing cultures and performance compared to unsupplemented cultures. Again, *C. ljungdahlii* ATF1 Sc was the only strain capable of producing ethyl acetate (Figure 1B and Figure S3). Ethyl acetate titers were slightly higher ($p = 0.05$) with the ethanol supplementation reaching 32.29 ± 1.03 mg/L, while the general performance in terms of acetate and ethanol titers remained similar ($p > 0.1$) (Figure 1C). Thus, *C. ljungdahlii* ATF1 Sc was the only strain capable of producing ethyl acetate, albeit at low titers. Noteworthy, ethyl acetate production was growth-dependent in this case, unlike in yeast [33], probably due to the lack of available acetyl-CoA in the stationary phase. Ethanol availability in early growth phases was shown only to slightly increase the production, and therefore, ethyl acetate production must be limited by other factors.

3.2 | Ethyl Acetate Production in Autotrophic Conditions

The production of ethyl acetate during gas fermentation from CO_2 and H_2 was evaluated with the aforementioned strains (Figure S4) expressing their corresponding AAT (ATF1 Sc, ATF2 Sc, ATF1 Km, and EAT1 Km). After 3 weeks of cultivation, no ethyl acetate was detected in any of the strains (limit of detection: 0.1 mg/L). In *C. ljungdahlii* ATF1 Sc cultures, which were able to produce ethyl acetate heterotrophically, acetate was continuously produced up to 1.33 ± 0.49 g/L, while ethanol reached 0.25 ± 0.04 g/L in the second week and remained stable afterwards. When compared to the heterotrophic growth on fructose, titers of acetate and especially ethanol as well as the OD_{600} were much lower ($p < 0.001$). However, the pH obtained at the end of both experiments was similar ($p > 0.1$). The vitamin folic acid (often referred to as folate) plays a big role as a cofactor of the methyl branch of the Wood-Ljungdahl pathway, and since the medium did not contain any source of folate, forcing its de novo biosynthesis by *C. ljungdahlii*, we decided to add it to promote the autotrophic metabolism and with that ethyl acetate production. With the addition of folate (Figure 2A), *C. ljungdahlii* ATF1 Sc produced 5.08 ± 0.31 mg/L of ethyl acetate autotrophically after 1 week, which increased to 7.38 ± 0.43 mg/L after the second week and remained stable for the third week. Formate addition (Figure 2A), which bypasses the first reaction of the methyl branch of the Wood-Ljungdahl pathway (CO_2 reduction to formate with the expense of reduced ferredoxin), resulted in similar ethyl acetate titers. In this case, *C. ljungdahlii* ATF1 Sc produced 6.57 ± 0.01 mg/L of ethyl acetate after 1 week and remained stable for the rest of the 3 weeks. Compared to the autotrophic cultivation without additions, more acetate was produced when formate was added ($p = 0.005$), while no significant increase was triggered by the addition of folate ($p > 0.1$). However, ethanol was produced at significantly higher titers in response to the addition of folate and formate ($p = 0.001$, $p = 0.002$; respectively). OD_{600} and pH were similar in the three conditions ($p > 0.1$). So, it is possible that the production of ethyl acetate following the addition of folate and formate was due to the increase in ethanol availability.

In parallel, we realized that the mitochondrial transit peptide of the enzyme EAT1 Km (Figure 2B) could be preventing proper expression of this enzyme. In order to resolve this issue, a truncated version of this enzyme was constructed (thereafter referred to as trEAT1 Km) and expressed in *C. ljungdahlii*. Growing on CO_2 and H_2 with folate supplementation, *C. ljungdahlii* trEAT1 Km produced 0.29 ± 0.06 mg/L of ethyl acetate autotrophically after one week. With fructose, ethyl acetate titers improved up to 2.88 ± 1.08 mg/L (Figure 2C), which were still not better than the autotrophic titers of *C. ljungdahlii* ATF1 Sc. Thus, even with ethyl acetate production enabled for trEAT1 Km, the production of ATF1 Sc was shown to be higher.

