Review

Advanced aspects of acetogens

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HIGHLIGHTS

- Presentation of the current taxonomy of known acetogens.
- Appearance of recently isolated and described acetogens.
- Discussion of the physiology and diversity of redox carriers in acetogens.
- Summary of metabolic engineering efforts using specific acetogens.
- Features of mixed and co-cultures as well as bioreactor technologies.

GRAPHICAL ABSTRACT
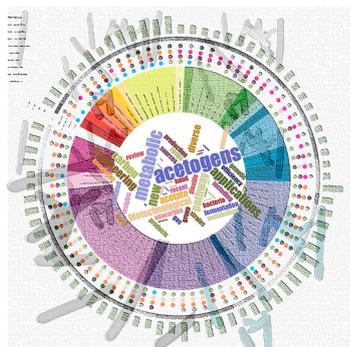
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ABSTRACT

Acetogens are a diverse group of anaerobic bacteria that are capable of carbon dioxide reduction and have for long fascinated scientists due to their unique metabolic prowess. Historically, acetogens have been recognized for their remarkable ability to grow and to produce acetate from different one-carbon sources, including carbon dioxide, carbon monoxide, formate, methanol, and methylated organic compounds. The key metabolic pathway in acetogens responsible for converting these one-carbon sources is the Wood-Ljungdahl pathway. This review offers a comprehensive overview of the latest discoveries that are related to acetogens. It delves into a variety of topics, including newly isolated acetogens, their taxonomy and physiology and highlights novel metabolic properties. Additionally, it explores metabolic engineering strategies that are designed to expand the product

range of acetogens or to understand specific traits of their metabolism. Lastly, the review presents innovative gas fermentation techniques within the context of industrial applications.

1. Introduction − Basic physiology of acetogens

Molecules containing only one single carbon atom (C_1 compounds) exist in gaseous or liquid form at standard atmospheric pressure (102 kPa) and temperatures below 100 °C. Examples include carbon dioxide (CO_2), carbon monoxide (CO), formaldehyde, formic acid, methane (CH_4), or methanol. Certain microbes are able to utilize these gaseous C_1 compounds as carbon and/or energy sources for growth or cell maintenance through various metabolic pathways. Microbes that are capable of producing organic carbon compounds from inorganic one-carbon (C_1) compounds alone are termed autotrophs, and this process is known as autotrophy. The concept of autotrophy was introduced by Wilhelm Pfeffer (1897), in association with oxygenic photosynthesis and CO_2 fixation in plants. Fischer et al. (1932) described an anaerobic mode of autotrophy, demonstrating that CO is partially converted into CH_4 via the water–gas shift reaction and CO_2 reduction driven by hydrogen (H_2) oxidation using mixed cultures of methanogens. They also observed the conversion of $H_2 + CO_2$ into acetate using anaerobic digested sludge (Fischer et al., 1932). In 1936, Klaas T. Wieringa isolated and described the first known autotrophic bacterium, *Clostridium aceticum*, able to grow on $H_2 + CO_2$ while producing acetate (Wieringa, 1936, 1939). Unfortunately, the strain was lost, hindering further research for decades (Bengelsdorf et al., 2018). In Fontaine et al. (1942) isolated a thermophilic bacterial strain from horse feces, which was formerly known as *Clostridium thermoaceticum* (now termed *Moorella thermoacetica*). This acetogen played a crucial role in illuminating the biochemical reactions (acetogenesis) required for bacterial anaerobic CO_2 fixation. One of the intriguing aspects of all acetogens is their ability to synthesize acetate and other two-carbon (C_2) and four-carbon (C_4) compounds from various C_1 sources such as CO_2 or CO, and from more complex methylated organic compounds. They utilize a metabolic pathway called the Wood-Ljungdahl pathway (WLP), which is also known as the reductive acetyl-CoA pathway, to convert C_1 carbon sources to acetyl-CoA, which is further converted to mainly acetate in most acetogens. During the last eight decades, numerous comprehensive reviews and book chapters have covered this subject extensively. For bacterial acetogenesis, recommended literature includes Ljungdahl (1986), Wood (1991), Ragsdale (2004), Ragsdale and Pierce (2008), Drake et al. (2008), Schuchmann and Müller (2014), and Müller (2019). Microbiologists consider the reductive acetyl-CoA pathway and the microbes utilizing it for H_2-dependent carbon and energy metabolism as ancient (Martin, 2020; Basen and Müller, 2017).

For acetogens, the conversion of $H_2 + CO_2$ into formate, acetate, and pyruvate involves around ten enzymes and almost as many organic cofactors. The reductive acetyl-CoA pathway stands out as the only metabolic route capable of both carbon reduction and energy conservation simultaneously. The pathway itself is ATP-neutral but is associated with chemiosmotic mechanisms that conserve energy. Moreover, among the seven known CO_2 fixation pathways it is the only one using CO as an intermediate. Its exergonic nature is based on generating carboxyl groups via CO reduction in the carbonyl branch. Bacterial acetogens employ tetrahydrofolate (THF, also abbreviated as H_4F) as a key C_1-transferring cofactor and ATP hydrolysis for CO_2 activation to form formyl-THF in the methyl branch of the WLP (Ljungdahl, 1986). The formyl group is reduced stepwise to a methyl group. The key enzyme of the WLP, CO dehydrogenase/acetyl-CoA synthase (COdh/Acs) then takes the methyl group and fuses it to [CO] and CoA to yield acetyl-CoA (Ragsdale, 2004). Acetyl-CoA then in part serves as a precursor for biomass, but is mainly catabolically converted to acetate by phosphotransacetylase (Pta) and acetate kinase (Ack), yielding ATP. Some acetogens also produce ethanol or butyrate from acetyl-CoA,

under certain conditions (Bertsch and Müller, 2015; Ragsdale, 2008). The energy required originates from the oxidation of H_2 to create an ion gradient across cell membranes. Therefore, acetogens harbor typically one of two different enzyme complexes depending on the mechanism that is used to create the ion gradient. One of these is the ferredoxin: NAD^+ oxidoreductase complex (also known as <u>Rhodobacter</u> <u>n</u>itrogen <u>fix</u>ation (Rnf) complex), which couples electron transfer from reduced ferredoxin to NAD^+ with electrogenic movement of Na^+ or H^+ out of the cell (Biegel and Müller, 2010). The other enzyme complex occurs mainly in thermophilic bacteria and depends on an energy-converting hydrogenase (Ech) complex, which couples H_2 metabolism directly to the electrochemical ion gradient across the membrane (Schoelmerich and Müller, 2019; Katsyv et al., 2021; Baum et al., 2024).

This review provides an overview of recent developments in the field of acetogenesis, including recently described bacterial species and new insights into novel metabolic properties with a focus on redox and energy metabolism, which are related to very specialized enzymes and enzyme complexes. Moreover, metabolic engineering approaches are discussed, which are aimed to broaden the product spectrum of acetogens, and thus opening new avenues for biotechnological applications. Finally, novel gas fermentation approaches in pure or mixed cultures are presented and discussed in an industrial context.

2. Recently isolated and described acetogens

In total, 138 strains within 26 bacterial families have been isolated since the first known autotrophic bacterium was described in 1936 (Section 1). Within the last 5 years up to 10 newly isolated acetogens were described from various habitats all over the world (Table 1), and presumably, there are more in nature to be found. Among them are acidophilic, alkaliphilic, halophilic, and thermophilic strains classified in different taxa. Here, we summarize the major physiological features of these new strains.

The genus *Aceticella* is the most recently added member to the family of *Thermoanaerobacteraceae* (Wiegel, 2015b). Currently, *Aceticella autotrophica*, which was isolated from a hot spring in Kamchatka, is the only representative of this genus (Frolov et al., 2023). The most closely related acetogen is *Thermoanaerobacter kivui*. Both species grow acetogenically with $H_2 + CO_2$, and *Ac. autotrophica* was adapted to grow on CO, in a similar manner as *T. kivui* (Weghoff et al., 2016). A major physiological difference between both species is *Ac. autotrophica*'s inability to grow organoheterotrophically. In fact, *Ac. autotrophica* represents the first isolated bacterium of an obligately autotrophic acetogen (Frolov et al., 2023). *Ac. autotrophica* is hypothesized to employ a hydrogen-dependent CO_2-reductase (HDCR) complex in the methyl branch of the WLP, and H_2 is supposed to be oxidized by an electron-bifurcating hydrogenase (Section 4). Most interestingly, Frolov et al. (2023) performed phylogenetic analyses on the *acs* genes. The origin of these genes that are key to acetogenesis is different in both species. Therefore, it was suggested that acetogenesis is a trait acquired by independent horizontal gene transfer events, rather than originating in the *Thermoanaerobacteraceae* (and lost by all the other non-acetogenic species).

The recently proposed family of *Alkalibacteraceae* comprises the two genera *Alkalibacter* and *Alkalibaculum* (Chuvochina et al., 2023). Only for the later genus, two CO-utilizing acetogens, *Alkalibaculum bacchi* (Section 3.2) and the recently described *Alkalibaculum sporogenes* are known (Khomyakova et al., 2020). *Al. sporogenes* is an alkaliphilic acetogen that was isolated from a mud volcano. It is motile by peritrichous flagella and slightly curved to rod-shaped, endospore-forming bacterium with optima for growth at 30 °C and pH at 8.0. The alkaliphilic

Table 1
Major physiological and genomic characteristics of acetogens (type and non-type strains) listed according taxonic families.

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Family Acetohalobiaceae												
Acetohalobium arabaticum	Z-7288	DSM 5501[T]	$H_2 + CO_2$, CO	acetate	38-40	7.6-8.0	Zhilina & Zavarzin, 1990	CP002105	no	2,469,596	36.6	Sikorski et al., 2010
Family Acetonemataceae												
Acetonema longum	APO-1	DSM 6540[T]	$H_2 + CO_2$	acetate, butyrate	30-33	7.8	Kane & Breznak, 1991	AFGF00000000	n.d.	4,323,011	50.4	Chen et al., 2011
Family Alkalibacteraceae												
Alkalibaculum bacchi	CP11	DSM 22112[T]	$H_2 + CO_2$, CO	acetate, CO_2, ethanol	37	8.0-8.5	Allen et al., 2010	QNRX00000000	n.d.	3,098,941	34.5	n.p.
Alkalibaculum sporogenes	M08 DMBT	KCTC 15840[T]	CO	acetate	30	8.0	Khomyakova et al., 2020	WHNX0000000	n.d.	3,505,994	32.3	Khomyakova et al., 2020
Family Calderihabitantaceae												
Calderihabitans maritimus	KKC1	DSM 26464[T]	CO	$H_2 + CO_2$, acetate	65	7.0-7.5	Yoneda et al., 2013	BDGJ00000000	n.d.	3,064,849	47.0	Omae et al., 2017
Family Carboxydocellaceae												
Carboxydocella sporoproducens	KarT	DSM 16521[T]	CO	$H_2 + CO_2$	60	6.8	Slepova et al., 2006	FUXM00000000	n.d.	2,578,924	49.1	n.p.
Carboxydocella thermautotrophica	41	DSM 12326[T]	CO	$H_2 + CO_2$	58	7.0	Sokolova et al., 2001	CP028514	yes	2,743,125	49.1	Toshchakov et al., 2018
Family Carboxydothermaceae												
Carboxydothermus ferrireducens	JW/AS-Y7	DSM 11255[T]	$H_2 + CO_2$, CO	n.r.	65	6.0-6.2	Slobodkin et al., 1997b; Slobodkin et al., 2006	ATYG00000000	n.d.	2,441,992	41.9	n.p.
Carboxydothermus hydrogenoformans	Z-2901	DSM 6008[T]	CO	$H_2 + CO_2$	70–72	6.8–7.0	Svetlichny et al., 1991	CP000141	no	2,401,520	42.1	Wu et al., 2005
Carboxydothermus islandicus	SET IS-9	DSM 21830[T]	CO	$H_2 + CO_2$	65	5.5-6.0	Novikov et al., 2011	BDJL00000000	n.d.	2,386,596	42.0	Fukuyama et al., 2018
Carboxydothermus pertinax	Ug1	DSM 23698[T]	$H_2 + CO_2$, CO	$H_2 + CO_2$	65	6.0-6.5	Yoneda et al., 2012	BDJK00000000	n.d.	2,465,639	40.7	Fukuyama et al., 2018
Carboxydothermus siderophilus	1315	DSM 21278[T]	CO + FeIII	$H_2 + CO_2$ + FeII	65	6.5-7.2	Slepova et al., 2009	no genome sequence				
Family Clostridiaceae												
"Clostridium autoethanogenum"	JA1-1	DSM 10061	$H_2 + CO_2$, CO	2,3-butanediol, acetate, ethanol	37	5.8-6.0	Abrini et al., 1994; Köpke et al., 2011	CP006763	no	4,352,205	31.1	Brown et al., 2015
Clostridium bovifaecis	BXX	JCM 32382[T]	$H_2 + CO_2$, CO	acetate	30	7	Zhu et al., 2018	CP046522	no	3,727,984	32.5	Yao et al., 2020
Clostridium carboxidivorans	P7	DSM 15243[T]	$H_2 + CO_2$, CO	acetate, ethanol, butyrate, butanol, hexanote, hexanol	38	5.0-7.0	Liou et al., 2005	CP011803-CP011804	yes	5,752,782	32.5	Li et al., 2015
"Clostridium coskatii"	*	PTA-10522	$H_2 + CO_2$, CO	acetate, ethanol	37	5.8-6.5	Zahn & Saxena, 2012	LROR00000000	n.d.	4,511,956	31.1	Bengelsdorf et al., 2016
Clostridium drakei	SL1	DSM 12750[T]	$H_2 + CO_2$, CO	acetate, ethanol, butyrate, hexanoate	30-37	5.4-7.5	Küsel et al., 2000; Liou et al., 2005; Gößner et al., 2008	JIBU00000000	n.d.	5,578,774	29.5	Jeong et al., 2014

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Table 1 (*continued*)

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Clostridium drakei	FP	DSM 14770	$H_2 + CO_2$, CO	acetate, butyrate	25-30	4.8-6.8	Gößner et al., 2008	no genome sequence				
Clostridium ljungdahlii	PETC	DSM 13528[T]	$H_2 + CO_2$, CO	2,3-butanediol, acetate, ethanol	37	6.0	Tanner et al., 1993; Köpke et al., 2010; Köpke et al., 2011	CP001666	no	4,630,065	31.1	Köpke et al., 2010
Clostridium luticellarii	FW431	DSM 29923[T]	$H_2 + CO_2$, CO	acetate, butyrate	37	6.5	Wang et al., 2015; Mariën et al., 2023	PVXP00000000	n.d.	3,754,778	35.0	Poehlein et al., 2018b
Clostridium magnum	WoBdP1	DSM 2767[T]	$H_2 + CO_2$	acetate	30-32	7.2	Schink, 1984; Bomar et al., 1991	LWAE00000000	n.d.	6,634,930	32.1	Uhlig et al., 2016
Clostridium muellerianum	P21	DSM 111390[T]	$H_2 + CO_2$, CO	acetate, butyrate, caproate, ethanol, hexanol	30-40	6.5	Doyle et al., 2022	JABBNI000000000	n.d.	5,645,749	29.5	Doyle et al., 2022
"Clostridium ragsdalei"	P11	DSM 15248	$H_2 + CO_2$, CO	2,3-butanediol, acetate, ethanol	37	6.3	Huhnke et al., 2008; Köpke et al., 2011; Bengelsdorf et al., 2016	LROS00000000	n.d.	4,424,992	31.0	Bengelsdorf et al., 2016
Clostridium scatologenes	*	DSM 757[T]	$H_2 + CO_2$, CO	acetate, ethanol, butyrate	37-40	n.r.	Küsel et al., 2000; Liou et al., 2005	CP009933	no	5,749,410	30.3	Zhu et al., 2015
Clostridium thailandense	PL3	DSM 111812[T]	$H_2 + CO_2$	acetate	30	7	Chaikitkaew et al., 2022	JAEEGC000000000	n.d.	6,484,535	31.1	Chaikitkaew et al., 2022
Clostridium sp.	AWRP	n.dp.	$H_2 + CO_2$, CO	2,3-butanediol, acetate, ethanol	37	6.0-6.5	Kwon et al., 2022	CP029758	no	4,579,114	31	Lee et al., 2019
Family Desulfotomaculaceae												
Desulfotomaculum nigrificans	CO-1-SRB	DSM 14880	CO	$H_2 + CO_2$, CO	55	6.8-7.2	Parshina et al., 2005; Visser et al., 2014	CP002736	no	2,892,255	46.5	Visser et al., 2014
Family Desulfovirgulaceae												
Desulfofundulus thermobenzoicus ssp. *thermosyntrophicus*	TPO	DSM 14055[T]	$H_2 + CO_2$	acetate	55	7.0-7.5	Plugge et al., 2002; Watanabe et al., 2018	WHYR00000000	n.d.	3,716,015	56.0	Bertran et al., 2020
Family Eubacteriaceae												
Acetobacterium bakii	Z-4391	DSM 8239[T]	$H_2 + CO_2$, CO	acetate	20	6.5	Kotsyurbenko et al., 1995;	LGYO00000000	n.d.	4,135,635	41.2	Hwang et al., 2015
Acetobacterium carbinolicum	WoProp1	DSM 2925[T]	$H_2 + CO_2$	acetate	27	7.0-7.2	Eichler and Schink, 1984; Schuppert and Schink, 1990	no genome sequence				
Acetobacterium dehalogenans	MC	DSM 11527[T]	$H_2 + CO_2$, CO	acetate	25	7.3-7.7	Traunecker et al., 1991; Kaufmann et al., 1998	AXAC00000000	n.d.	4,050,072	43.8	n.p.

4

Table 1 (*continued*)

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Acetobacterium fimetarium	Z-4290	DSM 8238^T	$H_2 + CO_2$, CO	acetate	30	7.5	Kotsyurbenko et al., 1995	WJBC00000000	n.d.	3,245,738	44.7	Ross et al., 2020
Acetobacterium malicum	MuME 1	DSM 4132^T	$H_2 + CO_2$	acetate	30	7.5-8.0	Tanaka and Pfennig, 1988	WJBE00000000	n.d.	4,086,418	43.7	Ross et al., 2020
Acetobacterium paludosum	Z-4092	DSM 8237^T	$H_2 + CO_2$, CO	acetate	20	7.0	Kotsyurbenko et al., 1995	WJBD00000000	n.d.	3,696,449	40.1	Ross et al., 2020
Acetobacterium tundrae	Z-4493	DSM 9173^T	$H_2 + CO_2$, CO	acetate	20	7.0	Simankova et al., 2000	WJBB00000000	n.d.	3,567,344	39.7	Ross et al., 2020
Acetobacterium wieringae	C	DSM 1911^T	$H_2 + CO_2$	acetate	30	7.6	Braun and Gottschalk, 1982	LKEU00000000	n.d.	3,895,828	44.1	Poehlein et al., 2016
Acetobacterium woodii	WB1	DSM 1030^T	$H_2 + CO_2$	acetate	30	7.6	Balch et al., 1977; Bache and Pfennig, 1981	CP002987	no	4,044,777	39.3	Poehlein et al., 2012
Acetobacterium sp.	Gra EG 12	DSM 2396	$H_2 + CO_2$	acetate	n.r.	n.r.	Schink and Stieb, 1983	no genome sequence				
Acetobacterium sp.	KoMAc1	DSM 5193	$H_2 + CO_2$	acetate	30	6.8-7.0	Schuppert and Schink, 1990	no genome sequence				
Acetobacterium sp.	LuPhet1	DSM 9077	$H_2 + CO_2$	acetate	25-30	7.0-7.5	Frings and Schink, 1994	no genome sequence				
Acetobacterium sp.	LuTria3	DSM 8909	$H_2 + CO_2$	acetate	25-30	7.1-7.4	Frings et al., 1994	no genome sequence				
Eubacterium aggregans	SR12	DSM 12183^T	$H_2 + CO_2$	acetate, formate	35	7.2	Mechichi et al., 1998	FNRK00000000	n.d.	2,833,471	48.6	n.p.
Eubacterium callanderi	FD	DSM 3662^T	MeOH	acetate, butyrate, hexanoat	37	7.0	Mountfort et al., 1988; Flaiz et al., 2024	JAVVAB000000000	n.d.	4,369,831	47.0	Flaiz et al., 2024
Eubacterium callanderi (formerly *Butyribacterium methylotrophicum*)	Marburg	DSM 3468	$H_2 + CO_2$, CO (after adaption), MeOH	acetate, butyrate, butanol, hexanoate	37-40	7.5	Lynd et al., 1982; Zeikus et al., 1980; Flaiz et al., 2024	CP132155-CP132156	yes	4,278,751	47.5	Flaiz et al., 2024
Eubacterium callanderi	2A	DSM 2593	MeOH	n.r	37-40	7.5	Flaiz et al., 2024	CP132135	no	4,612,903	46.9	Flaiz et al., 2024
Eubacterium callanderi	llA	DSM 2594	MeOH	n.r	37-40	7.5	Flaiz et al., 2024	CP132136	no	4,612,907	46.9	Flaiz et al., 2024
Eubacterium callanderi	G14	DSM 107592	MeOH	n.r	37-40	7.5	Flaiz et al., 2024	CP132137	no	4,409,437	47.5	Flaiz et al., 2024
Eubacterium callanderi	KIST612	n.dp.	$H_2 + CO_2$, CO (after adaption), MeOH	acetate, butyrate, butanol, hexanoate	37-40	7.5	Flaiz et al., 2024	CP002273	no	4,316,707	47.5	Roh et al., 2010
Eubacterium limosum	n.r.	DSM 20543^T	$H_2 + CO_2$, MeOH, MeOH + HCOOH	acetate, butyrate, butanol, hexanoate	39	7.0-7.2	Eggerth, 1935; Flaiz et al., 2024; Genthner et al., 1981; Genthner and Bryant, 1982	CP019962	no	4,422,837	47.2	Song and Cho, 2015
Eubacterium limosum	B2	n.dp.	$H_2 + CO_2$, $H_2 + CO_2 + CO$, MeOH	acetate, butyrate, hexanoate	37	7.0	Flaiz et al., 2021	CP097376	no	4,421,327	47.2	Pregnon et al., 2022
Eubacterium maltosivorans	YI	DSM 105863^T	$H_2 + CO_2$, CO	acetate, butyrate	37	7.0	Feng et al., 2022	CP029487	no	4,337,501	47.8	Feng et al., 2022

Table 1 (*continued*)

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Eubacterium maltosivorans	32	DSM 20517	MeOH	acetate, butyrate, hexanoate	37	7.0	Flaiz et al., 2024	CP132138	no	4,152,794	47.6	Flaiz et al., 2024
Eubacterium maltosivorans	SA11	n.dp.	$H_2 + CO_2$, MeOH	acetate, butyrate	40	7.0	Flaiz et al., 2024; Kelly et al., 2016	CP011914	no	4,150,332	47.4	Kelly et al., 2016
Family Filifactoraceae												
Acetoanaerobium noterae	NOT-3	ATCC 35199[T]	$H_2 + CO_2$	acetate	37	7.6-7.8	Sleat et al., 1985	FUYN00000000	n.d.	2,814,227	33.4	n.p.
Acetoanaerobium sticklandii [a]	HF	DSM 519[T]	n.r.	acetate	37	7.6-7.8	Stadtman & McClung, 1957; Galperin et al., 2016	FP565809	no	2,715,461	31.0	Fonknechten et al., 2010
Family Halobacteroidaceae												
Fuchsiella alkaliacetigena	Z-7100	DSM 24880[T]	$H_2 + CO_2$	acetate	40	8.8-9.3	Zhilina et al., 2012	JALKBZ000000000	n.d.	2,873,640	36.0	Huang et al., 2022
Fuchsiella ferrireducens	Z-7101	DSM 26031[T]	$H_2 + CO_2$	acetate	30-37	9.8	Zhilina et al., 2015	no genome sequence				
Family Holophagaceae												
Holophaga foetida [a]	TMBS4	DSM 6591[T]	n.r.	n.r.	28-32	6.8-7.5	Liesack et al., 1994	AGSB00000000	n.d.	4,127,237	63.0	Anderson et al., 2012
Family Lachnospiraceae												
Acetitomaculum ruminis	139 B	DSM 5522[T]	$H_2 + CO_2$, CO	acetate	37-42	6.8	Greening & Leedle, 1989	FOJY00000000	n.d.	3,082,721	34.2	Seshadri et al., 2018
Blautia coccoides [a]	CLC-1	DSM 935[T]	$H_2 + CO_2$	acetate	37[b]	7.0[b]	Kaneuchi et al., 1976; Liu et al., 2008; Liu et al., 2015	CP136422	n.d.	6,097,890	45.5	Böer et al., 2024a
Blautia coccoides	GA-1	n.dp.	$H_2 + CO_2$	acetate	37[b]	7.0[b]	Liu et al., 2015	no genome sequence				
Blautia hydrogenotrophica	S5a33	DSM 10507[T]	$H_2 + CO_2$	acetate	35-37	6.6	Bernalier et al., 1996; Liu et al., 2008	CP136423-CP136424	yes	3,590,609	45.1	Böer et al., 2024a
Blautia luti	BInIX	DSM 14534[T]	formate+ CO_2+CO	acetate	37[a]	n.r.	Simmering et al., 2002; Liu et al., 2008; Trischler et al., 2022	AP028156	no	3,741,599	42.9	Fukuoka et al., 2023
Blautia producta	U-1	DSM 3507	$H_2 + CO_2$, CO	acetate	37	6.5-7.0	Lorowitz & Bryant, 1984; Geerligs et al., 1987; Liu et al., 2008;	no genome sequence				
Blautia schinkii	B	DSM 10518[T]	$H_2 + CO_2$	acetate	39	6.3-6.8	Rieu-Lesme et al., 1996; Liu et al., 2008	JNKJ00000000	n.d.	6,679,327	45.6	n.p.
Blautia sp.	Nab1	DSM 12568	$H_2 + CO_2$	acetate	37-40	n.r.	Rieu-Lesme et al., 1996	no genome sequence				
Blautia wexlerae	WAL 14507	DSM 19850[T]	formate+ CO_2+CO	acetate	37[a]	7.0[b]	Liu et al., 2008; Trischler et al., 2022	CP102267	no	4,685,728	41.5	Cheng et al., 2022
Marvinbryantia formatexigens	I-52	DSM 14469[T]	$H_2 + CO_2$, formate	acetate	37[b]	7.0[b]	Wolin et al., 2003; Wolin et al., 2008	ACCL00000000	n.d.	4,548,960	49.6	n.p.

Table 1 (*continued*)

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Family Moorellaceae												
Moorella caeni	AMP	DSM 21394[T]	CO	$H_2 + CO_2$	60-65	6.9	Jiang et al., 2009; Vecchini Santaella et al., 2023	MDDC00000000	n.d.	2,567,468	56.05	Redl et al., 2020
Moorella carbonis	ACPs	DSM 116161	CO	$H_2 + CO_2$	60	7.5	Böer et al., 2024b	CP136420	no	2,773,270	55	Böer et al., 2024b
Moorella glycerini[a]	JW/AS-Y6	DSM 11254[T]	n.r.	acetate	58	6.3-6.5	Slobodkin et al., 1997a	CP046244	yes	3,559,463	54.7	Redl et al., 2020
Moorella humiferrea	LNE	DSM 117358	$H_2 + CO_2$, CO	acetate	n.r.	n.r.	Böer et al., 2024b	CP136418	No	2,572,492	54	Böer et al., 2024b
Moorella humiferrea	OCP	DSM 117359	CO	$H_2 + CO_2$	n.r.	n.r.	Böer et al., 2024b	CP136421	No	2,414,751	54	Böer et al., 2024b
Moorella mulderi	TMS	DSM 14980[T]	$H_2 + CO_2$	acetate	65	7.0	Balk et al., 2003	LTBC00000000	n.d.	2,999,839	54.5	Castillo Villamizar and Poehlein, 2016
Moorella stamsii	E3-O	DSM 26217[T]	CO	$H_2 + CO_2$	65	7.5	Alves et al., 2013	PVXL00000000	n.d.	3,328,173	53.8	Poehlein et al., 2018a
Moorella sulfitireducens	SLA38	DSM 111068[T]	CO	acetate	60	6.5-7.0	Slobodkina et al., 2022	JANRHG000000000	n.d.	2,888,417	53.5	Slobodkina et al., 2022
Moorella thermoacetica	CO-adapted strain	DSM 2955[T]	$H_2 + CO_2$, CO	acetate, ethanol	55	6.9	Kerby & Zeikus, 1983	CP012370	no	2,623,349	55.81	Bengelsdorf et al., 2015b
Moorella thermoacetica	Fontaine	DSM 521[T]	$H_2 + CO_2$, CO	acetate	55	6.9	Fontaine, et al., 1942; Andreesen et al., 1970; Reed, 1985; Gößner et al., 1999; Daniel et al., 1990	CP012369	no	2,527,564	56.0	Poehlein et al., 2015a
Moorella thermoacetica (formerly *M. thermoautotrophica*)	Inf1	DSM 7417	$H_2 + CO_2$	acetate	56-60	5.7	Rijssel et al., 1992	MDDE00000000	no	2,585,122	55.87	Redl et al., 2020
Moorella thermoacetica	99-78-22	ATCC 31490	n.r.	n.r.	n.r.	n.r.	Schwartz & Keller 1982	VCDV00000000	no	2,616,798	55.81	Redl et al., 2020
Moorella thermoacetica	Fontaine	ATCC 35608[T]	$H_2 + CO_2$, CO	acetate	55-60	n.r.	Kerby & Zeikus, 1983	VCDW00000000	no	2,611,625	55.83	Redl et al., 2020
Moorella thermoacetica	*	ATCC 39073	$H_2 + CO_2$, CO	acetate	n.r.	n.r.	Daniel et al., 1990	CP000232	no	2,628,784	55.79	Pierce et al., 2008
Moorella thermoacetica	6	DSM 11768	n.r.	n.r.	n.r.	n.r.	Redl et al., 2020	MIHH00000000	no	2,851,436	55.66	Redl et al., 2020
Moorella thermoacetica	ET-5a	DSM 12797	$H_2 + CO_2$	acetate	n.r.	n.r.	Gößner et al., 1999	MIIF00000000	no	2,746,010	55.54	Redl et al., 2020
Moorella thermoacetica	PT-1	DSM 12993	n.r.	n.r.	n.r.	n.r.	Drake & Daniel, 2004	MDDD00000000	no	2,648,948	55.74	Redl et al., 2020
Moorella thermoacetica	G-20	DSM 6867	n.r.	n.r.	n.r.	n.r.	Parekh & Cheryan, 1991	MDDB00000000	no	2,617,097	55.83	Redl et al., 2020
Moorella thermoacetica (formerly *M. thermoautotrophica*)	JW701/3	DSM 103132 (DSM 1974)	$H_2 + CO_2$	n.r.	n.r.	n.r.	Redl et al., 2020; Arahal et al., 2022	CP017019	no	2,976,077	55.10	Redl et al., 2020

Table 1 (*continued*)

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Moorella thermoacetica (formerly *M. thermoautotrophica*)	JW701/3	DSM 103284 (DSM 1974)	$H_2 + CO_2$	n.r.	n.r.	n.r.	Redl et al., 2020; Arahal et al., 2022	CP017237	no	2,560,375	55.94	Redl et al., 2020
Moorella thermoacetica	KAM	n.dp.	$H_2 + CO_2$, CO	acetate, ethanol	60	7	Böer et al, 2024c	CP136425	no	2,629,105	55.5	Böer et al, 2024c
Moorella thermoacetica	BGP	n.dp.	$H_2 + CO_2$, CO	acetate, ethanol	65	8	Böer et al, 2024c	CP136416	no	2,800,819	55.5	Böer et al, 2024c
Moorella thermoacetica	COM	n.dp.	$H_2 + CO_2$, CO	acetate, ethanol	60	8	Böer et al, 2024c	CP136551	no	2,536,557	55.5	Böer et al, 2024c
Moorella thermoacetica	MBA	n.dp.	$H_2 + CO_2$, CO	acetate, ethanol	60	7.5	Böer et al, 2024c	CP136417	no	2,751,603	56	Böer et al, 2024c
Family *Natranaerofabaceae*												
Natranaerofaba carboxydovora	ANCO1	DSM 108926[T]	CO	acetate, formate	48–50	9.5–9.7	Sorokin et al., 2020	CP054394	no	3,264,732	35.2	Sorokin et al., 2020
Family *Natronincolaceae*												
Andreesenella acetica	Wieringa	DSM 1496[T]	$H_2 + CO_2$, CO	acetate	30	8.3	Wieringa, 1936; Adamse, 1980; Lux & Drake, 1992; Braun et al., 1981	CP009687-CP009688	yes	4,207,069	35.3	Poehlein et al., 2015b, 2015c
Andreesenella formicoacetica	A 1	DSM 92[T]	CO	acetate, formate	37	8.1	Andreesen et al., 1970; Lux & Drake, 1992	CP020559	no	4,586,728	35.5	Karl et al., 2017
Andreesenella formicoacetica	5(1)b	DSM 94	n.r.	n.r.	37	n.r.	Andreesen et al., 1970	no genome sequence				
Andreesenella formicoacetica	12(1)c	DSM 95	n.r.	n.r.	37	n.r.	Andreesen et al., 1970	no genome sequence				
Andreesenella formicoacetica	12(3)b	DSM 96	n.r.	n.r.	37	n.r.	Andreesen et al., 1970	no genome sequence				
Andreesenella formicoacetica	26(1)a	DSM 97	n.r.	n.r.	37	n.r.	Andreesen et al., 1970	no genome sequence				
Andreesenella formicoacetica	29(2)b	DSM 98	n.r.	n.r.	37	n.r.	Andreesen et al., 1970	no genome sequence				
Andreesenella formicoacetica	21(1)b	DSM 99	n.r.	n.r.	37	n.r.	Andreesen et al., 1970	no genome sequence				
Andreesenella formicoacetica	34(1)a	DSM 100	n.r.	n.r.	37	n.r.	Andreesen et al., 1970	no genome sequence				
Family *Oxobacteraceae*												
Oxobacter pfennigii	V5-2	DSM 3222[T]	$H_2 + CO_2$, CO	acetate, butyrate	36-38	7.3	Krumholz & Bryant, 1985	LKET00000000	n.d.	4,510,552	39.0	Bengelsdorf et al., 2015a
Family *Peptostreptococcaceae*												
Clostridioides difficile	630	DSM 27543	$H_2 + CO_2$	acetate	37[b]	5.9[b]	Köpke et al., 2013	CP010905	no	4,290,252	29.1	Riedel et al., 2015
Clostridioides difficile	AA1	DSM 12056	$H_2 + CO_2$	acetate	35-40	6.8	Köpke et al., 2013; Rieu-Lesme et al., 1998	no genome sequence				
Clostridioides difficile	A90	DSM 12057	$H_2 + CO_2$	acetate	35-40	6.8	Köpke et al., 2013; Rieu-Lesme et al., 1998	no genome sequence				

Table 1 (*continued*)

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Terrisporobacter glycolicus [a],[b]	*	DSM 1288[T]	$H_2 + CO_2$	acetate	30[b]	7[b]	Böer et al., 2023	CP117523	yes	4,039,277	28.7	Böer et al., 2023
Terrisporobacter glycolicus	RD-1	DSM 13865	$H_2 + CO_2$	acetate	37-40	7.0-7.5	Küsel et al., 2001; Gerritsen et al., 2014	no genome sequence				
Terrisporobacter mayombei	SFC-5	DSM 6539[T]	$H_2 + CO_2$	acetate	33	7.3	Kane et al., 1991; Gerritsen et al., 2014	CP101637	yes	4,064,271	29.0	Böer et al., 2023
Terrisporobacter petrolearius [a]	LAM0A37	JCM 19845[T]	$H_2 + CO_2$	acetate	30[b]	7[b]	Deng et al., 2015; Böer et al., 2023	CP102984	yes	4,124,338	29.0	Böer et al., 2023
Terrisporobacter petrolearius	HSE	n.dp.	$H_2 + CO_2$	acetate	25	7[b]	Böer et al., 2024e	CP154619-CP154621	yes	4,115,567	29.0	Böer et al., 2024e
Terrisporobacter petrolearius	ELB	n.dp.	$H_2 + CO_2$	acetate	25	8	Böer et al., 2024e	CP154622-CP154624	yes	4,031,631	29.0	Böer et al., 2024e
Terrisporobacter petrolearius	DSL	n.dp.	$H_2 + CO_2$	acetate	30	7	Böer et al., 2024e	CP154616-CP154618	yes	4,138,706	29.0	Böer et al., 2024e
Terrisporobacter vanillatitrophus	COM	DSM 116160	$H_2 + CO_2$	acetate, ethanol	35	7.5	Böer et al., 2024e	CP154625-CP154628	yes	4,015,703	29.0	Böer et al., 2024e
Family Sporomusaceae												
Desulfosporomusa polytropa	STP3	DSM 12907[T]	$H_2 + CO_2$	acetate	n.r.	n.r.	Sass et al, 2004	no genome sequence				
Sporomusa acidovorans	Mol	DSM 3132[T]	$H_2 + CO_2$	acetate	35	6.5-7.0	Ollivier et al., 1985	LSLL00000000 CP155571	n.d.	6,060,615	44.6	Humphreys et al., 2017a; Böer et al., 2024d
Sporomusa aerivorans	TmAO3	DSM 13326[T]	$H_2 + CO_2$	acetate	30	7.0	Boga et al., 2003	CP156926-CP156927	yes	6,496,238	46.5	Böer et al., 2024d
Sporomusa carbonis	ACPt	DSM 116159	CO	$H_2 + CO_2$	40	7	Böer et al., 2024d	CP155570	no	4,144,804	46.2	Böer et al., 2024d
Sporomusa malonica	WoG12	DSM 5090[T]	$H_2 + CO_2$	acetate	28-32	7.3	Dehning et al., 1989	FWXI00000000 CP155572	no	5,191,417 5,312,210	44.5	Böer et al., 2024d
Sporomusa ovata	H1	DSM 2662[T]	$H_2 + CO_2$	acetate	34	6.3	Möller et al., 1984	ASXP01000008 CP146301	no	5,383,666 5,433,971	43.3	Poehlein et al., 2013; Böer et al., 2024c
Sporomusa ovata	H6	DSM 2663	$H_2 + CO_2$	acetate	37	6.5	Möller et al., 1984	no genome sequence				
Sporomusa paucivorans	X	DSM 3697[T]	$H_2 + CO_2$	acetate	34	6.7	Hermann et al., 1987	CP155574	no	4,787,310	47.3	Böer et al., 2024d
Sporomusa rhizae	RS	DSM 16652[T]	$H_2 + CO_2$	acetate	35	7.5	Gößner et al., 2006	CP156925	no	5,829,736	43.5	Böer et al., 2024d
Sporomusa silvacetica	DG-1	DSM 10669[T]	$H_2 + CO_2$	acetate	25-30	5.5-7.7	Kuhner et al., 1997	LSLK00000000 CP155573	no	6,046,356	43.0	Humphreys et al., 2017b; Böer et al., 2024d
Sporomusa sphaeroides	E	DSM 2875[T]	$H_2 + CO_2$	acetate	35-37	6.5	Möller et al., 1984	LSLJ00000000 CP146991-CP146992	yes	4,956,256	47.2	Castillo Villamizar et al., 2017; Böer et al., 2024c
Sporomusa termitida	JSN-2	DSM 4440[T]	$H_2 + CO_2$, CO	acetate	30	7.2	Breznak et al., 1988	CP036259	yes	5,316,008	49.0	Poehlein et al., 2020
Sporomusa sp.	Nile	DSM 3300	$H_2 + CO_2$	acetate	33-37	6.3	Möller et al., 1984	no genome sequence				