3.3 | Fumarate as an Additional Electron Acceptor

In heterotrophic conditions, CO_2 released from fructose catabolism acts as an electron acceptor through the Wood-Ljungdahl pathway positively influencing acetate synthesis and contributing to the turnover of reducing equivalents. However, due to the very tight stoichiometry of acetogenesis, CO_2 availability could be limited considering the high headspace volume and the poor mixing of the liquid with the gas phase, even when high CO_2 solubility is expected. In order to test this idea and its effect on performance and ethyl acetate production, the addition of another electron acceptor, namely fumarate was explored. Fumarate could act as an electron acceptor in case of CO_2 limitation, if the genes CLJU_RS11215 and CLJU_RS11225, encoding for a fumarate reductase, could form a functional enzyme. It can also act as an electron and carbon source through the branched tricarboxylic acid cycle, in case of fructose limitation as the preferred carbon source.

The biological standard potential ($E^{\circ\prime}$) of CO_2 reduction to acetate is −290 mV, while that for reduction of fumarate to succinate is +33 mV [34]. Therefore, fumarate should be the preferred choice as electron acceptor, over CO_2. However, the tight regulation and the importance of the Wood-Ljungdahl pathway for the turnover of reducing equivalents and redox balancing suggest that the reduction of CO_2 is not merely about thermodynamic favorability, but also plays a crucial role in cellular metabolism and energy conservation. This multivalent function of fumarate was first evaluated with the wild-type strain of *C. ljungdahlii*, which was cultured heterotrophically on fructose with fumarate, fructose alone, and fumarate alone (Figure 3A). Fumarate supplementation led to a 2.2-fold increase in acetate titers ($p = 0.06$) compared to fructose alone. Succinate titers increased 5.5-fold, but this change was not statistically significant ($p > 0.1$). On the other hand, growth on fumarate alone led to lower OD_{600} ($p = 0.03$) and pH drop ($p = 0.03$) compared to the other conditions, without producing ethanol or succinate. In all conditions, CO_2 and H_2 were produced heterotrophically (Figure S5), supporting the idea that not all reducing equivalents were recycled through CO_2 reduction in the Wood-Ljungdahl pathway, but rather through H_2 formation. H_2 production was found to be higher when growing on fructose alone ($p = 0.06$), which would indicate that fumarate indeed acts as an assistant electron acceptor, reducing the formation of H_2.

Next, the ethyl acetate producing strain *C. ljungdahlii* ATF1 Sc was cultured heterotrophically on fructose with fumarate supplementation, on fumarate alone and mixotrophically on CO_2 and H_2 with fumarate supplementation (Figure 3B). As expected, fumarate slightly improved the performance when growing on