Table 1 (*continued*)

Organism	Strain designation	culture collection number	Substrate	Products	optimal growth temperature [°C]	optimal pH	Reference	Accession number	Plasmids	Size [bp]	G+C [mol %]	Reference genome publication
Sporomusa sp.	DMG58	DSM 3301	$H_2 + CO_2$	acetate	35-39	6.2	Möller et al., 1984	no genome sequence				
Sporomusa sp.	An4	DSM 21435	$H_2 + CO_2$, CO	acetate	37	7	Balk et al., 2010	CTRP00000000	n.d.	5,305,280	43.5	Visser et al., 2016
Family Thermosinaceae												
Thermosinus carboxydivorans	Nor1	DSM 14886[T]	CO	$H_2 + CO_2$	60	6.8-7.0	Sokolova et al., 2004	AAWL00000000	n.d1	2,889,774	51.5	n.p.
Family Thermacetogeniaceae												
Thermacetogenium phaeum	PB	DSM 26808[T]	$H_2 + CO_2$	acetate	58	6.8	Hattori et al., 2000	CP003732	no	2,939,057	53.9	Oehler et al., 2012
Family Thermincolaceae												
Thermincola carboxydiphila	2204	DSM 17129[T]	CO	$H_2 + CO_2$	55	8.0	Sokolova et al., 2005	no genome sequence				
Thermincola ferriacetica	Z-0001	DSM 14005[T]	CO	$H_2 + CO_2$	57-60	7.0–7.2	Zavarzina et al., 2007	LGTE00000000	n.d.	3,178,727	45.5	Lusk et al., 2015
Family Thermoanaerobacteraceae												
Aceticella autotrophica	3443-3Ac	DSM 108286[T]	$H_2 + CO_2$	acetate	46-50	6	Frolov et al., 2023	CP060096	no	2,267,618	33.0	Frolov et al., 2023
Caldanaerobacter subterraneus ssp. *pacificus*	JMT	DSM 12653[T]	CO	$H_2 + CO_2$	70	6.8-7.1	Sokolova et al., 2001; Fardeau et al., 2004	ABXP00000000	n.d.	2,391,417	37.5	Sant'Anna et al., 2015
Thermoanaerobacter kivui	LKT-1	DSM 2030[T]	$H_2 + CO_2$	acetate	66	6.4	Daniel et al., 1990; Leigh et al., 1981; Leigh & Wolfe, 1983	CP009170	no	2,397,824	35.0	Hess et al., 2014
Thermoanaerobacter thermohydrosulfuricus ssp. *carboxydovorans*	TLO	DSM 15750	CO	$H_2 + CO_2$	70	6.3-6.8	Balk et al., 2009	no genome sequence				
Family Thermolithobacteraceae												
Thermolithobacter carboxydivorans	R1	DSM 7242[T]	CO	$H_2 + CO_2$	70	6.8-7.0	Sokolova et al., 2006	no genome sequence				
Family Treponemataceae												
Treponema primitia	ZAS-2	DSM 12427[T]	$H_2 + CO_2$	acetate	30	7.2	Graber et al., 2004; Graber & Breznak, 2004	CP001843	no	4,059,867	50.8	Rosenthal et al., 2011

Abbreviations: n.d, not determinded. n.dp., not deposited; n.r., not reported; n.p. no published article; [a] no growth on gas reported, but all Wood-Ljungdahl pathway genes found in the genome; [b] condition not described as optimal but used in the reference; * no strain designation.

bacterium (NaCl optimum of 1 %) grows using CO, glucose, fructose, mannose, xylose, 3,4-dimethoxybenzoic acid, and 2-methoxyphenol, while yeast extract stimulates growth. The main fermentation product is acetate, but also trace amounts of H_2 and CO_2. This strain can not grow on $H_2 + CO_2$. Several electron acceptors, such as sulfate, thiosulfate, nitrate, or fumarate were tested with 3,4-dimethoxybenzoic acid as an electron donor, but no growth was detected. This strain can grow on aromatic compounds but due to the lack of genes encoding Bzd- or Bam-type benzoyl-CoA reductases in the genome, only the methyl groups serve as substrates via methyltransferase systems.

Four new clostridial strains were described in the family *Clostridiaceae* (Section 3.1.) *Clostridium bovifaecis* was isolated from cow manure (Zhu et al. 2018). This Gram-positive, spore-forming and rod-shaped bacterium grows best at 30 °C with a pH of 7 and 1 %. (w/v) NaCl. This bacterium shows a formate-dependent acetogenic growth behaviour and uses also mannose, galactose, D-fructose, 2-methoxyethanol, 1,2-propanediol, glycerol, pyruvate and formate, with acetate as main fermentation product. The addition of yeast extract or casein supports growth. Yao et al. (2020) showed that the gene encoding the formate dehydrogenase (Fdh) is missing in the genome of *C. bovifaecis*. Fdh catalyzes the CO_2 reduction to formate as the initial step of the methyl branch of the WLP, and thus explains the formate-dependent acetogenesis. *Clostridium luticellarii*, which also belongs to the family of *Clostridiaceae*, was assumed to be an acetogen (Wang et al., 2015; Petrognani et al., 2020), and this was was finally proven by Mariën et al. (2023). This bacterium was isolated from mud cellars, is Gram-positive, and forms curved rods, endospores, and peritrichous flagella. The optimal growth temperature is 37 °C, with an optimal pH value of 6.5. This bacterium grows well on palatinose, L-fucose, b-hydroxybutyric acid, L-rhamnose, and a-ketobutyric acid and is used for aromatic liquor production due to its ability to produce isobutyric acid. Petrognani and co-workers (Petrognani et al., 2020) showed the production of butyric and isobutyric acid during growth on methanol, but also on $H_2 + CO_2$ via the WLP at a pH of ≤ 5.5 and by increasing acetic acid concentrations (Petrognani et al., 2020). *Clostridium muellerianum* and *Clostridium thailandense* are two just recently described acetogens isolated from old hay (Doyle et al., 2022) and peatland soil (Chaikitkaew et al., 2022), respectively. Both microbes are Gram-positive bacteria and rod-shaped cells, while *C. thailandense* forms endospores, but interestingly, in contrast to other clostridia, *C. muellerianum* does not. Both strains can metabolize $H_2 + CO_2$ and a variety of sugars. *C. muellerianum* can also grow using CO. Main fermentation product of both strains is acetate, but *C. muellerianum* also produces butyrate, caproate, ethanol, and hexanol.

Three novel *Moorella* species were described recently: *Moorella caeni*, *Moorella carbonis*, and *Moorella sulfitireducens*. *M. caeni* (strain AMP) was originally described as *M. thermoacetica*, but recently reclassified by Vecchini Santaella et al. (2023). Whole genome comparisons by Redl et al. (2020) already showed that strain AMP does not belong to the species *M. thermoacetica* and suggested reclassification. *M. caeni* was isolated from a thermophilic methanogenic bioreactor that was fed with sludge of a pilot plant, treating wastewater from a paper mill with methanol as substrate (Jiang et al., 2009). This species showed an atypical metabolism as it did not use $H_2 + CO_2$, but CO and yielded H_2 and CO_2 as final products (water–gas shift reaction; Section 3.6). *M. caeni* is rod-shaped and Gram-positive with an optimal growth at 60–65 °C and a pH of 6.9. This microbe can also grow on formate, pyruvate, lactate, mannose, vanillin, and vanillate, and uses thiosulfate as an electron acceptor (Jiang et al., 2009). *M. carbonis* (strain ACPs) was isolated from the covering soil of an active charcoal burning pile and showed optimal growth at 60 °C and pH 7.5. The cells are Gram-positive, spore-forming rods that utilize fructose, xylose, ribose, galactose, mannose, rhamnose, myo-inositol, sucrose, raffinose, melezitose, formate, pyruvate, lactate, vanillate, syringate, dimethylglycine, betaine, methanol, and 1,2-propanediol for growth. Like *M. caeni*, *M. carbonis* was found to not use $H_2 + CO_2$ for growth and to transform CO to $H_2 + CO_2$ via the water–gas shift reaction (Böer et al., 2024b).

Another recently described species is *M. sulfitireducens* was isolated from a thermal spring. Cells are Gram-positive and rod-shaped, showing optimal growth at 60 °C and at pH of 6.5–7.0 (Slobodkina et al., 2022). This bacterium is able to grow on CO with or without thiosulfate as electron acceptor. Yeast extract for micronutrients and sulfite as electron acceptors are required for growth on $H_2 + CO_2$.

Natranaerofaba carboxydovora was isolated from sediments of hypersaline soda lakes with CO as substrate. This moderately thermophilic and haloalkaliphilic microbe grows best at 48 °C, 4 M NaCl, and a pH of 9.7. Growth is dependent on the addition of yeast extract. *Na. carboxydovora* can grow on CO, formate, and pyruvate, but not on $H_2 + CO_2$. In contrast to other thermophilic acetogens, the genome of *Na. carboxydovora* does not encode genes for an Ech-complex, but genes for the Rnf-complex are present and are probably used for energy metabolism conservation (Schuchmann and Müller, 2014).

Terrisporobacter vanillatitrophus (strain COM) has recently been isolated from compost. The cells are Gram-positive, spore-forming rods that grow optimally at 35 °C and pH 7.5. Substrates used for growth comprise glucose, xylose, fructose, sorbitol, mannitol, cellobiose, maltose, raffinose, melezitose, dextrin, formate, vanillate, and 1,2-propanediol. The conversion of $H_2 + CO_2$ to acetate and small amounts of ethanol mainly occurs during the stationary growth phase (Böer et al., 2024e).

Sporomusa carbonis (strain ACPt) was isolated from the covering soil of an active charcoal burning pile and showed optimal growth at 40 °C and pH 7. Cells were found to be Gram-positive curved rods that utilized ribose, fructose, glucose, sucrose, lactose, melezitose, pyruvate, vanillate, syringate, and methanol for growth. In contrast to all other described *Sporomusa* species this strain did not utilize $H_2 + CO_2$ for growth, but was found to convert CO mainly into $H_2 + CO_2$ via the water-gas shift reaction (Böer et al., 2024d). This strain is one of the rare mesophilic isolates of a carboxydotrophic hydrogenogen being unable to grow at temperatures higher than 50 °C.

3. Taxonomy and physiology of acetogens

The reductive acetyl-CoA pathway or WLP is possibly the oldest mode of microbial energy conservation and CO_2 fixation (Wimmer et al., 2021). It is present in acetogens as well as in methanogens and is spread all over different microbial families and phyla (Schuchmann and Müller, 2014). Isolates of true acetogens in the strictest sense (*i.e.* thriving on the conversion of $H_2 + CO_2$ to acetyl-CoA via the WLP (and then further to acetate)) are only found in the domain *Bacteria*. Reports on metagenomes found evidence for archaeal variants of acetogenesis in the domain *Archaea*, but the respective microbes have not yet been cultivated (He et al., 2016; Loh et al., 2021). *Archaeoglobus fulgidus*, *Methanosarcina acetivorans*, and an engineered strain of *Methanosarcina acetivorans* have been shown to thrive on CO conversion to acetate (Henstra et al., 2007; Rother and Metcalf, 2004; Schöne et al., 2022). In the following, we focus only on microbes belonging to the domain *Bacteria*, and we include obligate carboxydotrophic as well as organotrophic/mixotrophic acetogens, which are not able to grow with $H_2 + CO_2$ alone. Except for two, all acetogens belong to the phylum *Bacillota* (formerly known as *Firmicutes*) and are assigned to the kingdom *Bacillati*. The remaining two are assigned to the phyla *Acidobacteriota* and *Spirochaetota*, which belong to the kingdom *Pseudomonadati* (Oren and Göker, 2024). Fig. 1 shows 83 strains as a consequence of a multilocus sequence analysis (MLSA) based on the core orthologous groups (56 OGs) of gene products derived by using the software tool Proteinortho (version 4.26). Strains were clustered into different clades, which correspond well with the respective taxonomic families. Moreover, specific features, such as genome size, G + C content, methanol utilization, preferred temperature range, spore-formation and Gram-staining, are presented. Table 1 provides the corresponding information regarding the genome sequences of acetogens presented in Fig. 1. To the best of our knowledge, we summarized all, thus far, isolated acetogens in Table 1, and provided detailed information concerning strain
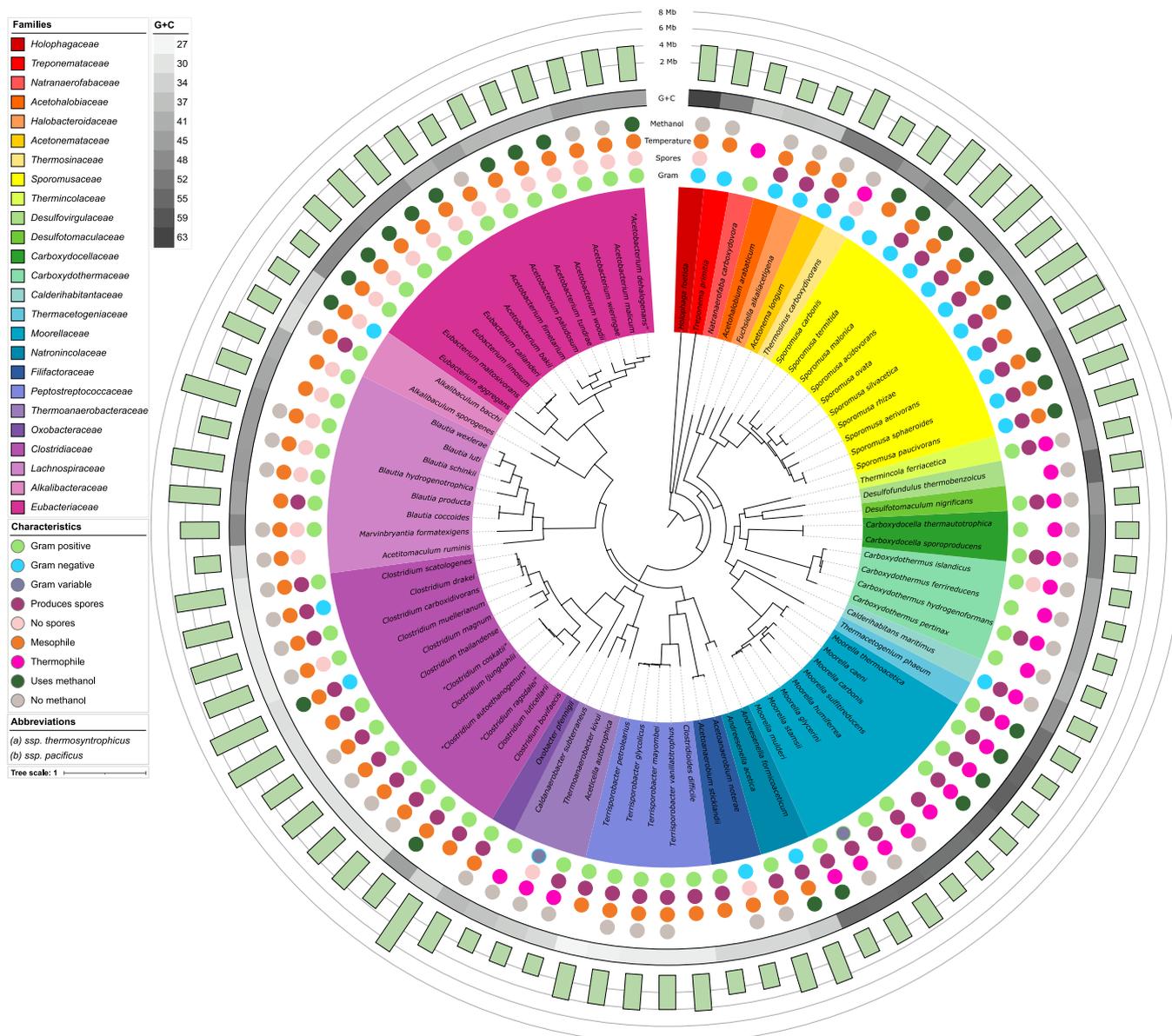
Fig. 1. MLSA protein sequence-based phylogenetic representation of acetogens and their main characteristics. The tree is based on the core orthologous groups (56 OGs) derived from the amino acid sequences using Proteinortho (v. 4.26) (Lechner et al., 2011). The MLSA-based tree was calculated in RAxML (v. 8.2.12) using the PROTGAMMAWAG substitution method and 500 bootstraps (Stamatakis, 2014). Gram-staining and spore-formation are based on experimental data from the literature. The optimum temperatures, genome size, and G + C content (mol %) of these 83 strains are specified in Table 1. Names indicated in quotes are not yet accepted as bacterial names in the Approved Lists of Bacterial Names and the respective validation list (https://lpsn.dsmz.de).

designation, culture collection number, genome, substrates, products, optimal growth temperature, and optimal pH.

3.1. Clostridiaceae

The family *Clostridiaceae* is a diverse group with more than 20 different genera, including, for instance, *Anaeromonas*, *Caloramator*, *Clostridium*, *Fonticella*, *Haloimpathiens*, *Hungatella*, and *Tindallia*. As of yet, acetogens have been solely found in the genus *Clostridium* (Table 1). These microbes are Gram-positive rods, spore-forming, and motile by flagella. Except for *Clostridium magnum*, all strains can grow on both H₂ + CO₂ and CO. *Clostridium ljungdahlii*, *"Clostridium autoethanogenum"*, *"Clostridium ragsdalei"* and *"Clostridium coskatii"* are all very closely related strains producing acetate, ethanol, and 2,3-butanediol. These strains have been isolated from chicken yard waste, rabbit feces, and sediment (Tanner et al., 1993; Abrini et al., 1994; Zahn and Saxena,

2012). However, *"C. coskatii"* produces considerably less ethanol compared to the other strains. Bengelsdorf and co-workers (2016) reported the lack of genes encoding aldehyde:ferredoxin oxidoreductase (AOR) in the genome of *"C. coskatii"*. *C. ljungdahlii* is also able to produce glycine and ethanolamine in considerable amounts (Romans-Casas et al., 2022; Boto et al., 2023). Ethanol production rates during growth on CO can be increased by additional sodium acetate in the medium (Schulz et al., 2023). *C. ljungdahlii*, *"C. autoethanogenum"*, and *"C. ragsdalei"* reduce acetone to isopropanol (Köpke et al., 2014). AOR and alcohol dehydrogenase are crucial for ethanol production, and inhibition of the bi-functional aldehyde/alcohol dehydrogenase (AdhE) led to enhanced ethanol production during autotrophic growth (Köpke et al., 2010; Liew et al., 2017). *C. ljungdahlii*, *"C. autoethanogenum"* and *"C. ragsdalei"* are also able to produce lactate by the reduction of pyruvate (Köpke et al., 2014; Kracke et al., 2016). *C. ljungdahlii*, *"C. ragsdalei"* and likely other acetogens can reduce carboxylic acids, such as

propionate, *n*-butyrate, *n*-pentanoate, and *n*-hexanoate to their corresponding alcohols *n*-propanol, *n*-butanol, *n*-pentanol, and *n*-hexanol, respectively (Perez et al., 2013; Isom et al.,2015; Diender et al., 2023). *Clostridium carboxidivorans*, *Clostridium drakei*, and *Clostridium scatologenes* are also closely related, showing > 99 % sequence similarity based on their 16S rRNA gene sequences (Liou et al., 2005; Bengelsdorf et al., 2013). These strains have been isolated from an agricultural-settling lagoons and acidic coal-mine ponds (Liou et al., 2005; Küsel et al., 2000). *C. carboxidivorans* has been used for the production of acetate, *n*-butyrate, ethanol and *n*-butanol from CO (Rajagopalan et al., 2022). This strain is able to, produce *n*-hexanol and *n*-hexanoate under increased molybdenum concentrations (Phillips et al., 2015; Ramió-Pujol et al., 2015). Recent studies reported an increased *n*-hexanol production with a temperature lowered to 30 °C and a CO gas content of 70 % (Kottenhahn et al., 2021; Oh et al., 2022). Much higher values (up to 8.45 g/L) could be achieved when *n*-hexanol was extracted from the fermentation process, with small amounts of oleyl alcohol and ethanol as byproducts (Kottenhahn et al., 2021; Oh et al., 2022). Ethanol and *n*-butanol production of *C. carboxidivorans* could be enhanced by mixotrophic cultivation with glucose and CO (Vees et al., 2022). As mentioned above, *C. scatologenes* and *C. drakei* also utilize $H_2 + CO_2$, but growth is very limited, and decreased after several subcultivations in the lab (Song et al., 2020). *C. drakei* was originally described as *C. scatologenes* (Küsel et al, 2000), but was reclassified by Liou et al. (2005) as *C. drakei*. A genome-scale metabolic model of *C. drakei* showed the interconnection of WLP with the glycine synthase-reductase pathway and reductive glycine pathway to fix C_1 compounds. (Song et al., 2020). *C. magnum* has been isolated from freshwater sediment. It can grow on $H_2 + CO_2$ (growth dependens on yeast extract), formate, and methanol. Interestingly, this microbe is able to fix molecular nitrogen (Bomar et al., 1991). Moreover, cells can tolerate low amounts of oxygen in the growth medium (Karnholz et al., 2002).

3.2. Alkalibacteraceae and Eubacteriaceae

The family *Alkalibacteraceae* was proposed by Chuvochina et al. (2023) and contains two acetogens that are assigned to genus *Alkalibaculum*. Allen et al. (2010) isolated three *Alkalibaculum bacchi* strains from livestock-impacted soil, and deposited strain CP11 as a type strain in public culture collections. Cells of CP11[T] stain Gram-negative, are non-spore-forming, and are motile rods. The optimum pH for growth is 8.0–8.5, and $H_2 + CO_2$, CO, and a variety of sugars are utilized to produce acetate and ethanol. *Al. bacchi* CP15 was investigated in a continuous syngas fermentation for the production of ethanol, but due to a contamination with *Clostridium propionicum*, the alcohols *n*-propanol and *n*-butanol occurred as additional products. This mixed culture formed a maximum of 8 g/L ethanol, 6 g/L *n*-propanol and 1 g/L *n*-butanol (Liu et al., 2014). Besides that, not much is known about *Al. bacchi*, except for the genome sequence of CP11[T] (Table 1) and the fact that genes encoding the WLP gene products are scattered over the bacterial chromosome (Esposito et al., 2019). The other species, *Al. sporogenes* (Khomyakova et al., 2020), is briefly described in Section 2.

The family of *Eubacteriaceae* contains bacterial species belonging to the genera *Acetobacterium*, *Eubacterium*, *Gallibacter*, *Pseudoramibacter*, and *Rhabdanaerobium*. Among those, bacterial strains belonging to the genera *Acetobacterium* and *Eubacterium* are known acetogens (Table 1). Respective strains have been isolated from various habitats, including anoxic freshwater sediments, tundra soil, sewage digesters and animal feces (Wade, 2015). As of yet, all species within the genera *Acetobacterium* are able to convert either $H_2 + CO_2$ or CO to acetate via the WLP. The genus *Acetobacterium* encompasses 9 species, but for most of them only basic metabolic features are described. Genome sequencing data are available for *Acetobacterium bakii*, *Acetobacterium dehalogenans*, *Acetobacterium malicum*, *Acetobacterium paludosum*, *Acetobacterium tundrae*, *Acetobacterium wieringae*, *Acetobacterium woodii*, and six additional metagenome-assembled genomes. Ross et al. (2020) conducted a

comparative genome analysis of 11 different *Acetobacterium* genomes and identified clade-specific metabolic potentials within members of this genus. *A. woodii* has been studied most detailed. It grows lithoautotrophically ($H_2 + CO_2$) as well as organoheterotrophically (fructose, glucose, lactate, glycerate, formate, and O-methylated aromatic compounds). Acetate is usually the only metabolic end product, but its metabolism was engineered to produce acetone, isopropanol, lactate, and poly-3-hydroxybutyrate (Hoffmeister et al., 2016; Mook et al., 2022; Höfele et al., 2023; Höfele and Dürre, 2023) (Section 5). *A. woodii* cells depend on Na^+ to generate an ion gradient across the cytoplasmic membrane via a membrane-bound ferredoxin:NAD^+ oxidoreductase and the Rnf complex. As a concequence, they possess an Na^+-dependent ATP synthase (Heise et al., 1989; Biegel and Müller, 2010; Müller et al., 2001), as explained in Section 4. The Rnf complex itself is supplied with reduced ferredoxin (Fd^{2-}) by an electron-bifurcating hydrogenase (Schuchmann and Müller, 2012). Thus, the combination of reactions of both protein complexes drives energy conservation (Section 1 and 4).

There are two noteworthy strains of *A. wieringae*, which are termed strain "JM" and strain "73–4-6p-4", but both are not available in public culture collections (Arantes et al., 2020). Strain JM grows by metabolizing a 100 % CO atmosphere (total pressure 170 kPa) without any yeast extract or formate supplementation and produces mainly acetate. Strain JM outcompetes the type strain with a shorter lag phase and a four times faster CO consumption rate. An electrotransformation protocol was developed for strain JM, and recombinant acetone production was achieved via plasmid-based expression of the required genes (Moreira et al., 2023). It has been demonstrated that the strain "73–4-6p-4" notably accelerates the corrosion of iron (Fe^0) when compared with abiotic controls (Philips et al., 2018). The increase in corrosion is presumably associated with free extracellular elements that catalyze the production of H_2 on the iron surface, or due to the maintenance of minimal H_2 concentrations on the iron surface caused by attached cells, and thereby promoting H_2 formation from a thermodynamic perspective.

Eubacterium limosum, *Eubacterium callanderi*, and *Eubacterium maltosivorans* are members of the family *Eubacteriaceae*. These strains share the ability to utilize C_1 sources and harbor genes of the WLP, the methyltransferase system, and the *bcs/hcs* operon in their genomes (Flaiz et al., 2024). Of the 54 validly published species in the genus *Eubacterium*, only the three mentioned before and *Eubacterium aggregans* (strain SR12[T] (DSM 12183[T])) are known acetogens. The history of strains classified as *E. limosum* is complex and confusing. Therefore, 11 closely related strains were reclassified by genomic, phylogenetic, and physiologic means (Flaiz et al., 2024). As a result of these analyses: (I) two strains were found to belong to the species *E. limosum* (DSM 20543[T] and 'B2' (not yet publicly avalible)); (II) six strains belong to the species *E. callanderi* ('FD' (DSM 3662[T]), 'Marburg' (formerly known as '*Butyribacterium methylotrophicum*' (DSM 3468), 'KIST612' (not yet publicly availaible), '2A' (DSM 2593), '11A' (DSM 2594), and 'G14' (DSM 107592)); and (III) three strains to the species *E. maltosivorans* ('YI' (DSM 105863[T]), '32' (DSM 20,517 formerly described as '*Butyribacterium rettgeri*' and *E. limosum*, respectively), and 'SA11' (not yet publicly availaible). Eight of these strains are genetically accessible. Members of these species exhibit diverse metabolic substrate consumption capabilities and are able to produce acetate, *n*-butyrate, *n*-hexanoate, and traces of *n*-butanol from various C_1 sources, such as from CO, $H_2 + CO_2$, methanol, and formate. *E. limosum*, *E. callanderi*, and *E. maltosivorans* may develop as biocatalysts to produce value-added products naturally or recombinantly due to their flexible metabolism and genetic accessibility (Section 5).

3.3. Lachnospiraceae

Lachnospiraceae is a family of bacteria with most of its representatives isolated from the gastrointestinal tract of mammals. They are strictly anaerobic and have a fermentative metabolism. Hexoses are

oxidized through glycolysis to pyruvate. Pyruvate can be reduced to lactate or phosphoenolpyruvate, or carboxylated to yield oxaloacetate, which is reduced to fumarate by enzymes of the reverse citrate cycle. In addition, pyruvate can be oxidized to acetyl-CoA and CO_2, whereby ferredoxin is reduced. Two moieties of acetyl-CoA can also be condensed to acetoacetyl-CoA and further reduced via hydroxybutyryl-CoA and crotonyl-CoA to butyryl-CoA, which is the precursor of *n*-butyrate. *n*-Butyrate is produced in two different ways: first, it is converted by phosphotransbutyrylase to butyryl-phosphate and then by butyrate kinase to *n*-butyrate, which is accompanied with the synthesis of one ATP. Alternatively, a butyryl-CoA:acetate acetyltransferase produces acetyl-CoA, and *n*-butyrate and the acetyl-CoA is converted by Pta and Ack to acetate and ATP. Thus, *Lachnospiraceae* have a pronounced fermentative metabolism producing short chain alcohols as well as short-chain carboxylic acids. Whereas the former are normally associated with negative effects on health and well-being of mammals, the latter, and especially *n*-butyrate, fumarate, and succinate, are seen to have very positive effects on health and well-being (Liu et al., 2021; Wei et al., 2023; Rowland et al., 2017).

Some members of the *Lachnospiraceae*, such as those in the genus *Blautia*, can reduce CO_2 as an electron acceptor to acetate via the WLP, thereby, integrating fermentation with anaerobic respiration for energy conservation. Consequently, they are recognized as typical acetogens (Trischler et al., 2022). Some, but not all, can grow autotrophically by producing acetate from H_2 + CO_2. This is well-known for *Blautia hydrogenotrophica* and *Blautia schinkii* (Bernalier et al., 1996; Rieu-Lesme et al., 1996). In contrast, *Blautia luti* and *Blautia wexlerae* do not grow with H_2 + CO_2 as substrates (Trischler et al., 2022). Inspection of the genome sequence revealed all genes of the WLP except genes encoding a Fdh and an electron-bifurcating hydrogenase, and enzymatic analysis confirmed this prediction (Trischler et al., 2022). The absence of a Fdh implies that formate is the starting point of the methyl branch of the WLP, and, indeed, genetic deletion of the HDCR in *T. kivui* or *A. woodii* abolished acetate formation from CO_2 with growth restored by the addition of formate (Jain et al., 2020; Moon et al., 2021). A technical problem is encountered when testing for autotrophic acetogenesis in a hydrogenase-free acetogen. This problem can be circumvented by using CO, because CO is an intermediate of the WLP, and thus a substrate for many acetogens. *B. luti* and *B. wexlerae* have an active COdh and resting cells perform acetogenesis from formate + CO + CO_2, demonstrating a functional WLP (Trischler et al., 2022). Bioinformatic analyses revealed that many *Blautia* strains as well as other gut acetogens, such as *C. bovifaecis* and *Marvinbryantii*, lack formate dehydrogenases and hydrogenases (Yao et al., 2020; Wolin et al., 2003). Thus, the use of formate instead of H_2 as an interspecies hydrogen and electron carrier seems to be more common in the gut microbiome.

Blautia spp. are gut bacteria and have many representatives which are characterized as acetogens. Interestingly, *Blautia* spp. seem to be directly linked to human health. Depletion of *Blautia* species in the human gut is not only associated with diseases, such as obesity or gut inflammation, but is also correlated to psychological diseases such as autism (Benítez-Páez et al., 2020; Johnson, 2020). Increased amounts of *Blautia* spp., on the other hand, are beneficial for human health and reduces, for example, lethality due to graft-versus-host disease (Jenq et al., 2015). Thus, acetogens are not only essential for the global carbon cycle but also for our well-being and health. We are merely at the start of exploring this promising field, which offers a novel perspective on the potential and importance of acetogens.

Gut acetogens are metabolically very flexible and may be used as new production platforms based on materials other than CO or CO_2 as feedstocks. Glycerol is a rather unusual growth substrate and converted to ethanol by only a few bacteria under anoxic conditions. Among these, the acetogen *B. schinkii* demonstrates a unique substrate-dependent product formation. At low glycerol concentrations, *B. schinkii* generates acetate and ethanol in nearly equal proportions. However, at high glycerol levels, ethanol becomes the sole product (Trischler et al., 2023).

Furthermore, while the WLP is active at low glycerol concentrations, high glycerol concentrations lead to the inactivation of the WLP. Cells are able to metabolize up to 1.5 M glycerol. Transcriptome and enzymatic analyses revealed the pathway of glycerol oxidation, and dihydroxyacetone (DHA) as an intermediate. Indeed, cells also grow on DHA (Trischler et al., 2023). Homoethanologenesis from glycerol by this gut acetogen and by gut bacteria in general is a novel feature, which might not only be of relevance for human health but is also interesting for biotechnological applications.

3.4. *Natronincolaceae* and proposal for the reclassification of *Clostridium aceticum* and *Clostridium formicoaceticum*

Clostridium aceticum (DSM 1496[T]), an anaerobic and endospore-forming microbe, was first isolated from sludge from a town canal in Wageningen in 1936. It was the first bacterial isolate that was known to form acetate autotrophically from H_2 + CO_2 (Wieringa, 1936). It was thought to be lost during World War II, but was recovered from a spore preparation by Braun et al. (1981). This bacterium grows also organoheterotrophically by using sugars, organic acids, and alcohols (Poehlein et al., 2015c). *Clostridium formicoaceticum* (DSM 92[T]), which is a mixotrophic homoacetogen, was first isolated in 1970 from sewers and ditches near Göttingen (Andreesen et al., 1970). It is an obligate anaerobic, mesophilic, motile, rod-shaped and spore-forming bacterium. Unlike its close relative *C. aceticum*, *C. formicoaceticum* is not able to grow at the expense of H_2 + CO_2, but by using CO. The genus name *Clostridium* is misleading for both bacterial strains. Reconstructions of phylogenetic trees based on 16S rRNA gene sequences and additional comparisons of strain-specific features showed that the validly described bacteria *Natronincola ferrireducens*, *Natronincola histidinovorans*, and *Anaerovirgula multivorans* are the closest phylogenetic relatives of both strains (Poehlein et al., 2015c; Bao et al., 2019; Pikuta et al., 2006). Pikuta et al. (2006) stated that *Av. multivorans* SCA[T] clearly differentiates from *C. formicaceticum* and *C. aceticum* based on a comparison of their phenotypic properties, fatty-acid profiles, and low levels of DNA–DNA hybridization between strain SCA[T] and the aforementioned two strains. Similarly, *C. formicoaceticum* and *C. aceticum* can also be differentiated from strains of the genera *Tindallia* and *Alkaliphilus*. A quick inspection of genome sequences revealed that *N. ferrireducens*, *N. peptidivorans*, *Alkaliphilus transvaalensis*, *Ap. peptidifermentans*, and *Ap. metalliredigens* contain genes encoding gene products that are requierd for the WLP as well as gene clusters encoding sodium/proton antiporters, which are similar to the ones from *C. aceticum*. Bacterial strains of the genera *Natronincola*, *Alkaliphilus*, *Anaerovirgula*, and *Serpentinicella* were assigned to the family *Natronincolaceae* (Chuvochina et al., 2023). All bacterial strains belonging to the mentioned genera are described as alkaliphilic anaerobes, but none as an acetogen. The ezAAI tool (Kim et al, 2021) was used to calculate the average amino acid identity (AAI) values of above mentioned relatives. A treshould of 76 % AAI has been used for genus delineation (Nicholson et al., 2020; Riesco and Trujillo, 2024). *C. formicaceticum* and *C. aceticum* are both above these treshold of 76 % AAI, while all other strains shown are below (Fig. 2). In conclusion, we suggest a reclassification of *C. aceticum* and *C. formicaceticum* into a novel genus. The proposed new genus name is *Andreesenella* and proposed names of the strains are *Andreesenella formicoacetica* (DSM 92[T], ATCC 35044[T]) and *Andreesenella acetica* (DSM 1496[T], ATCC 27076[T]).

3.5. Description of *Andreesenella* gen. nov.

Andreesenella (An.dree.sen.el'la. N.L fem. n. *Andreesenella*, named in honor of Jan R. Andreesen).

In general, cells are obligately anaerobic, alkaliphilic, mesophilic, spore-forming, and rod-shaped. Cells are Na$^+$-dependent and use the F_1F_0-type ATP synthase and Rnf complex for energy conservation. Metabolic end products are acetate and, under respective growth conditions, formate and ethanol. The type species of the genus *Andreesenella*
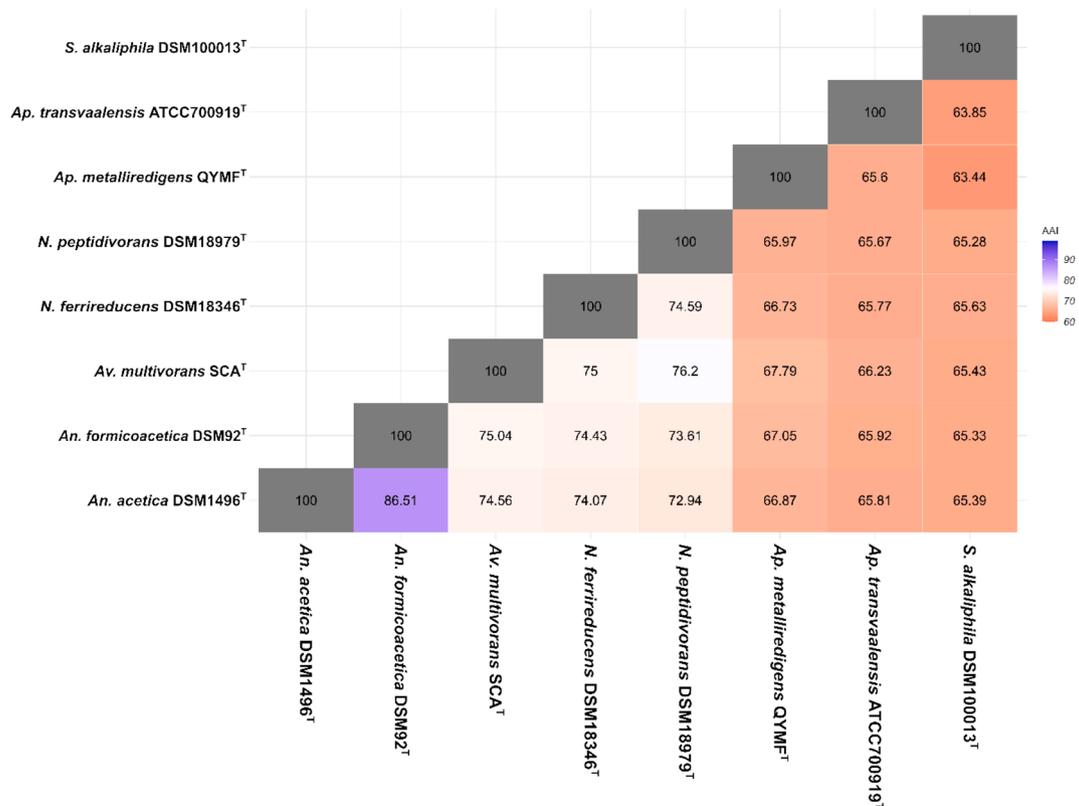
	An. acetica DSM1496^{T}	An. formicoacetica DSM92^{T}	Av. multivorans SCA^{T}	N. ferrireducens DSM18346^{T}	N. peptidivorans DSM18979^{T}	Ap. metalliredigens QYMF^{T}	Ap. transvaalensis ATCC700919^{T}	S. alkaliphila DSM100013^{T}
S. alkaliphila DSM100013^{T}								100
Ap. transvaalensis ATCC700919^{T}							100	63.85
Ap. metalliredigens QYMF^{T}						100	65.6	63.44
N. peptidivorans DSM18979^{T}					100	65.97	65.67	65.28
N. ferrireducens DSM18346^{T}				100	74.59	66.73	65.77	65.63
Av. multivorans SCA^{T}			100	75	76.2	67.79	66.23	65.43
An. formicoacetica DSM92^{T}		100	75.04	74.43	73.61	67.05	65.92	65.33
An. acetica DSM1496^{T}	100	86.51	74.56	74.07	72.94	66.87	65.81	65.39

AAI
90
80
70
60

Fig. 2. Heat map of AAI genome comparison: average amino acid identity (AAI) was calculated using ezAAI (Kim et al., 2021).

is *Andreesenella formicoacetica* (DSM 92^{T}).