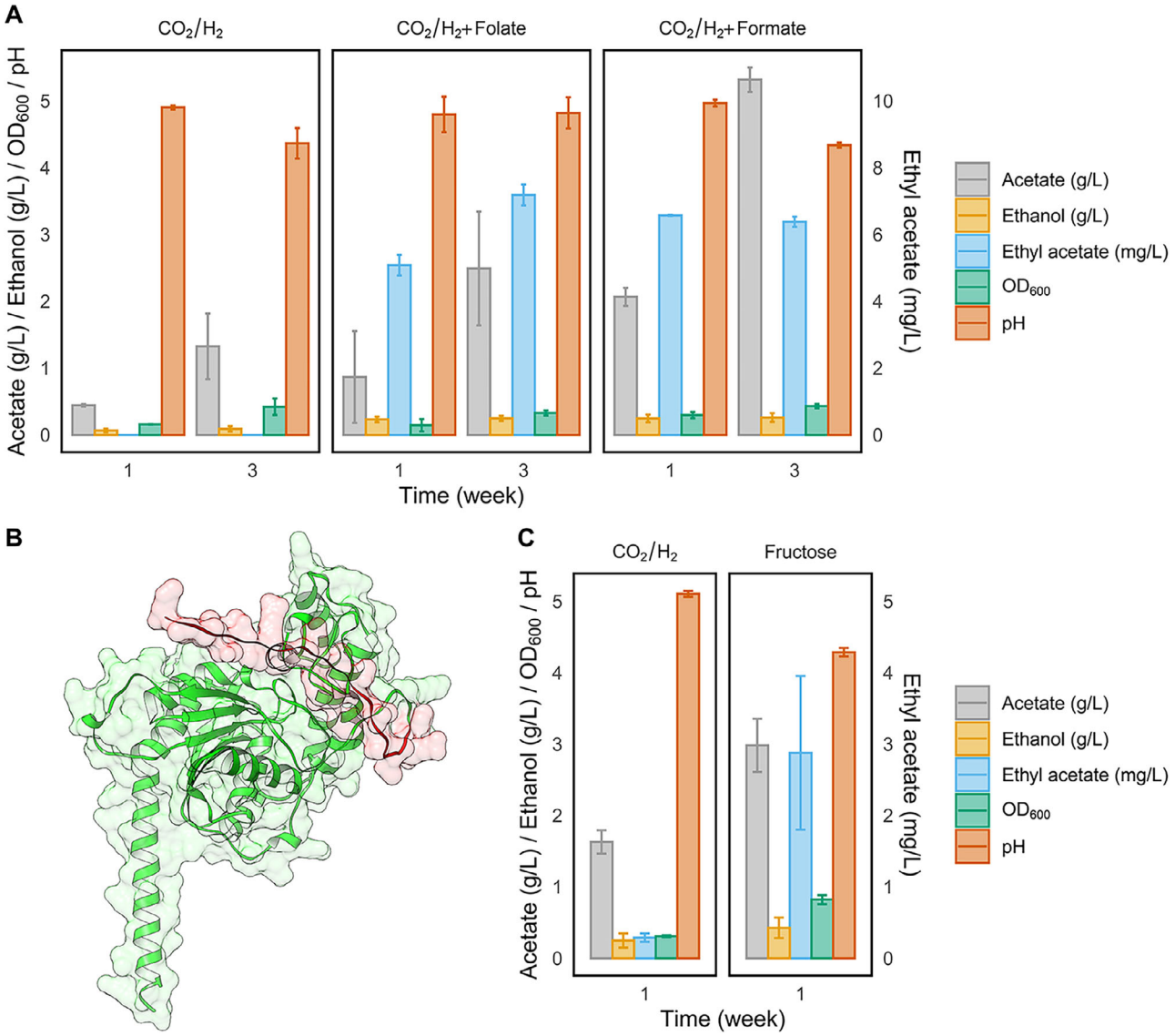

FIGURE 2 | Ethyl acetate production by *C. ljungdahlii* under autotrophic conditions. (a) Ethyl acetate production in autotrophic conditions using the strain *C. ljungdahlii* ATF1 Sc. Performance differences due to the supplementation of folate (0.1 mg/L) and formate (0.1 g/L) were evaluated. Key parameters are reported for 1 and 3 weeks after the start of the cultivation. The CO_2/H_2 (20:80 v/v; 2.5 bar) atmosphere was replaced weekly to avoid gaseous substrate limitation. Data from the second week is omitted here for the sake of clarity. (b) Predicted protein structure model of EAT1 Km, indicating the predicted mitochondrial transit peptide in red. The structural model was obtained from AlphaFoldDB (Uniprot ID: W0T4A7). (c) Ethyl acetate production by *C. ljungdahlii* trEAT1 Km growing on CO_2/H_2 with folate supplementation (0.1 mg/L), compared to heterotrophic growth on fructose. Key parameters are reported for 1 week after the start of the cultivation. The experiments were all conducted in triplicates ($n = 3$) and mean ± standard deviation is represented.

fructose compared to the unsupplemented cultivation in terms of acetate ($p = 0.03$) and ethyl acetate ($p = 0.03$) production, which reached 4.14 ± 0.29 g/L and 37.51 ± 9.44 mg/L, respectively, after 1 week. Contrary to before, ethyl acetate titer improvement seemed to not be linked to increased ethanol availability, which titers did not improve ($p > 0.1$). Succinate was produced at low titers (52.33 ± 5.13 mg/L) and fumarate was hardly consumed during the experiment (>4 g/L not consumed), indicating that fumarate probably acted just as a backup electron acceptor when CO_2 was scarce. Since ethyl acetate titers increased with fructose and fumarate together, the experiment was also performed with the other three generated strains, but again none of them was able to produce ethyl acetate (Figure S6). When fumarate was provided as the sole carbon and electron source, very poor growth was observed (as with the wild-type), but no acetate was detected (<1 mg/L). Just minor amounts of ethanol (0.24 ± 0.04 g/L) and ethyl acetate (3.82 ± 3.31 mg/L) were produced after 1 week, while fumarate was barely consumed again (>4 g/L not consumed) and succinate was produced at 21.67 ± 5.13 mg/L. Under mixotrophic conditions, with CO_2 and H_2 along with fumarate, *C. ljungdahlii* ATF1 Sc produced 1.50 ± 0.35 g/L of acetate, 0.05 ± 0.03 g/L of ethanol and 6.86 ± 0.26 mg/L of ethyl acetate after one week. In the presence of CO_2 and H_2 as electron acceptor and additional electron donor, respectively, fumarate was converted mainly to acetate (as with the wild-type on fumarate alone). OD_{600} and ethyl acetate titers were slightly higher than when fumarate

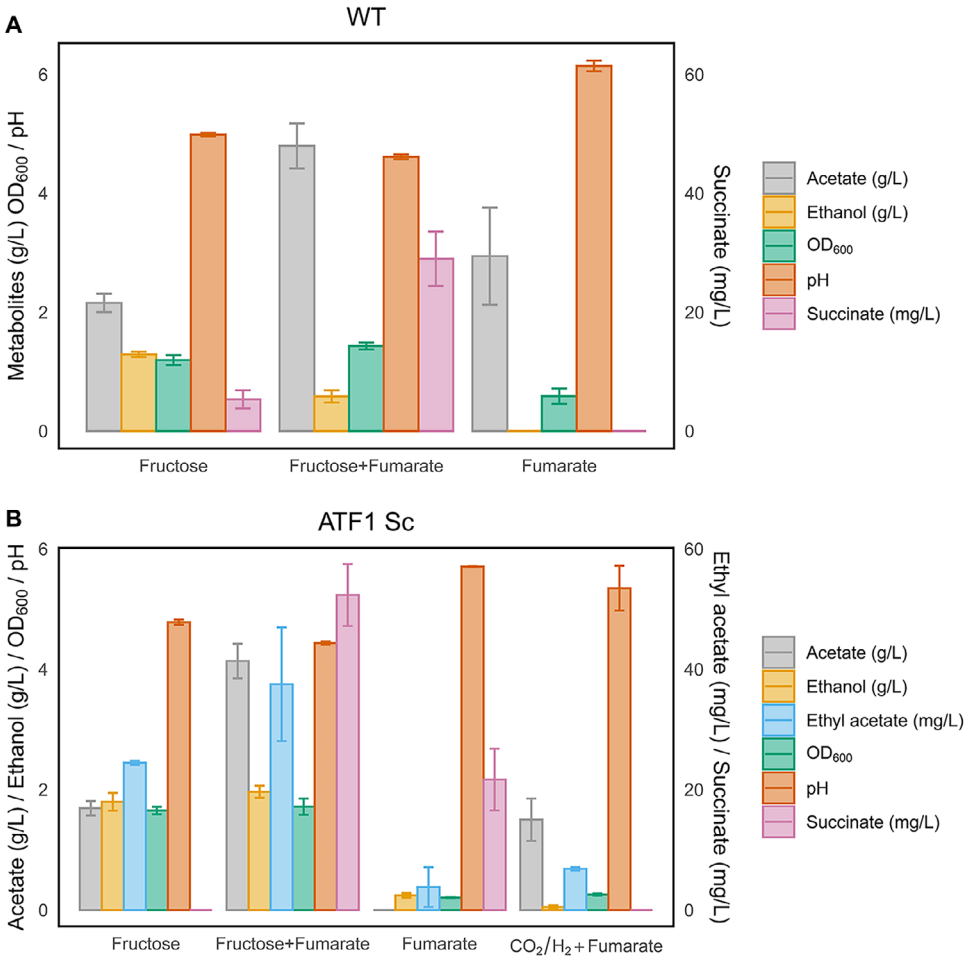

FIGURE 3 | Double role of fumarate in the metabolism of *C. ljungdahlii*. (a) Evaluation of the role of fumarate as electron acceptor and as electron and carbon source in *C. ljungdahlii* wild-type in combination with fructose and alone. Key parameters are reported for 1 week after the start of the cultivation. (b) Effect of fumarate addition on ethyl acetate production by the strain *C. ljungdahlii* ATF1 Sc. Performance differences due to the supplementation of fumarate in heterotrophic and mixotrophic conditions were evaluated. Key parameters are reported for 1 week after the start of the cultivation. The experiments were all conducted in triplicates ($n = 3$) for 1 week. Mean ± standard deviation is represented.