3.6. Emended description of Andreesenella formicoacetica (ex Andreesen 1970)

Andreesenella formicoacetica comb. nov. (for.mi.co.a.ce'ti.ca. N.L. neut. n. *acidum formicum*, formate; N.L. neut. n. *acidum aceticum*, acetate; N.L. fem. adj. *formicoacetica*, pertaining to formate and acetate).

Basonym: *Clostridium formicoaceticum* Andreesen et al. 1970 (Approved Lists 1980). We are aware of the proposed correction of *formicoacetica* to *formicaceticum* in the LPSN website, but this correction destabilizes nomenclature. It may be possible based on Rule 61 of the ICNP, but it contravenes Principle 1(1).

The description is as given by Andreesen et al. (1970) with the following amendments and modifications. *An. formicoacetica* produces acetate as the sole metabolic product from various carbon sources, including sugars, glycerol, formate, and CO. Its 4.59 Mbp genome contains a highly conserved WLP gene cluster with the same arrangement as in other acetogens, including *Andreesenella acetica*, *C. carboxidivorans*, and *C. ljungdahlii* (Bao et al., 2019). For energy conservation, *An. formicoacetica* does not contain all genes needed for the synthesis of cytochromes or quinones required to generate a proton gradient. Instead, cells harbor the Rnf system and a Na^{+}-translocating ATP synthase. Cells grown under organoheterotrophic or lithoautotrophic conditions are dependent on Na^{+}-ions. Furthermore, *An. formicoacetica* encodes genes for acetaldehyde dehydrogenases, alcohol dehydrogenases, and aldehyde oxidoreductases, which could convert acetyl-CoA *via* acetaldehyde to ethanol. *An. formicoacetica* is the type species of the genus *Andreesenella*.

3.7. Emended description of Andreesenella acetica (ex Wieringa 1940)

Andreesenella acetica comb. nov. (a.ce'ti.ca. N.L. neut. n. *acidum aceticum*, acetate; N.L. fem. adj. *acetica*, pertaining to acetate). Basonym:

Clostridium aceticum (*ex* Wieringa 1940) Gottschalk and Braun 1981.

The description is as given by Braun et al. (1981) with the following amendments. The genome of *Andreesenella acetica* (Wieringa) has been completely sequenced and consists of a 4.2 Mbp chromosome and a small circular plasmid of 5.7 kbp. It contains a Rnf complex and a Na^{+}-translocating ATP synthase for energy conservation. Its genome harbors all genes required for the biosynthesis of a cytochrome *c*, but no genes required for the biosynthesis of quinones were found. Malate and fumarate are used to produce acetate and succinate, respectively (Poehlein et al., 2015a). Furthermore, certain fermentation conditions lead to formate or ethanol formation and consumption, respectively (Mayer et al., 2018; Arslan et al., 2019).

3.8. Filifactoraceae and Peptostreptococcaceae

Acetogens in the *Peptostreptococcaceae* family can be found in the genera *Clostridioides* and *Terrisporobacter*. The genus *Acetoanaerobium* is a member of the family *Filifactoraceae* (Chuvochina et al., 2023). Acetogens of these three genera are characterized by their inability to grow with CO, but they utilize H$_2$ + CO$_2$. Moreover, they are often dependent on the addition of yeast extract or peptone to achieve substantial growth during acetogenesis.

The genus *Acetoanaerobium* consists of the three non-spore-forming species: *Acetoanaerobium noterae*, *Acetoanaerobium pronyense* and *Acetoanaerobium sticklandii* (formerly *Clostridium sticklandii*) (Galperin et al., 2016; Rainey, 2015). However, only the genomes of *Aa. noterae* and *Aa. sticklandii* encode all genes required for the WLP and only *Aa. noterae* was shown to utilize H$_2$ + CO$_2$. Genome comparisons of the type strains from *Aa. sticklandii* and *Aa. noterae* showed that these strains belong to one phylogenomic cluster belonging to the same species. Therefore, a reclassification of *Aa. noterae* strains was suggested to be a member of the species *Aa. sticklandii* (Böer et al., 2024e). *Aa. noterae* produced more biomass if cells were cultivated under of N$_2$ + CO$_2$ atmosphere, compared to cell suspensions under an atmosphere of H$_2$ + CO$_2$ in the

respective yeast extract supplemented medium. Anyhow, with an $H_2 + CO_2$ atmosphere *Aa. noterae* cells produced higher amounts of acetate and showed a biphasic growth compared to respective cell suspensions under a $N_2 + CO_2$ atmosphere. An initial rapid growth phase was followed by a slower second growth phase where H_2 uptake was increased (Sleat et al., 1985).

The genus *Clostridioides* contains the two species *Clostridioides difficile* and *Clostridioides mangenotii* of which only the former species comprises strains that utilize $H_2 + CO_2$ (Lawson et al., 2016). The type strain of *Cl. difficile* DSM 1296[T] was shown to have lost the ability to grow autotrophically, but the strains *Cl. difficile* DSM 27543, DSM 12056, and DSM 12057 were demonstrated to metabolize $H_2 + CO_2$ by producing acetate (Köpke et al., 2013). Strains losing the ability to utilize $H_2 + CO_2$ are hypothesized to retain the WLP as an electron sink to dispose reduction equivalents during the fermentation of amino acids by the Stickland reaction (Gencic and Grahame, 2020). As an autotrophic pathogen, *Cl. difficile* is not suitable for applications in biotechnology.

The genus *Terrisporobacter* contains the species *Terrisporobacter glycolicus*, *Terrisporobacter mayombei*, *Terrisporobacter petrolearius, Terrisporobacter hibernicus,* and *Terrisporobacter vanillatitrophus* (Deng et al., 2015; Gerritsen et al., 2014; Mitchell et al., 2023; Böer et al., 2024e). From the five type strains, only *Ts. mayombei* DSM 6539[T] was proven to convert $H_2 + CO_2$ into acetate (Kane et al., 1991; Böer et al., 2024e). Furthermore, the non-type strains *Ts. glycolicus* DSM 13865 and *Ts. glycolicus* ATCC 29797 were described to grow with $H_2 + CO_2$ (Küsel et al., 2001; Ohwaki et al., 1977). Analysis of *Terrisporobacter* genomes showed that genes encoding gene products required for autotrophic growth are present in every strain. However, the ability to grow by WLP was lost by some strains (Böer et al., 2023). *Ts. glycolicus* is often discussed as an emerging pathogen, and thereby would not be available for biotechnological applications (Cheng et al., 2016; Syuhadah et al., 2020). However, phylogenomic analysis of *Terrisporobacter* genomes demonstrated that isolates classified as *Ts. glycolicus* in the past should be reassigned to the non-pathogenic species *Ts. petrolearius* (Böer et al., 2023). The type strain of *Ts. petrolearius* is described to be unable to use $H_2 + CO_2$ for growth. In contrast three H_2-utilizing *Ts. petrolearius* strains (DSL, HSE and ELB) were isolated from digested sludge of a waste water treatment plant, horse feces, and river marsh sediment (Böer et al., 2024e).

3.9. Moorellaceae

M. thermoacetica (formerly *Clostridium thermoaceticum*) is one of the most prominent acetogens, as it has been used by H. G. Wood and L. Ljungdahl to examine the reactions of the methyl and carbonyl branch of the WLP (Ragsdale, 1997). Most species in the the genus *Moorella* are mostly acetogenic species, which mainly differ in their ability to utilize gaseous substrates (Wiegel, 2015a). *M. thermoacetica* has been described to utilize both $H_2 + CO_2$ and CO for growth (Kerby & Zeikus, 1983). *M. sulfitireducens* was shown to grow on CO with or without thiosulfate, while growth on $H_2 + CO_2$ was dependent on the addition of sulfite as an electron acceptor (Slobodkina et al., 2022; Section 2). The species *Moorella glycerini, Moorella stamsii, M. caeni*, and *M. carbonis* were described to utilize only CO (Myers et al., 2023; Slobodkin, 1997a; Böer et al., 2024b). However, the major end product of CO conversion by *M. stamsii, M. caeni,* and *M. carbonis* is $H_2 + CO_2$ (water–gas shift reaction), while other CO-oxidizing species of the *Moorella* genus produce acetate as the major fermentation product (Alves et al., 2013; Jiang et al., 2009; Vecchini Santaella et al., 2023; Böer et al., 2024b). The species *M. mulderi* was described to only utilize $H_2 + CO_2$ (Balk et al., 2003). The type strain of *Moorella humiferrea* neither utilizes $H_2 + CO_2$, or CO for growth (Nepomnyashchaya et al., 2012; Alves et al., 2013), however, two strains were isolated that are able to grow autotrophically with gaseous substrates (Table 1). *M. humiferrea* OCP was found to be a carboxydotrophic hydrogenogen converting CO into $H_2 + CO_2$ and *M. humiferrea* LNE was described to produce acetate from CO as well as

from $H_2 + CO_2$ (Böer et al., 2024b). *Moorella thermoautotrophicum* (formerly known as *Clostridium thermoautotrophicum*) has been described as separate species within the genus *Moorella* (Wiegel et al. 1981; Rijssel et al., 1992). The strains DSM 7417 and the type strain DSM 1974[T] were deposited in public type culture collections. In 2020, Redl et al. (2020) sequenced the genomes of both strains and showed that, on the one hand, the deposited culture of DSM 1974[T] is a mixed culture consisting of at least two different strains (cultures at DSMZ and ATCC) and, on the other hand, that DSM 1974[T] and DSM 7417 belong to the species *M. thermoacetica*. They also resubmitted the two strains that were isolated from the mixed culture under DSM 103132 and DSM 103284 and proposed reclassification of DSM 7417. However, Rainey et al., 1993 submitted a partial 16S rRNA gene sequence of *M. thermoautotrophica*, which exhibited only 91 % sequence similarity to the 16S rRNA gene sequence of *M. thermoacetica* DSM 512[T]. This sequence indicated that in the past *M. thermoautotrophica* seems to have existed but was lost over the last 27 years. Consequently, *M. thermoautotrophica* is considered a *nomen confusum* and placed on the list of rejected names (Arahal et al., 2022). Several strains of *M. thermoacetica* were described to produce acetate as the sole product from $H_2 + CO_2$. However, it was reported that the strains Y72, HUC22-1, KAM, BGP, COM, MBA and DSM 2955[T] also produce small amounts of ethanol when cultivated with $H_2 + CO_2$ (Sakai et al., 2005; Inokuma et al., 2007; Kimura et al., 2016; Böer et al., 2024b). Methanol utilization has been reported for *M. thermoacetica*, *M. mulderi, M. stamsii, M. caeni,* and *M. carbonis*. All genomes of strains from the *Moorella* genus encode both cytochromes and quinones (Rosenbaum and Müller, 2021). This is a rare feature for acetogens and was only detected in strains of the *Sporomusa* genus (Section 3.4; Das et al., 2003; Möller et al., 1984). The physiological role of cytochromes and quinones in acetogens remains unclear. However, they are hypothesized to provide additional routes for energy conservation as alternative systems to the Ech complex in *Moorella* (Rosenbaum and Müller, 2023). Examples for these systems are subunits of the heterodisulfide reductase together with two methylene-THF reductase subunits (MetFV-HdrABC-MvhD) or the FixABC-complex, which are present in every strain of the genus *Moorella* (Kremp et al., 2022; Rosenbaum and Müller, 2021). The majority of carboxydotrophic hydrogenogens show a conserved gene cluster encoding a monofunctional CODh (*cooS*) in synteny with genes for a membrane-associated Ech complex (Diender et al., 2015; Fukuyama et al., 2020). Carboxydotrophic hydrogenogenic strains in the *Moorella* genus were found to differ from other carboxydotrophic hydrogenogens, as they encode an Ech complex in synteny to *cooS* that is structurally more similar to the Ech complex found in acetogenic *Moorella* strains (Böer et al., 2024b).

3.10. Sporomusaceae

The family of *Sporomusaceae* comprises bacterial species belonging to the genera *Anaeroarcus, Anaeromusa, Anaerosinus, Anaerospora, Anaerosporomusa, Dendrosporobacter, Desulfosporomusa, Methylomusa, Pelorhabdus, Pelosinus, Propionispora, Psychrosinus, Sporolituus, Sporomusa*, and *Thermosinus*. However, known acetogens are only found in the genera *Thermosinus* and *Sporomusa* (Table 1), the latter contains nine different described species. These strains have been isolated from a variety of different sources such as effluent of an alcohol distillation industry, gut of termites, anoxic mud from a polluted freshwater creek, Leine or sugar beet leaf silage, sediment, forest soil, and from roots (Ollivier et al., 1985; Boga et al., 2003; Breznak et al., 1988; Hattori et al., 2013; Dehning et al., 1989; Möller et al., 1984; Hermann et al., 1987; Kuhner et al., 1997; Gößner et al., 2006). All *Sporomusa* are slightly curved or banana-shaped rods with lateral flagella and form colonies showing a brownish color. Cells have a multi-layered cell wall and stain mostly Gram-negative. Except for *Sporomusa paucivorans*, all strains form endospores (Hermann et al., 1987). For all *Sporomusa* species, membrane bound b-type and c-type cytochromes as well as ubiquinones have been reported (Möller et al., 1984, Rosenbaum and

Müller, 2021). Optimal growth temperatures vary between 25–37 °C, with optimal pH values of 6.3–7.5. For most *Sporomusa* species only their basic metabolic features are described. All members of the genus are able to convert $H_2 + CO_2$ into acetate via the WLP, with supplementation of yeast extract supporting growth. CO utilization is only known for *S. ovata* and *S. termitida*. A variety of different sugars, methylated compounds, betaine, lactate, and lots of other substrates can be used by the different species (Hermann et al., 1987; Breznak et al., 1988). Exceptions are *S. termitida,* which only utilizes mannitol and *S. paucivorans,* which does not metabolize any sugar. The species *S. carbonis* (Section 2) differs from other described acetogenic *Sporomusa* species by its inability to grow on $H_2 + CO_2$. By now, *S. carbonis* is the only carboxydotrophic hydrogenogenic strain in the genus *Sporomusa* converting CO to $H_2 + CO_2$ as major product (Böer et al., 2024d).

Although *S. sphaeroides* is the type species of this genus, more is known about metabolic traits of *S. ovata.* Both strains are able to grow on $H_2 + CO_2$, betaine, N,N-dimethylglycine, sarcosine, DL-lactate, pyruvate, L-alanine, methanol, ethanol, *n*-propanol, *n*-butanol, 1,2-propanediol, and 2,3-butanediol. N,N-dimethylethanolamine, trimethylamine, glycerol, DL-glycerate, 3-hydroxybutyrate, L-serine, and ethylene glycol can only be metabolized by *S. sphaeroides,* whereas fructose only by *S. ovata.* The main fermentation product of both strains is acetate, with traces of ethanol. Kremp and coworkers (2020) proposed a new type of electron-bifurcating, ferredoxin-dependent transhydrogenase − Stn; the *Sporomusa* type of NADH-dependent reduced ferredoxin:NADP + oxidoreductase (Nfn). From a genomic context, the genes encoding this enzyme complex (*stnABC*) are located directly downstream of gene cluster encoding the genes for enzymes of the WLP (Poehlein et al., 2013; Kremp et al., 2020; Böer et al., 2024d). Kremp et al. (2020) proposed NADH-dependent NADP$^+$ reduction with parallel oxidation of reduced ferredoxin. Several *Sporomusa* species have been used for microbial electrosynthesis, but *S. ovata* showed the best performance in acetate production during microbial electrosynthesis that was coupled with CO_2 reduction, followed by *A. woodii* (Section 3.2) and *C. ljungdahlii* (Section 3.1) (Nevin et al., 2010; Nie et al., 2013; Aryal et al., 2017; Das et al., 2020). For the latter two species, genetic tools have been available for several years, but recently Tremblay and Zhang (2024) also published a protocol for electroporation of plasmids into *S. ovata.* They also created a recombinant strain able to produce acetone from CO_2 and developed a tool for genome editing.

3.11. Selected acetogens from different families

The family *Acetonemataceae* comprises two genera, namely *Acetonema* and *Anaerosporomusa.* For the genus *Acetonema*, only one species is described, *Acetonema longum*, which is an acetogen isolated from the termite gut, (Kane and Breznak, 1991). This rod-shaped microbe stains Gram-negative, forms endospores, is motile, and has an optimal growth temperature between 30–33 °C with an optimal pH of 7.8. Cells grow using $H_2 + CO_2$, glucose, ribose, fructose, mannose, rhamnose, mannitol, citrate, pyruvate, oxaloacetate, fumarate, *n*-propanol, ethylene glycol, and 3,4,5-trimethoxybenzoate, with acetate and *n*-butyrate as main fermentation products. It does not use sulfate or nitrate as electron acceptors. *At. longum* is known to form polyphosphate storage granules containing phosphorus, magnesium, and oxygen during sporulation (Tocheva et al., 2013). This microbe forms microcompartments during growth on 1,2-propandiol or rhamnose (Tocheva et al., 2014).

The family *Halobacteroidaceae* comprises halophilic bacteria, which are mostly found in salt caverns and other high-salt environments, such as the Dead Sea (Bordenave et al., 2013). Both acetogens and methanogens highly impact the carbon storage capacity of salt caverns. The genus *Acetohalobium* comprises solely of the species *Acetohalobium arabaticum*, which is an acetogen capable of carbon fixation from $H_2 + CO_2$, CO, and formate (Zhilina and Zavarzin, 1990). Because the species is halophilic, it requires a NaCl concentration between 1.7 M to 4 M but a neutral pH for growth (Zavarzin and Zhilina, 2015). In contrast, the halophilic acetogens *Fuchsiella alkaliacetigena* and *Fuchsiella ferrireducens* require, besides a high NaCl concentration, an alkaline pH of 8.5–10.5 for growth (Zhilina et al., 2012; Zhilina et al., 2015; Huang et al., 2022; Zhilina, 2018). Both acetogens of the family *Halobacteroidaceae* metabolize $H_2 + CO_2$ to form acetate as the sole product (Zhilina et al., 2012; Zhilina et al., 2015). *F. ferrireducens* reduces different iron-containing compounds with ferric iron as an electron electron acceptor (Gracheva et al., 2017). *Natranaerofaba carboxydovora* is another haloalkaliphilic CO-utilizing acetogen but is assigned to the family *Natranaerofabaceae* (Sorokin et al., 2020). Further details are given in Section 2.