alone was used ($p = 0.05$). On the contrary, ethanol titers were slightly lower ($p = 0.05$). Ethyl acetate titers were comparable to those obtained autotrophically with the addition of folate and formate ($p > 0.1$), even when ethanol titers were extremely low in this case, indicating that the changes in ethyl acetate titers could be attributed to general redox balance and homeostasis, rather than ethanol availability as previously suggested. Overall, these findings suggest that ethyl acetate production is severely limited by the availability of acetyl-CoA, which is linked to central metabolism and redox balance.

3.4 | Scouting the Promiscuity of AATs

As evidenced by the results presented so far, the production of ethyl acetate using AATs was not characterized by high titers or yields. And our further analysis indicates that this seems to be linked to the fact that AAT activity is closely linked to central metabolism and is energetically expensive. In addition, the activity of these enzymes may not be very specific and other esterified products may be produced. To assess this, the five AAT-expressing strains and the wild-type strain were cultured in headspace vials to analyze volatiles produced by all strains using headspace GC-MS.

In a global partial least squares discriminant analysis (PLS-DA) of all detected compounds, the scores plot showed a clear separation between the different strains along the primary components (Figure 4A), which implied relevant differences in their volatile production. The normalized peak intensities of the main volatiles identified in the samples were statistically analyzed using ANOVA to determine significant differences in volatile levels among the strains. The ANOVA results, as shown in Figure 4B, revealed several volatiles with statistically significant changes ($p < 0.01$), indicating that these metabolites were differentially produced depending on the strain, and thus, on the AAT expressed. A tentative identification of volatiles using GC-MS was performed. Metabolite assignments were made based on their m/z values and comparison of their mass spectra with the NIST database, allowing volatile categorization into general metabolite classes. However, these identifications were not definitely confirmed and should be considered preliminary. Some of the database hits referred to esterified compounds, which align with the concept of enzyme promiscuity, suggesting that the

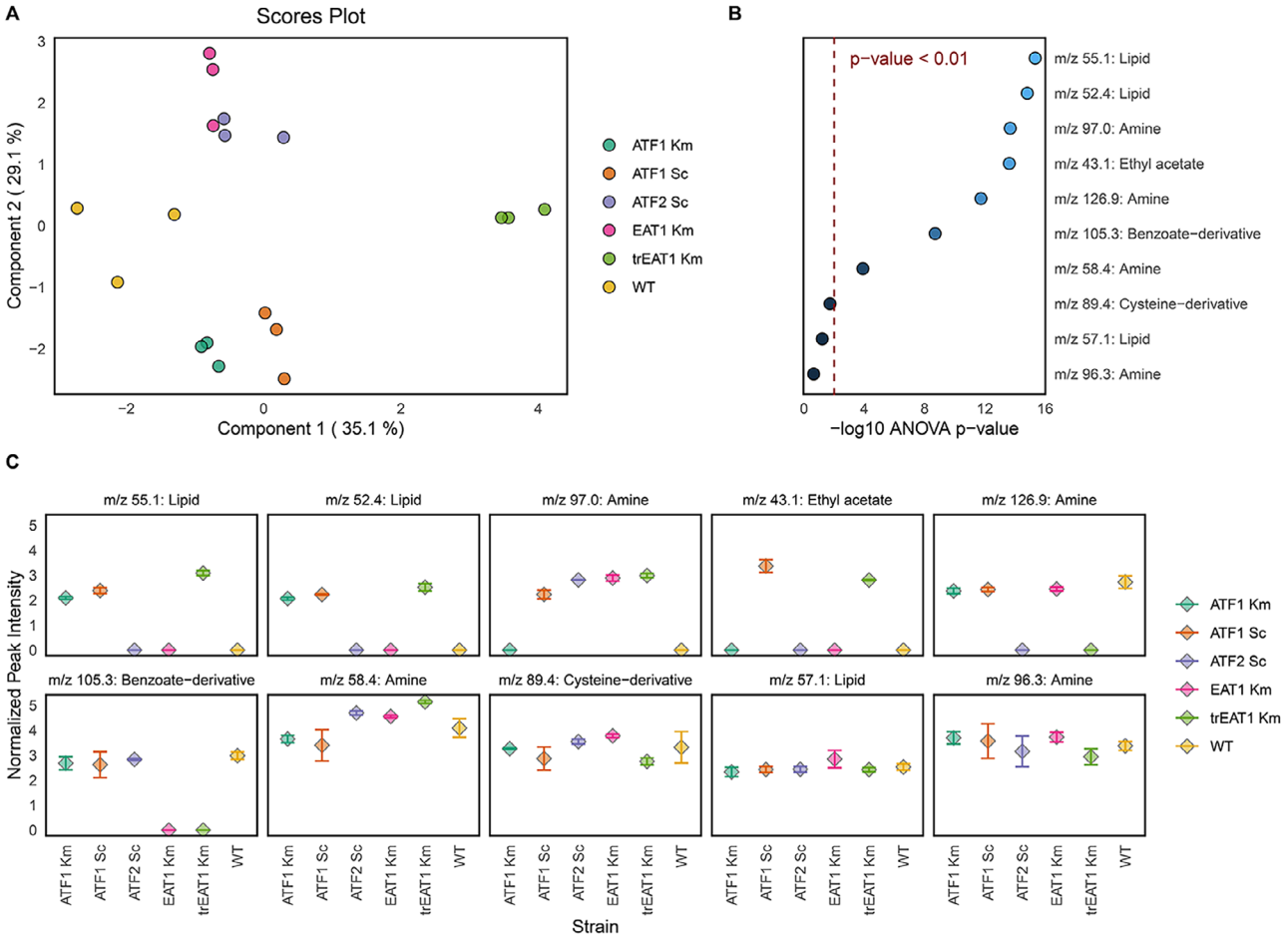

FIGURE 4 | Volatile production in the AAT-expressing and the wild-type *C. ljungdahlii* strains. (a) Scores plot of partial least squares discriminant analysis (PLS-DA), showing the two main components and the proportion of the total variance in the data explained by each component. Each dot represents a replicate of the corresponding strain (color-specific). (b) ANOVA results for each of the volatiles included in the analysis. A threshold of 0.01 (p value) was chosen to determine statistical significance. (c) Log-normalized peak intensity variation among strains of the volatiles included in the analysis. Each diamond represents the mean relative abundance of a specific metabolite, with error bars indicating the standard deviation. Metabolite names only indicate a general metabolite description, based on best MS spectra matches against the NIST database. The experiments were all conducted in triplicates ($n = 3$) for 1 week.

AATs involved may catalyze the formation of multiple esterified products. The most interesting metabolites in this regard were the ones found to be uniquely produced in AAT-expressing strains (Figure 4C). Specifically, three metabolites, one amine (m/z 97.0) and two lipids (m/z 52.4 and m/z 55.1), were always detected in strains capable of producing ethyl acetate. Others were also found in certain nonproducing strains. Besides this, we also detected several relevant volatiles, which are also produced by the wild-type strain. Interestingly, two metabolites that were also found in the wild-type (m/z 105.3 and m/z 126.9), disappeared entirely in some AAT-expressing strains, suggesting a possible metabolic shift or suppression in this metabolic background.

4 | Discussion

Following the evaluation of five different AATs, only the heterologous expression of ATF1 from *S. cerevisiae* and trEAT1 Km from *K. marxianus* led to the production of ethyl acetate. The best producing strain *C. ljungdahlii* ATF1 Sc was able to produce up to 37.51 ± 9.44 mg/L (0.43 ± 0.12 mM) of ethyl acetate in serum bottles under heterotrophic conditions with fructose and fumarate, and up to 7.38 ± 0.43 mg/L (0.08 ± 0.005 mM) of ethyl acetate under autotrophic conditions with CO_2 and H_2 (in the presence of folate). These concentrations are very low, even for *C. ljungdahlii*, although they are in line with other attempts of producing substances that are not native to the genus *Clostridium* or related genera [22, 23]. During the conduct of this study, other researchers published similar results for *C. autoethanogenum* [25], in which up to 26.43 mg/L of ethyl acetate was produced when grown on CO. However, there are notable differences between the two studies: our work focuses on *C. ljungdahlii*, instead of *C. autoethanogenum*, and demonstrates ethyl acetate production using a combination of CO_2 and H_2, which represents a more energy-limited feedstock compared to CO. Due to the enormous energy limitations that acetogenic bacteria are subjected to, it has been proven in the past that diverting the metabolic flux from the production of acid and alcohols (i.e., acetate and ethanol) is really challenging. However, this seems to not be the case for nonacetogenic *Clostridium* spp., since *Clostridium saccharoperbutylacetonicum* was successfully engineered for the production of butyl acetate and butyl butyrate in the gram