Holophaga foetida was isolated during enrichment cultivation targeted to obtain *A. woodii* (Bak et al., 1992). The strain represents the only species in this genus and is assigned to the family *Holophagaceae* (Liesack et al., 1994). The strain fixes carbon from CO and $H_2 + CO_2$ using the WLP but does not use H_2 as an electron donor (Anderson et al., 2012). Besides its acetogenic properties, *H. foetida* is known for the degradation of methoxylated aromatic compounds and inorganic sulfides to dimethylsulfide and methanethiol (Kreft and Schink, 1993). Therefore, it uses sulfide as a methyl acceptor, which is unique in the group of demethylating acetogens.

The only member of the family *Thermacetogeniaceae* is *Thermacetogenium phaeum*, which was isolated in co-culture with a thermophilic hydrogenotrophic methanogen (Hattori et al., 2000). *Th. phaeum* grows syntrophically in co-culture with methanogens by acetate oxidation, but is also able to grow with $H_2 + CO_2$ or CO in pure culture producing acetate, though with very low specific growth rates of ∼ 23 h (Oehler et al., 2012; Hattori et al. 2000). Acetate activation likely involves the initial reduction of acetate to acetaldehyde by an aldehyde:ferredoxin oxidoreductase (AOR), followed by oxidation to acetyl-CoA, which is then split into a methyl and a [CO]-group in a reversal of the COdh/Acs reaction (Keller et al., 2019). Furthermore, *Th. phaeum* grows with pyruvate or methylotrophic with several alcohols and methoxylated monoaromates. It is unable to utilize sugars.

Within the family *Thermoanaerobacteraceae*, the genus *Thermoanaerobacter* contains, next to many organotrophic fermentative species, only two known acetogens (Table 1). *T. kivui*, which is the thermophilic acetogen with the highest optimum-growth temperature of 66 °C (Leigh et al., 1981). Isolated from mud samples of Lake Kivu, cells are capable of growing using several sugars, pyruvate, and formate in addition to $H_2 + CO_2$, without any requirement for added vitamins. *T. kivui* can not grow solely on CO, but cells utilize CO as part of a gas-mix with H_2 and CO_2 (Daniel et al., 1990; Kevbrina and Pusheva, 1996). *T. kivui* cells were adapted to grow exclusively on CO through serial passaging (Weghoff and Müller, 2016). The highly ordered surface layer surrounding *T. kivui* cells has also been characterized extensively (Lupas et al., 1994). More insight regarding its physiology and genetic engineering capabilities are presented in Section 4 and 5.5, respectively.

The genus *Treponema* within the family *Spirochaetaceae* is known to contain human pathogens (e.g., *Treponema pallidum*, *Treponema denticola*, and *Treponema carateum*), which cause syphilis, periodontal disease, and pinta (*i.e.*, chronic skin lesions that occur primarily in young adults), respectively (Pillay, 2013; Buyuktimkin et al., 2019). In contrast, *Treponema primitia* is a non-pathogenic microbe, which grows in the guts of termites (Graber et al., 2004). In its natural habitat *Tn. primitia* utilizes H_2 that is provided from symbiotically living protozoa together with CO_2 to produce acetate, which serves as the major energy source in the termite *Zootermopsis angusticolis* (Murphy et al., 2008). Growth of *Tn. primitia* is hindered by CO, but the acetogen was proven to be oxygen-tolerant to a maximal concentration of 0.5 % (v/v) (Graber and Breznak, 2004).

4. Diversity of redox carriers used in WLP

While the core enzymes and molecular intermediates of the WLP are well characterized, details of the electron carriers involved, and in some

cases the exact mechanism of chemiosmotic energy conservation remain unclear. One challenge is the remarkable diversity of acetogens. While pure acetogenesis consists of converting $H_2 + CO_2$ to acetate via the WLP, most acetogens can use a variety of other growth substrates, and a few produce a broader spectrum of end products. There is also a great deal of diversity in lifestyle, with some species seemingly optimized for growth on gases expressing WLP proteins at extremely high levels (Valgepea et al., 2022). Other species are optimized for a heterotrophic lifestyle and may prefer to make use of alternative soluble electron acceptors such as nitrate, or even amino acids, in which case the WLP is typically expressed at much lower levels and acts as a supplemental electron sink to improve the energetics of overall metabolism (Seifritz et al., 1993; Gencic and Grahame, 2020).

Species also vary in the specific redox carriers that are used to shuttle electrons to the WLP, with some species preferring NADH, NADPH, Fd^{2-}, or even H_2 as reducing equivalents for specific reactions (Fig. 3). The carbonyl branch of the WLP consists of CO_2 reduction to CO via COdh, with ferredoxin as the electron donor. The methyl branch of the WLP reduces CO_2 to a methyl-group coupled to a corrinoid iron sulfur protein (CoFeSP), requiring three reduction steps and input of one ATP. The first step, which is oxidation of CO_2 to formate, is usually carried out by a Fdh, which is NADPH-dependent in *M. thermoacetica* and "*C. autoethanogenum*", where it forms a complex with electron bifurcating Fe-NADPH-dependent hydrogenase (Yamamoto et al., 1983; Wang et al., 2013). One of *C. ljungdahlii*'s three Fdh candidates is similar to non-acetogenic clostridial enzymes requiring ferredoxin, but the more

likely candidate for acetogenesis appears to use NADH (Köpke et al., 2010; Moon et al., 2021). Alternatively, HDCR can directly reduce CO_2 using hydrogen in *A. woodii* and *T. kivui* (Schuchmann and Müller, 2013; Schwarz et al., 2018). The next crucial reduction step is carried out by methylene-THF dehydrogenase (FolD), which is NADH-dependent in *A. woodii*, and NADPH-dependent in *M. thermoacetica* (Ragsdale and Ljungdahl, 1984; O'Brien et al., 1973). Methylene-THF is further reduced by a methylene-THF reductase (MTHFR) complex, which consists at a minimum of the two subunits MetV and MetF, but in some cases these appear to associate with other enzyme subunits. For example, in *M. thermoacetica* MetFV in complex with HdrABC and MvhD is suggested to couple methylene-THF reduction to an unknown electron acceptor via flavin-based electron bifurcation, possibly using electrons from NADH. A similar enzyme complex of *S. ovata* is proposed to be associate with the cell membrane, possibly electrons from quinones and confurcating electrons from NADH as well (Mock et al., 2014; Kremp et al., 2022). There is evidence that a much smaller complex in *A. woodii*, consisting of MetFV with RnfC2, reduces methylene-THF with electrons from NADH, but no evidence of electron bifurcation was found (Bertsch et al., 2015). The MetFV of *C. ljungdahlii* accepts electrons from ferredoxin without the need for other subunits, and without evidence for electron bifurcation (Yi et al., 2021).

The WLP must be coupled to a membrane gradient to allow for energy conservation, and here again, there is considerable diversity among acetogens. All species contain a transmembrane complex that uses reduced ferredoxin to pump ions across the cytoplasmic membrane. In
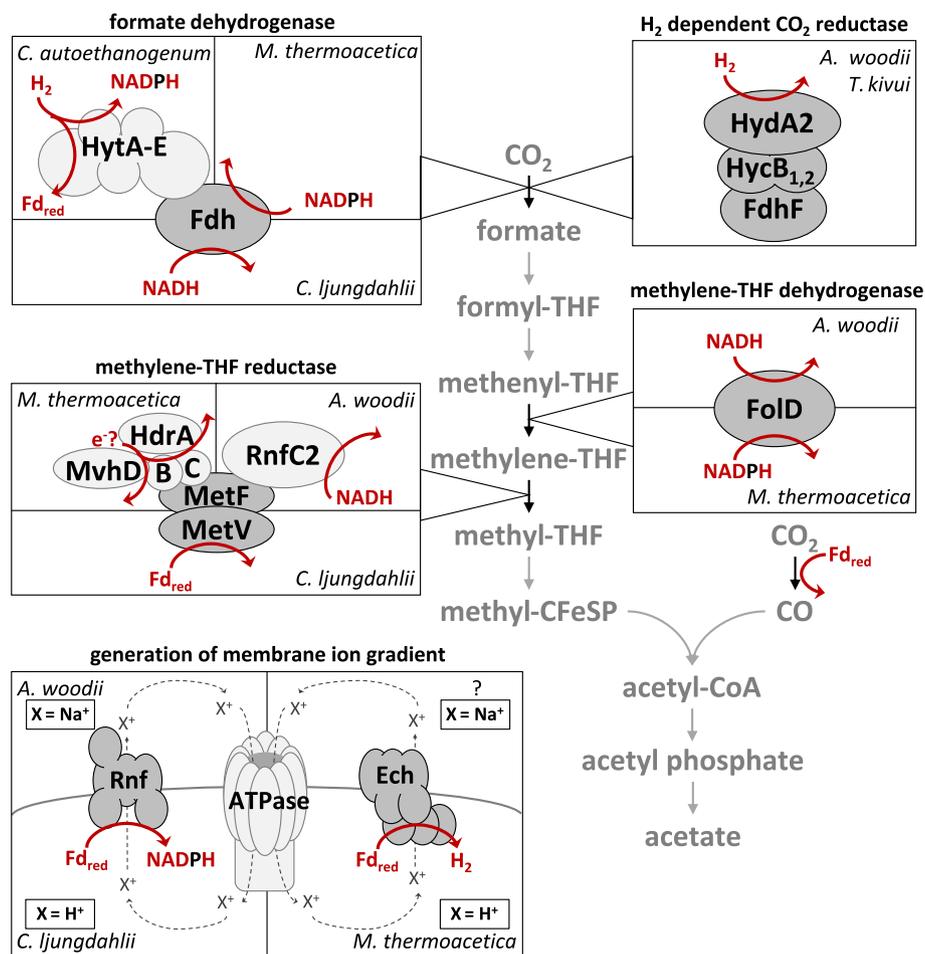
Fig. 3. Diversity of redox carriers in acetogens. Red arrows indicate known redox carriers for specific enzymes of acetogenesis, for simplicity only the reduced form of each redox carrier is shown. The subunits of the core enzyme that are catalyzing each redox step are shown in dark gray, and any species-specific associated enzymes in light gray. Cation transport across the membrane is shown as X^+, with the specific cation boxed next to the species name. e^- indicates specific reducing equivalent is unknown.

most species, the Rnf complex couples the transfer of ions across the membrane to reduction of NAD$^+$, using reduced ferredoxin (Biegel and Müller, 2010). Alternatively, an Ech complex transports ions while using ferredoxin to reduce molecular hydrogen (Katsyv and Müller, 2022). The nature of the ion transported varies by species, with *A. woodii*'s Rnf transporting Na$^+$, while *C. ljungdahlii*'s version transports H$^+$, and the Ech complex of *M. thermoacetica* transports H$^+$ as well (Schuchmann and Müller, 2014). Most Ech-containing acetogens contain two copies of the Ech gene cluster, and in the case of *T. kivui*, there is biochemical evidence to suggest that both Na$^+$ and H$^+$ could be transported, but genomic evidence suggests its ATP synthase is H$^+$-dependent (Schoelmerich and Müller, 2019; Hess et al., 2014). In fact, there is genomic evidence for all four combinations of Ech/Rnf-dependent acetogens with Na$^+$/H$^+$-dependent ATP synthases, although, in theory, an Na$^+$/H$^+$ antiporter could couple ATP synthesis to an Ech/Rnf complex transporting the other ion (Rosenbaum and Müller, 2021).

Another aspect that has recently received considerable attention is the role of two genes of the glycine cleavage system, dihydrolipoyl dehydrogenase *lpdA* and glycine cleavage system H protein *gcvH*, which are often co-localized within a tightly clustered group of WLP genes, especially in *Clostridia* sp. (Poehlein et al., 2015a). Recent evidence for the role of these genes in acetogens include; (1) reverse function of the glycine cleavage system for amino acid biosynthesis and reductant disposal under starvation conditions in Stickland fermenting bacteria (Gencic and Grahame, 2020); (2) part of the glycine synthase carbon fixation pathway, functioning complementary to the WLP to allow reductant balancing and greater metabolic flexibility (Song et al., 2020); and (3) as donor for an electron-bifurcation reaction with methylene-THF reductase, allowing coupling of this step to a transmembrane proton gradient (Öppinger et al., 2021).

The reasons for all this physiologic diversity are not entirely clear but presumably reflect the environments and growth conditions that a specific strain will thrive under, as well as the appropriateness of any specific strain for industrial applications. For example, dramatic differences in thermodynamic efficiency are evident when comparing the threshold H$_2$ level (the H$_2$ partial pressure below which acetogenesis is not possible) across species, which is used as a proxy for the relative thermodynamic efficiency of a particular acetogen's metabolism. The H$_2$ thresholds vary widely, from 200 to 2000 Pa for *Clostridia* species, 20 to 50 Pa for *Acetobacterium*, and under 15 Pa for *Sporomusa* species (Munoz and Philips, 2023). Based on this, it appears that members of *Clostridia* are more appropriate for syngas fermentation, because they can make use of high gas pressures for better growth yields and product generation, while *Sporomusa* strains are most efficient for microbial electrosynthesis applications (Munoz and Philips, 2023).

5. Metabolic engineering of acetogens

Several acetogens have been metabolically engineered and various plasmid-based genetic tools have been implemented into their respective genetic toolboxes (Table 2). Currently, *A. woodii, A. wieringae,* "*C. autoethanogenum*", "*C. coskatii*", *C. ljungdahlii, E. callanderi, E. limosum, E. maltosivorans,* and the two thermophiles *M. thermoacetica* and *T. kivui* are genetically accessible. From these, *A. woodii,* "*C. autoethanogenum*", *C. ljungdahlii, E. limosum,* and the thermophile *T. kivui* have been studied most. For those, the main biobricks in the development of respective plasmids are origins of replication (replicons), constitutive and inducible promoters, enzyme- and fluorescence-based reporter proteins, genetic elements for gene deletions and insertions, as well as non-native pathways for recombinant production of biocommodities, which will be discussed in this Section (Table 2).

5.1. Acetobacterium woodii

Acetobacterium woodii was the first acetogen that was transformed with foreign plasmid DNA (Strätz et al., 1994). Despite this early

Table 2
Available tools and respective purposes for genetic engineering in acetogens.

tool	purpose	literature
A. woodii		
Homologous recombination (suicide plasmids)	Gene deletions and insertions*	Westphal et al., 2018; Wiechmann et al., 2020; Moon et al., 2021; Moon and Müller, 2021; Mook et al., 2022; Baker et al., 2022; Moon et al., 2023a; Moon et al., 2023b; Moon et al., 2023c; Chowdhury et al., 2020;
CRISPR-Cas[#]	Gene deletions and insertions	Poulalier-Delavelle et al., 2023
Reporter proteins (β-glucuronidase, FAST)	Biobricks screening as promoter studies; tagging proteins of interest; cell labeling for co-culture experiments	Beck et al., 2019; Mook et al., 2022; Poulalier-Delavelle et al., 2023; Flaiz et al., 2022
"*C. autoethanogenum*" and *C. ljungdahlii*		
ClosTron	Gene deletions	Liew et al., 2017
Homologous recombination (suicide plasmids)	Gene deletions and insertions	Leang et al., 2013; Ueki et al., 2014; Liew et al., 2017
Transposon based	Insertion of large gene clusters	Philipps et al., 2019
CRISPR-Cas9	Gene deletion	Nagaraju et al., 2016; Huang et al., 2016; Seys et al., 2020; Poulalier-Delavelle et al., 2023
CRISPR-Cas12a	Gene deletion	Zhao et al., 2019;Klask et al., 2022; Dykstra et al. 2022
CRISPRi	Gene deactivation	Woolston et al., 2018; Fackler et al., 2021
CRISPR based base-editing	Single nucleotide gene manipulation	Xia et al., 2020; Seys et al., 2023
Reporter proteins (β-glucuronidase, chloramphenicol acetyltransferase, FbFPs, FAST, HaloTag, SNAP-tag)	Biobrick screening, promoter studies; Tagging proteins of interest; cell labeling for co-culture experiments	Banerjee et al., 2014; Huang et al., 2016; Nagaraju et al., 2016; Molitor et al., 2016; Yang et al., 2017; Woods et al., 2022; Charubin et al., 2020a; Charubin et al., 2020b
E. limosum* and *E. callanderi		
CRISPR-Cas9	Gene deletions	Shin et al., 2019; Jeong et al., 2020
RelBE-family toxin-antitoxin	Gene deletions	Millard et al., 2023
Homologous recombination (linear DNA)	Gene deletions[+]	Sanford et al., 2023
CRISPRi	Gene deactivation	Shin et al., 2023
Reporter proteins (β-glucuronidase, chloramphenicol acetyltransferase, eGFP, FbFPs, FAST, HaloTag, SNAP-tag)	Biobrick screening including promoters, 3′ UTRs, and 5′UTRs Tagging proteins of interest Cell labeling for co-culture experiments	Jeong et al., 2020; Sanford and Woolston, 2022; Song et al., 2022; Shin et al., 2019; Wang et al., 2021; Flaiz et al., 2021; Flaiz et al., 2022; Flaiz et al., 2024
M. thermoacetica		
Thermostable replicating plasmid	Plasmid-based gene expression	Bourgade et al., 2022b
Homologous recombination (suicide plasmids)	Gene deletions and insertions	Kita et al., 2013; Iwasaki et al., 2013; Iwasaki et al., 2017; Rahayu et al., 2017; Kato et al., 2021
T. kivui		
Thermostable replicating plasmid	Plasmid-based gene expression	Katsyv et al., 2021; Dietrich et al., 2022;

Table 2 (*continued*)

tool	purpose	literature
Homologous recombination (suicide plasmids)	Knockout strains	Katsyv and Müller, 2022 Basen et al., 2018; Moon et al., 2019; Jain et al., 2020; Schwarz et al., 2020
Reporter proteins (FAST)	Promoter studies	Hocq et al., 2023

A. woodii ΔpyrE was used as parental strain for further deletions/insertions in all instances.
\# making use of its endogenous CRISPR/Cas system
\+ by integrating antibiotic resistance genes

milestone, it took another 20 years to develop the first genetic tools for this strain (Straub et al., 2014). Although the initial report of transformation described both electroporation and conjugation, today electroporation is the method of choice to engineer *A. woodii*. The protocol currently used is based on that described for *C. ljungdahlii* (Leang et al., 2013). In recent years, the genetic toolbox of *A. woodii* has been improved by the implementation of various biobricks, such as promoters, reporter proteins, genome engineering elements, and replicons to enable physiological studies or expression of recombinant metabolic pathways.

Nowadays, four replicons are regularly used in *A. woodii*, including the replicon of plasmid pIP404 from *Clostridium perfringens*, but also several replicons of the *Clostridium-E. coli* shuttle plasmid series pMTL80000 (Heap et al., 2009). This includes replicons from plasmids of *Clostridium botulinum* (pBP1), *Clostridium butyricum* (pCB102), and *Cl. difficile* (pCD69). Although copy numbers were not yet experimentally determined, it was suggested that the use of different replicons impacted recombinant acetone production (Hoffmeister et al., 2016). While the chosen replicon affects the plasmid copy number, promoter activities highly regulate gene expression. Therefore, the choice of suitable promoters for heterologous gene expression is crucial. The identification of suitable constitutive or inducible promoters can be cumbersome as the number of reporter proteins applicable for anaerobes to perform respective enzyme activity assays is scarce. Beck and co-authors assembled a promoter library of one constitutive and four inducible promoters and determined their activity using the enzymatic β-glucuronidase reporter protein assay (Beck et al., 2019). The importance of controlled gene expression was showcased by the expression of genes encoding the arginine deiminase (ADI) pathway.