per liter range from carbohydrates [24]. Similarly, high titers of a broad spectrum of esters were produced in modified *E. coli* strains [32]. In contrast, the yeast *K. marxianus*, as a native producer of ethyl acetate, is capable of synthesizing up to 0.5 g/g biomass/h, which is far from the figures of any other biological platform for the production of ethyl acetate [35]. Nevertheless, exploring the autotrophic production of ethyl acetate and other compounds from gaseous substrates would have the advantage of using virtually unlimited, renewable, nonfood feedstocks, highlighting the importance of gas fermentation research as the future of chemical production.

As possible reasons for the challenging production of ethyl acetate, a low ethanol availability during lag and early exponential phases (acidogenesis) was hypothesized initially. Ethanol supplementation slightly increased ethyl acetate titers, but the level of increase was not correlated with the amount of supplemented ethanol. This was also shown for *C. autoethanogenum* for which only a small improvement was achieved with ethanol addition [25]. Here, fumarate was shown to boost ethyl acetate production in a similar magnitude to ethanol, indicating that besides ethanol availability, the bottleneck could be a problem of diverting flux through a reaction that relies on acetyl-CoA in very energy-limiting growth conditions. Acetogenic bacteria like *C. ljungdahlii* are experts in energy saving, even when growing heterotrophically. Their metabolic pathways, particularly the Wood-Ljungdahl pathway, are tightly controlled and highly efficient. These bacteria precisely regulate their energy use, ensuring no unnecessary expenditure. This strict regulation allows them to maximize energy conservation, converting substrates into essential compounds with minimal ATP investment, and thriving even in resource-limited environments [36]. Further genetic engineering could be applied to effectively divert metabolic flux toward ethyl acetate. For instance, in our recent attempts to enhance ethyl acetate production, we tried to express the acyl-CoA synthetase *fadK* from *E. coli* K12 into *C. ljungdahlii* ATF1 Sc, which should convert acetate back to acetyl-CoA by investing ATP to increase acetyl-CoA availability. However, this modification led to the generation of nonviable transformants (data not shown). Alternatively, other approaches like the use of acid-catalyzed esterification or other enzymes such as lipases or esterases offer distinct advantages in terms of reaction conditions, specificity and substrate versatility compared to AAT [37–39], which should be explored.

Despite these limitations, we could also show that the enzyme promiscuity of AATs has facilitated the production of a diverse yet specific range of tentative esterified compounds, highlighting the potential of AAT-based bioprocesses. This promiscuity, characterized by the ability of the enzyme to act on a variety of substrates, not only broadens the spectrum of esterified products but also enhances the potential for the production of novel compounds with high economic and functional value. However, we acknowledge that in many bioprocesses, the generation of multiple products can present challenges, particularly in terms of product recovery and purification. Therefore, while AATs promiscuity offers intriguing opportunities for expanding the product portfolio, its application requires careful consideration and optimization to mitigate such challenges and ensure process efficiency.

As a concluding remark, ethyl acetate was produced as a proof of principle through the heterologous expression of AATs leading to low titers that could be explained by the limited energy and redox state of *C. ljungdahlii*, which forces the metabolic flux toward the production of the most energetically efficient metabolites. The effect of the addition of fumarate in *C. ljungdahlii* cultures was never explored before and led to rearrangements in the metabolic flux caused by its double role as electron acceptor and electron donor. Finally, AAT promiscuity was shown to be key to expand the spectrum of esterified compounds, enhancing its potential for generating high-value products.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.