Screening of biobricks, such as promoters using enzymatic reporter assays, is time intensive, cannot be performed in real-time, and compels cell lysis. An elegant way to overcome these obstacles is the use of fluorescent reporter proteins. Most fluorescent reporters such as GFP or mCherry, are oxygen-dependent. However, several oxygen-independent reporters were recently described to be functional in strictly anaerobic species, including flavin mononucleotide-based fluorescent proteins (FbFPs), HaloTag, SNAP-tag, or the fluorescent activating and absorption shifting tag (FAST) (Streett et al., 2021). Due to its small size, FAST can also be used to generate fluorescent fusion proteins. In the case of *A. woodii*, the lactate dehydrogenase LdhD from *Leuconostoc mesenteroides* was tagged and expressed to quantify LdhD-producing and non-producing cells (Mook et al., 2022). Production of the fusion protein resulted in a heterogenous population, which was also observed in other reports where non-tagged variants of FAST were expressed in a plasmid-based manner (Mook et al., 2022; Flaiz et al., 2022; Poulalier-Delavelle et al., 2023). Thus far, explanations for this phenomenon are often based on speculations about plasmid instability or insufficient gene expression. A first glimpse to prove this assumption was provided by Poulalier-Delavelle and co-authors, who integrated FAST at the *pheA* locus into the genome of *A. woodii*. Consequently, the production of genomically integrated FAST resulted in a homogenous population. However,

fluorescence intensity massively decreased (Poulalier-Delavelle et al., 2023).

The most often-reported genomic engineering tool that is used to generate *A. woodii* mutant strains is based on two subsequent single crossover events, which are controlled by using an antibiotic resistance cassette as initial selection for the first crossover and *pyrE* combined with uracil/5-fluoroorotic acid (FOA) as a counterselection marker for the second crossover recombination (Westphal et al., 2018; Baker et al., 2022). This method was used to generate the parental mutant strain *A. woodii ΔpyrE*, and the function of the native *pyrE* was also restored in this mutant (Baker et al., 2022). Later, the *A. woodii ΔpyrE* mutant was used to generate further gene deletions to investigate fundamental metabolic features focusing on the WLP, lactate production, or alcohol metabolism. As a proof-of-concept, the physiological role of the Rnf complex in *A. woodii* was investigated. Deletion of genes encoding the Rnf complex revealed that this respiratory enzyme is not essential for growth on fructose but essential for autotrophic growth and growth with low-energy substrates (Westphal et al., 2018). Genes *hydBA* of the hydrogenase operon were deleted to investigate H_2 production by *A. woodii*. Respective cells lost the ability to grow on $H_2 + CO_2$. It was concluded that intermediate H_2 production is essential for growth on organic substrates, because it is required for CO_2 reduction by the HDCR (Wiechman et al., 2020).

A. woodii was genetically engineered to produce formate by a deletion of the *hdcr* gene cluster. This abolished the growth of $H_2 + CO_2$ but also of fructose; however, growth could be reconstituted by the addition of formate. It was shown that the respective deletion mutant had the ability to produce formate from alternative electron donors such as caffeate (Moon et al., 2023a). In contrast, deletion of genes *metFV* of the methyl branch of the WLP again abolished growth on C_1 compounds including $H_2 + CO_2$, while growth on fructose was still possible (Moon et al., 2023b). A further way of formate production was presented by the deletion of genes potentially encoding a formyl-THF synthase and a formate transporter. Double deletions impaired growth, abolished formate consumption, and resulted in formate production from $H_2 + CO_2$ (Moon et al., 2021). Furthermore, the lactate metabolism was investigated by creating the deletion mutant *A. woodii ΔlctCDEF*. Because these genes were essential for lactate metabolism, growth with this substrate was abolished. The authors hinted that the regulatory Lct system has the potential to be used as a tightly regulated lactate-inducible system (Schoelmerich et al., 2018). By generating a triple deletion mutant *via* combining three of the aforementioned knockouts, *A. woodii ΔhydBA/hdcr/lctbcd* was designed to produce lactate in resting cell experiments from various methyl groups and CO (Moon et al., 2023c). Alcohol metabolism was investigated by creating several *adh* deficient mutant strains. Deletion of the bifunctional aldehyde/alcohol dehydrogenase resulted in the loss of the ability to grow with ethanol as substrate (Baker et al., 2022). The deletion of the gene encoding the alcohol dehydrogenase Adh4 resulted in impaired growth on 1,2-propanediol and *n*-propanol production (Chowdhury et al., 2020). While the deletion of *adhE3* or *adhE6* did not affect ethanol formation, deletion of *adhE4* resulted in improved ethanol production from fructose in Na^+-depleted medium, which has the potential for biotechnological applications (Moon and Müller, 2021). An additional genomic engineering tool was recently presented in the form of the endogenous Type I-B CRISPR-Cas system of *A. woodii*, which was used to perform both gene deletions but also insertions. Usage of the endogenous CRISPR-Cas system circumvents possible toxic effects of non-native Cas9 proteins. After identification of the native PAMs and leader sequences, the system was used to generate *pyrE*, *pheA*, and *hsdR1* deletion mutants. Moreover, the gene encoding FAST was integrated into the *pheA* locus as stated previously (Poulalier-Delavelle et al., 2023).

A. woodii is developing to be a promising biocatalyst to produce various biocommodities from gaseous feedstock such as $H_2 + CO_2$, methanol, or formate. The first milestone was achieved through the plasmid-based production of acetone from $H_2 + CO_2$. A synthetic

acetone production operon with genes of *Clostridium acetobutylicum*, which were controlled by the thiolase promoter P*thlA* from the respective strain, was designed (Hoffmeister et al., 2016). Later, it was shown that by the addition of a secondary alcohol dehydrogenase acetone was partially further converted to isopropanol (Höfele et al., 2023). The heterologous expression of genes encoding the reverse β-oxidation (hexanoyl-CoA synthesis (*hcs*) operon) of *C. carboxidivorans* enabled the production of *n*-butyrate and *n*-hexanoate from $H_2 + CO_2$ (Wirth and Dürre, 2021). A similar set of genes encoding the butyryl-CoA synthesis (*bcs*) operon of *E. callanderi* was expressed in *A. woodii*, which resulted in *n*-butyrate production from methanol (Chowdhury et al., 2022). Isobutanol production was achieved by heterologous expression of a ketoisovalerate ferredoxin oxidoreductase from *Clostridium thermocellum* and a bifunctional aldehyde/alcohol dehydrogenase from *C. acetobutylicum*. Isobutanol production was feasible from fructose, however, production from $H_2 + CO_2$ was only possible after the addition of the precursor of ketoisovalerate (Weitz et al., 2021). The biodegradable plastic poly-3-hydroxybutyrate (PHB) was recombinantly produced from $H_2 + CO_2$ by expressing a genetic operon initially designed to produce PHB in *C. ljungdahlii* and "*C. coskatii*" (Höfele and Dürre, 2023; Flüchter et al., 2019). The mutant strain *A. woodii* Δ*lctCDEF* was used as the basis for recombinant lactate production. Plasmid-based but also genomically integrated *ldhD* expression resulted in lactate production from $H_2 + CO_2$ (Mook et al., 2022). Plasmid-based expression was further improved by exchanging the weak P*tet* with the stronger P*bgaL* promoter. Yet, the highest reported recombinant lactate production from $H_2 + CO_2$ was achieved with the respective strain (Mook et al., 2022). This lactate-producing strain was further applied in a co-culture to produce *n*-hexanoate from $H_2 + CO_2$. The engineered lactate-producing *A. woodii* strain provided the substrate for *C. drakei* which further converted lactate to *n*-hexanoate (Herzog et al., 2023; Mook et al., 2024).

5.2. "Clostridium autoethanogenum" and Clostridium ljungdahlii

Although *A. woodii* was the first acetogen that was genetically modified, "*C. autoethanogenum*" and *C. ljungdahlii* were developed into the acetogenic model systems with the largest genetic toolset to date after the first description of a transformation procedure in 2010 (Köpke et al., 2010). The transfer of plasmid DNA into the cells can be achieved by either electroporation or conjugation with different *E. coli* strains (Köpke et al., 2010; Leang et al., 2013; Molitor et al., 2016; Liew et al., 2017; Klask et al., 2022). The most widely applied replicons are from the pMTL80000 series (Heap et al., 2009), but also other replicons were successfully applied, such as the aforementioned pIP404 replicon region (Banerjee et al., 2014). From the investigated plasmids, the segregational stability of the origins of replication (ORIs) was partly tested, which provides choices of more or less stable plasmid systems for "*C. autoethanogenum*" and *C. ljungdahlii* (Heap et al., 2009). A whole variety of different promoters is available for "*C. autoethanogenum*" and *C. ljungdahlii*, including constitutive and inducible versions (Heap et al., 2009; Banerjee et al., 2014; Nagaraju et al., 2016; Zhao et al., 2019; Seys et al., 2020; Bourgade et al., 2022a). The strength of these promoters was tested with a set of reporter genes, such as chloramphenicol acetyltransferase (Yang et al., 2017), β-glucuronidase (Banerjee et al., 2014), and the anaerobic fluorescence markers FbFPs, FAST, HaloTag, and SNAP-tag (Molitor et al., 2016; Charubin et al., 2020b). The fluorescence markers were further applied to investigate co-cultures of *C. acetobutylicum* and *C. ljungdahlii in vivo* (Charubin et al., 2020a; Charubin et al., 2020b).

Genome-engineering technologies are well-developed and include ClosTron (Heap et al., 2010), allele-coupled exchange (Leang et al., 2013), and various CRISPR/Cas-based methods. The CRISPR/Cas-based methods include gene deletion with Cas9 (Nagaraju et al., 2016; Huang et al., 2016; Seys et al., 2020), Cas12a (Zhao et al., 2019; Klask et al., 2022), and the endogenous CRISPR/Cas machinery in "*C.

autoethanogenum" (Poulalier-Delavelle et al., 2023), as well as CRISPRi with deactivated enzyme variants (Woolston et al., 2018; Fackler et al., 2021), and even base-editing tools (Xia et al., 2020; Seys et al., 2023). Those tools allow the implemention of more complex metabolic engineering strategies by enabling deletions, insertions, and point mutations. Furthermore, a transposon-based technique to introduce large gene clusters into the genome of *C. ljungdahlii* was described (Philipps et al., 2019).

While other acetogens, such as *A. woodii* and *E. limosum*, catch up in terms of the available genetic tool set, the developed methodology for "*C. autoethanogenum*" and *C. ljungdahlii* led to the broadest application for recombinant chemical production. The first effort to develop a heterologous pathway for chemical production in the acetogen *C. ljungdahlii* resulted in the production of *n*-butanol. Köpke et al. (2010) introduced the natural *n*-butanol synthesis pathway from *C. acetobutylicum* into *C. ljungdahlii*. Following this proof-of-concept, the recombinant chemical production spectrum of *C. ljungdahlii* and "*C. autoethanogenum*" in the peer-reviewed literature has been expanded to acetone (Banerjee et al, 2014; Jones et al., 2016; Liew et al., 2022), *n*-butyrate (Ueki et al., 2014; Huang et al., 2019), 3-hydroxybutyrate (Jones et al., 2016; Woolston et al., 2018; Flüchter et al., 2019), ethylacetate (Dykstra et al., 2022), isobutanol (Weitz et al., 2021; Hermann et al., 2021), isoprene (Diner et al, 2018), isopropanol (Bengelsdorf et al., 2016; Liew et al., 2022), mevalonate (Diner et al., 2018), poly-3-hydroxybutyrate (de Souza Pinto Lemgruber et al., 2019; Flüchter et al., 2019), *n*-butanol, and *n*-hexanol (Lauer et al. 2022). Most notably, acetone and isopropanol were produced at up to 3 g/L h^{-1} with 90 % selectivity, following a considerable metabolic engineering effort (Liew et al., 2022). The study by Liew et al. (2022) provides the example of the first recombinant chemical production on pilot scale using syngas fermentation technology. In combination with next-level synthetic biology approaches, such as cell-free extracts (Karim et al. 2020; Krüger et al., 2020; Vögeli et al., 2022), multi-omics investigations (Richter et al., 2016; Valgepea et al., 2022), and genome-scale metabolic modeling (Nagarajan et al., 2013; Marcellin et al., 2016; Liu et al., 2019; Pavan et al., 2022), further breakthroughs can be expected.

5.3. Eubacterium limosum and related species

The interest in *E. limosum* increased in recent years and so has the number of available genetic tools. The often synonymously treated strains of *E. limosum* and *E. callanderi* showcase the best developed molecular toolboxes of strains belonging to *Eubacterium* species. However, first genetic tools were also described for *E. maltosivorans* (Flaiz & Sousa, 2024; Flaiz et al., 2024). As of yet, eight different *Eubacterium* strains are genetically accessible, including the *E. limosum* strains DSM 20543[T] and B2, the *E. callanderi* strains DSM 3662[T], KIST612, DSM 3468, DSM 2593, DSM 2594, and *E. maltosivorans* DSM 20517 (Song et al., 2018; Jeong et al., 2020; Flaiz et al., 2021; Wang et al., 2021; Flaiz et al., 2024). All respective reports for these *Eubacterium* strains are based on different electroporation protocols.

Stable plasmid maintenance and replication were achieved by using the Gram-positive replicon region of the *C. perfringens* plasmid pIP404 (Song et al., 2018; Jeong et al., 2020) but also by using various replicons of plasmids of the pMTL80000 series (Heap et al., 2009). While using plasmids encoding the replication region of pIP404 resulted in both high transformation efficiencies and high plasmid copy numbers in *E. limosum*, plasmids of the pMTL80000 series encoding replicons of pBP1 or pCB102 tend to have low efficiencies, and copy numbers (Shin et al., 2019; Sanford and Woolston, 2022). Interestingly, transformation efficiencies were high when using replicon regions of pCB102 and low with the replicon of pIP404 in *E. callanderi* DSM 3468 (Wang et al., 2021).

Screening of biobricks including promoters, 3′ untranslated regions (UTRs), and 5′UTRs was achieved by applying enzymatic but also fluorescent reporter proteins for different *Eubacterium* strains. The

enzymatic β-glucuronidase assay was applied for *E. callanderi* KIST612 to determine the strength of four native promoters and the chloramphenicol acetyltransferase assay in *E. limosum* to compare promoter activities (Jeong et al., 2020; Sanford and Woolston, 2022). Further studies showcased that various fluorescent reporter proteins can be applied in *Eubacterium* strains to determine promoter strength in a time saving manner. This included traditional oxygen-dependent fluorescent reporters, such as GFP, but also a variety of oxygen-independent reporters including FbFPs, different FAST variants, the HaloTag, and the SNAP-tag. eGFP was used to assess the strength of promoters combined with different 5′UTRs (Song et al., 2022). Although functional in *E. limosum*, authors switched to oxygen-independent reporters for further experiments. Therefore, the HaloTag and SNAP-tag were combined to form a dual fluorescent reporter system to determine transcript termination strength, which was indicated by the ratio of the determined orthogonal fluorescence of both reporters (Song et al., 2022). FbFP derivatives were used to identify a tightly controlled tetracycline-inducible promoter system to express Cas9 in *E. limosum* and to compare promoter activities in *E. callanderi* (Shin et al., 2019; Wang et al., 2021). FAST was used to determine the strength of various promoters but was also used to generate fluorescent fusion proteins to track protein production during growth (Flaiz et al., 2021). The determined overall fluorescence indicated that C- or N-terminally tagged enzymes were produced during cultivation. Recently it was hinted that promoter strength might influence population heterogeneity. It was shown that the production of FAST using the strong and constitutively active P_{fd} promoter of *C. ljungdahlii* not only results in strong fluorescence, but also in a homogeneously fluorescent population (Flaiz et al., 2024).

It was discussed that population heterogeneity can presumably be caused by plasmid instability. To overcome this issue, genomic engineering approaches for *Eubacterium* strains were achieved by making use of its native homologous recombination machinery, CRISPR-Cas based tools, as well as a RelBE-family toxin-antitoxin-based tool (Sanford et al., 2023; Shin et al., 2019; Jeong et al., 2020; Millard et al., 2023). The functionality of the CRISPR-Cas9-mediated homologous recombination was shown by the deletion of the genes *folD* or *acsC* of the WLP (Shin et al., 2019). Both knockout mutants lost the ability of autotrophic growth. In *E. callanderi*, CRISPR-Cas9 was used to generate an uracil auxotrophic and 5-FOA resistant *pyrF* deletion mutant (Jeong et al., 2020). Markerless deletions of genes of the histidine biosynthesis and the methanol methyltransferase were achieved in *E. limosum* by using a native RelBE-family toxin-antitoxin of *E. callanderi* as anhydrotetracycline-inducible counterselection marker (Millard et al., 2023). *E. limosum*'s native homologous recombination machinery was used to create gene knockouts by transforming linear DNA to exchange the gene of interest with an antibiotic resistance cassette (Sanford et al., 2023). While the deletion is not seamless, it is a fast and easy way to generate knockout mutants. Authors used this method to identify and delete genes responsible for the production of an extracellular polymeric substance to improve handling of this strain (Sanford et al., 2023). Knockdowns of genes using CRISPRi can be a powerful tool to assess gene functions. All genes of the *E. limosum* genome were targeted for transcriptional suppression to identify genes essential for autotrophic growth. Moreover, transcriptional repression using the designed CRISPRi library identified genes that improved autotrophic growth. Thus, the inhibition of three of these genes enhanced growth and productivity (Shin et al., 2023).

While the number of available tools has increased rapidly in recent years, the number of recombinantly produced products is quite narrow, and only traces have been produced thus far. This includes the production of acetone, *n*-butanol, and acetoin. Both acetone and *n*-butanol production were achieved recombinantly from methanol. Acetone production was achieved by heterologous expression of genes of the acetone production operon of *C. acetobutylicum*, which was already used to produce acetone with *A. woodii* (Flaiz et al., 2021). *n*-Butanol production was achieved in *E. limosum* and *E. callanderi* by recombinant expression

of the gene encoding a bifunctional aldehyde:alcohol dehydrogenase from *C. acetobutylicum* (Flaiz et al., 2021; Humphreys et al., 2022). Acetoin was produced from $H_2 + CO_2$ by heterologous production of acetolactate synthase and acetolactate decarboxylase from *Bacillus subtilis* and *Aeromonas hydrophila* (Song et al., 2020). Acetoin production was improved by applying CRISPRi as mentioned before (Shin et al., 2023).

5.4. Moorella thermoacetica

A genetic system for *M. thermoacetica* ATCC 39073, based on an uracil auxotrophic Δ*pyrF* strain has been developed, which relies on expression of three methylase genes from *M. thermoacetica* in *E. coli* to methylate cloning plasmids and avoid digestion by the restriction-modification system in *M. thermoacetica* (Kita et al., 2013). The methylated insertion plasmids contain regions homologous to the genome site for insertion or deletion and were transformed into *M. thermoacetica* by electroporation. The promoter of the native glyceraldehyde-3-phosphate dehydrogenase led to strong recombinant gene expression and was used later to control expression of a thermostable *kanR* gene from a *Streptococcus* plasmid, conferring kanamycin resistance (Kita et al., 2013; Iwasaki et al., 2013). This relatively basic toolkit of two selective markers and one strong constitutive promoter was adequate for several metabolic engineering efforts. By replacing native *pta* genes with a *ldh* from *Thermoanaerobacter pseudoethanolicus*, the metabolic end-product spectrum was shifted from exclusively acetate to almost entirely lactate (Iwasaki et al., 2017). A similar experiment deleting the *pta* genes and over-expressing a native aldehyde dehydrogenase (*aldh*) resulted in a strain that made primarily ethanol (Rahayu et al., 2017). Recombinant expression of four genes for acetone production (*thl, ctfA, ctfB,* and *adc*), coupled with deletion of the *pta* gene, with the highest affinity for acetyl-CoA, resulted in acetone production with fructose or $H_2 + CO_2$ as substrate (Kato et al., 2021). Acetone production from $H_2 + CO_2$ was later enhanced using supplemental electron acceptors in this engineered *M. thermoacetica* strain (Takemura et al., 2023).

While all previous work had been done using insertion plasmids, a replicating plasmid system for *M. thermoacetica* DSM 521[T] was developed more recently, which involved testing a variety of putative thermostable Gram-positive replicons (Bourgade et al., 2022b). Plasmids were methylated using native *M. thermoacetica* DSM 521[T] methylases expressed in *E. coli*, and cells were transformed by electroporation, but due to challenges with plating, selection for transformed cells was performed in liquid media. The resulting replicating plasmid was used to recombinantly express an alcohol dehydrogenase using the native promoter of the signal recognition particle *ffh*, resulting in ethanol production.

5.5. Thermoanaerobacter kivui

A shuttle plasmid based on a native *Thermoanaerobacter saccharolyticum* plasmid combined with kanamycin resistance and a pUC origin of replication was shown to replicate in related *Thermoanaerobacter* and *Thermoanaerobacterium* species, which all took up DNA naturally without chemical or electrical transformation (Shaw et al., 2010). The plasmid (pMU131) also functioned in *T. kivui* and was used to generate an uracil auxotrophic Δ*pyrE* strain for subsequent genomic manipulations (Basen et al., 2018). The pMU131 backbone was also used in another report, but there were indications that the plasmid is not segregationally stable in *T. kivui*, particularly at high temperatures (Hocq et al., 2023).

To date nearly all engineering in *T. kivui* relies on the same three promoters. The promoters from the kanamycin resistance gene in pMU131 (P_{kan}) and from *Thermoanaerobacter* sp. X514's gyrase gene ($P_{gyr\text{-}X514}$) have been used to express selection genes *kanR* and *pyrE*. For expression of recombinant proteins, the native promoter region of *T. kivui*'s very strongly expressed surface layer protein (P_{SLP}) has been

used. The fluorescent reporter system FAST was used to characterize several additional promoters, of which the promoter from *T. kivui*'s native phosphotransacetylase gene (P$_{pta}$) was the strongest, leading to expression nearly twice as high as P$_{SLP}$ (Hocq et al., 2023).

Recombinant expression of several native *T. kivui* proteins has been carried out with pMU131 using a kanamycin selection marker. Thus, investigations of *T. kivui*'s native his-tagged pyruvate ferredoxin oxidoreductase (PFOR), the native HDCR, and several mutated variants thereof, as well as a subunit of the membrane-associated energy converting hydrogenase complex (Ech2C) were performed to characterize these enzymes (Katsyv et al., 2021; Dietrich et al., 2022; Katsyv and Müller, 2022). Moreover, a *T. kivui* Δ*pyrE* mutant strain was used to generate stable genomic knockouts by uracil/5-FOA selection. Knockouts include a mannitol dehydrogenase (*mtlD*) essential for growth on mannitol, the four-gene HDCR operon, as well as an eight-gene operon coding for the Ech2 complex (Moon et al., 2019; Jain et al., 2020; Schwarz et al., 2020; Baum et al., 2024).

While recombinant product formation using metabolic-engineered *T. kivui* strains has not yet been reported, the native cells have been used in several proof of principle experiments. For example, *T. kivui* cells can act as whole-cell-catalysts to convert $H_2 + CO_2$ to formate, if high carbonate concentrations are used to inhibit the native production of acetate (Schwarz and Müller, 2020). *T. kivui* cells were also shown to use intermittent electrical energy (as might be available from renewable sources powered by sun or wind) to reduce CO_2 to acetate by taking up H_2 that is produced at cathodes of microbial electrochemical technologies (Deutzmann et al., 2022). Given the recent progress in genetic tools, improvements to these results with genetically engineered strains seems likely in the near future.

6. Mixed and co-cultures supplied with $H_2 + CO_2$ and/or CO

As discussed in previous sections, acetogens have been isolated from diverse natural environments (*e.g.*, gut, anaerobic sludges, hydrothermal vents or salt lakes). In their original niches they live together with other microbes leading to symbiotic relationships. These associations can provide acetogens with essential nutrients (auxotrophies), deplete acetogenic products (therefore creating a thermodynamic benefit for acetogens), or aid in the removal of inhibitors and toxic compounds. However, they also introduce challenges, such as substrate competition, particularly with hydrogenotrophic or carboxydotrophic sulfate-reducers or methanogens. Conversely, acetogens contribute positively to microbial communities; for instance, in CO-rich environments, they detoxify the surroundings, facilitating the thriving of other microbes. Inspired by nature, scientists try to exploit the potential of mixed cultures for the conversion of autotrophic substrates ($H_2 + CO_2$ and CO) to products of interest, as reviewed below.

Acetogens typically exhibit a limited product spectrum, primarily producing acetate and ethanol (Table 1). Some acetogens, such as *C. luticellarii*, *C. carboxidivorans*, and *E. limosum*, can also produce iso- and *n*-butyrate and/or iso- and *n*-butanol. Genetic engineering approaches have been used to broaden the range of products from acetogens (Section 5), but a less explored alternative is the utilization of mixed culture approaches (Parera Olm and Sousa, 2022; Diender et al., 2021). These approaches encompass both: (1) open (natural) mixed cultures (also referred to as open cultures, reactor microbiomes, natural microbiomes, microbial communities, and microbial consortia); and (2) defined synthetic co-cultures. The term "synthetic co-cultures" is used, given that these microbial consortia are purposefully designed and constructed. Synthetic co-cultures may incorporate natural microbes only, but the inclusion of genetically engineered microbes is also possible. In contrast to monocultures of acetogens, mixed cultures hold the potential to yield a more diverse array of products by combining autotrophic and heterotrophic metabolisms (Fig. 4). Additionally, synthetic co-cultures offer possibilities for a more precisely tailored product range, helping to minimize unwanted side products – a potential downside of open mixed cultures. Despite the laboratory advances in synthetic co-cultivation for the conversion of $H_2 + CO_2$ and CO, their practical application remains underdeveloped.

The first synthetic co-culture for the conversion of CO and $H_2 + CO_2$ + CO mixtures was described by Diender et al. (2016). This co-culture was composed of "*C. autoethanogenum*" and a chain-elongator, *Clostridium kluyveri*. Products generated by the acetogen, (*i.e.,* acetate and ethanol), were utilized by *C. kluyveri* to produce medium-chain carboxylic-acids, such as *n*-butyrate and *n*-hexanoate. Additionally, the production of *n*-butanol and *n*-hexanol was observed at later stages, resulting from the reduction of the acids by the acetogen. Several other studies have followed up, with distinct acetogens and also the inclusion of engineered strains in the co-culture systems (Table 3).

While co-cultures of acetogens and *C. kluyveri* typically yield even-chain carboxylic acids and alcohols (mainly C4 and C6 molecules), exploration into odd-chain compounds holds promise. Moreira et al. (2021) demonstrated that pairing an acetogen, specifically *A. wieringae* strain JM, with propiogenic bacteria, such as *Anaerotignum neopropionicum*, leads to the production of the C3 carboxylic acid, propionate. Propionate, in turn, is a suitable electron acceptor for ethanol-driven chain elongation by *C. kluyveri* (Bornstein et al., 1948). It has been shown that a co-culture of *A. neopropionicum* and *C. kluyveri* fed with ethanol results in the production of *n*-pentanoate/*n*-pentanol (C5) and *n*-heptanoate/*n*-heptanol (C7) (Parera Olm and Sousa, 2023). Therefore, a three-species co-culture involving *A. wieringae*, *A. neopropionicum*, and *C. kluyveri* could be envisioned to facilitate the production of longer, odd-chain molecules, directly from CO and/or H_2 + CO_2 (Parera Olm and Sousa, 2023). However, this co-culture produces
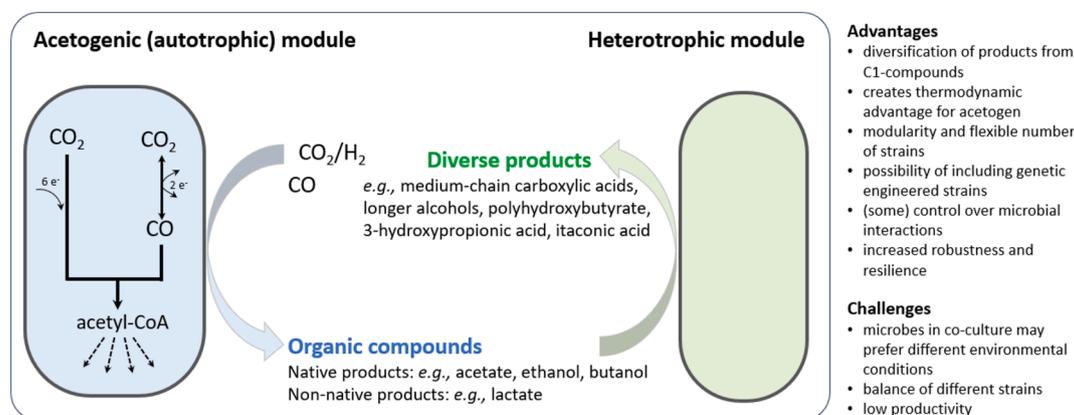

Fig. 4. Concept of a modular synthetic microbial co-culture for the conversion of $H_2 + CO_2$ and/or CO via intermediate organic compounds to diverse added-value products.

Table 3

Examples of chain elongation cocultures converting $CO_2/H_2/CO$.

Co-culture composition	Cultivation mode	T (°C)	pH	Substrates (% v/v)	Maximum metabolite concentration / Productivity	Ref.
Ethanol-driven chain elongation						
"*C. autoethanogenum*" + *C. kluyveri*	serum bottles	37	6–7.2	H_2/CO	Butyrate 2.27 g/L Caproate 1.16 g/L Butanol 0.43 g/L Hexanol 0.41 g/L	Diender et al., 2016
C. ljungdahlii + *C. kluyveri*	continuous stirred tank reactor (with continuous gassing)	37	6	$H_2/CO_2/CO$ (35:5:60)	Butyrate 2.8 g/L, 2.7 g/L day^{-1} Caproate 1.4 g/L, 1.3 g/L day^{-1} Butanol 4.2 g/L Hexanol 4.7 g/L, 0.54 g/L day^{-1} Octanol[a] 0.78 g/L, 2 mg/L day^{-1}	Richter et al., 2016
"*C. autoethanogenum*" + *C. kluyveri*	continuous stirred tank reactor (with continuous gassing)	37	6.2	H_2/CO (53:47)	Butyrate 1.1 g/L, 0.55 g/L day^{-1} Caproate 0.82 g/L, 0.41 g/L day^{-1}	Diender et al., 2019
C. carboxidivorans + *C. kluyveri*	batch stirred tank reactor (with continuous gassing)	37	6.0	CO/CO_2 (80:20)	Butyrate 0.31 g/L Caproate 0.36 g/L Butanol 0.84 g/L Hexanol 0.24 g/L,	Bäumler et al., 2023
C. aceticum + *C. kluyveri*	continuous stirred tank reactor		6.6–7.5	$H_2/CO_2/CO/N_2$ (15:5:30:50) + ethanol	Butyrate 7.0 g/L Caproate 8.2 g/L Butanol 0.72 g/L	Fernández-Blanco et al., 2022
A. woodii + *C. kluyveri*	bioelectrochemical system	(not indicated)	7	electric potential (−1 V) + 1.5 g/L ethanol	Butyrate 0.51 g/L Caproate 0.76 g/L	Zhang et al., 2023c
A. wieringae JM + *Anaerotignum neopropionicum*	serum bottles	30	7–7.2	$CO_2/N_2/CO$ (20:30:50)	Propionate 1.8 g/L Isovalerate 0.41 g/L	Moreira et al., 2021
Lactate-driven chain elongation						
A. woodii [P$_{bgaL}$_ldhD_NFP] + *C. drakei*	stirred tank reactor with AiO electrode	30	7	$CO_2 + H_2$	Caproate 0.11 g/L, 0.10 g/L day^{-1}	Herzog et al., 2022

[a] Octanol concentration refers to the condensate of the gas stripping system.

a mixture of even-chain (C4 + C6) and odd-chain (C5 + C7) carboxylates/alcohols. Steering the culture towards specific products poses a challenge, as does maintaining a balanced growth between the different species in the co-culture.

Most of the synthetic co-cultures for C_1-based chain elongation rely on ethanol as an intermediary product. However, by genetically engineering *A. woodii* to produce lactate from $H_2 + CO_2$, Mook et al. (2022) opened up possibilities for lactate-based chain elongation. Herzog et al. (2022) used these engineered strains to introduce a novel method for chain elongation, offering greater flexibility compared to ethanol-driven processes. While *C. kluyveri* is currently the sole culturable bacterium capable of chain elongation from ethanol, its high optimal pH requirement limits its compatibility with acetogens. In contrast, the higher diversity of lactate-based chain elongators increases the likelihood of finding optimal pairs for the process.

Beyond carboxylic acids, researchers have concentrated efforts on advancing co-cultivation for the production of other value-added compounds. Cestellos-Blanco et al. (2022) studied a co-culture composed of the acetogen *S. ovata* and the photoheterotrophic, diazotrophic *Rhodopseudomonas palustris nifA** mutant, aiming to concurrently fix CO_2 and N_2. In this co-culture, the acetate produced by *S. ovata* from $CO_2 + H_2$ is used by *R. palustris* to fuel N_2 fixation (producing NH_3) and, eventually, the accumulation of intracellular polyhydroxybutyrate (PHB). Conversely, the NH_3 generated by *R. palustris* can be utilized by *S. ovata* as a nitrogen source. Exploiting the versatile capabilities of *S. ovata*, which can also accept reducing equivalents from a poised cathode, the authors assessed the co-culture's performance in a

bioelectrochemical system. However, despite this innovative approach, the yield of PHB production by *R. palustris* was not reported for $H_2 + CO_2$ grown co-cultures and remains low in the bioelectrochemical systems, with only a 0.8 % carbon yield observed. In another study, Cha et al. (2021) constructed a synthetic co-culture of *E. limosum* with genetically engineered *E. coli* for the production of 3-hydroxypropionic acid (3-HP) and itaconic acid (ITA). Co-cultures were fed with CO, serving as energy and carbon source for *E. limosum*. Two *E. coli* strains were tested: the CL strain, which is a 3-HP producer, and strain WCIAG4, which produces ITA. Utilization of acetate by *E. coli* strains in anoxic conditions necessitated the addition of trimethylamine N-oxide as electron acceptor. Interestingly, co-cultivation with either *E. coli* strain led to a significant improvement of CO consumption rates by *E. limosum*, and this improvement could be positively correlated with the increasing densities of the *E. coli* strains. Although 3-HP and ITA production from CO alone were observed in the co-cultures, the yields remained relatively low compared to aerobic processes with *E. coli* strains on acetate.

Looking ahead, synthetic microbial co-cultures involving acetogens are a promising avenue for the diversification of products derived from the fermentation of C_1-substrates. They offer robustness and resilience surpassing monoculture approaches, yet with a complexity more manageable than mixed culture systems. The frontier of synthetic microbial communities beckons hybrid solutions combining native and engineered microbes. This will entail multifaceted efforts, ranging from the adequate selection of microbes for constructing synthetic co-cultures and potent need to isolat of novel microbes having new metabolisms which can be than adapted to specific co-culture conditions.

Furthermore, the development of genetic tools to engineer these microbes, and finally, the optimization of co-cultivation strategies are necessary.

7. Gas fermentation at an industrial scale

A biotechnological production platform called gas fermentation helps achieve at least four sustainable development goals (SDGs) set by the UN (Bengelsdorf and Dürre, 2017). Waste and greenhouse gasses are transformed into common chemicals and fuels through this process, which has a favorable impact on the global climate. We will explain gas fermentation, specifically when acetogens are being used, and its inherent advantage over continuous fermentation with liquid substrates. Thereby, the most important terms and concepts are presented. Moreover, we will discuss several promising and already-used bioreactor configurations and why these types are being used at an industrial scale. Finally, this section ends with examples of a successful translation of syngas fermentation at the industrial scale.

7.1. Gas fermentation

Conventional continuous fermentation with, for example, chemostats is operated by supplying a substrate (carbon and energy source, as well as all macro- and micronutrients) in a liquid form. For such fermentation, it is important to maintain a cell-residence time that is equal (or longer) than the cell-doubling time to prevent the washing out of the cell-culture. When a substrate is in a diluted form and limiting the growth of cells, the increase in the liquid-flow rate (*i.e.*, an increase in the dilution rate [day^{-1}]) with the same substrate concentration will lead to an increase in the volumetric production rates until the cells are washed out (Kuenen, 2019). Adding a cell guard (*i.e.*, a membrane filter) to maintain a high concentration of cells by recirculating them while removing only the liquid effluent could further increase production rates. However, this is different for gas fermentation because the carbon and energy sources ($H_2 + CO + CO_2$ [syngas] or $H_2 + CO_2$) are in the influent gas and not dissolved in the liquid broth containing all the other nutrients required for cell growth (Schill et al., 1996). An increase in the volumetric loading rate would, by simply increasing the gas flow rate, not lead to an increase in the dilution rate. When the product of gas fermentation is a gas, then the cell-residence time may be completely uncoupled from the cell-doubling time (Schill et al., 1996). However, for a liquid product, such as acetate or ethanol, a higher dilution rate may be necessary to prevent product inhibition, resulting in a cell-guard requirement to maintain high concentrations of cells (Richter et al., 2013). Here, we focus on acetate and ethanol production with acetogens; thus, a cell guard would likely be necessary.

Ultimately, the transfer rate of the gasses $CO + H_2$, which are characterized by a relatively low maximum solubility, into solution is the limiting factor regarding the volumetric production rates for gas fermentation (Munasinghe and Khanal, 2010; Martín and Grossmann, 2013). Here, we will discuss gas fermentation by gas bubbling into solution, while we will not discuss trickle-bed bioreactors with thin layers of liquids or hollow-fiber bioreactors with biofilms (Strübing et al., 2017; Munasinghe and Khanal, 2010). The gas-transfer rate (GTR) is approximated by the volumetric mass transfer coefficient (k_La) and the difference in the concentration of the specific gas at the gas/liquid interface (C_1) and the bulk liquid (C_2) via Eq. (1) (Schill et al., 1996; Keebler et al., 2024):

$$GTR = k_L a \times (C_1 - C_2) \tag{1}$$

The k_La depends on the characteristics of the: fermentation broth; mixing intensity; configuration, scale, and operating conditions of the bioreactor; and gas diffusivity, while the product, such as ethanol, also has an effect (Keebler et al., 2024; Puiman et al., 2022). The k_L represents the rate of molecular diffusion through the gas/liquid interface, and a represents the surface area of the interface. Measurements of the

k_La for syngas-fermentation conditions with CO showed a relatively low level of 25 h^{-1} by performing the experiments without microbial cells in distilled water (Puiman et al., 2022). However, using real microbial media broth with adapted and active microbial acetogens, it was shown that the true k_La was much higher and estimated to be 375 h^{-1} for *C. ljungdahlii*, and must be even higher to explain the industrial volumetric production rate of 1–10 g L^{-1}h^{-1} for ethanol (Martin et al., 2015; Gaddy et al., 2007).

There are two pertinent reasons for a superior k_La when biology is present. First, certain gas-fermenting microbial cells have relatively low threshold concentrations for $CO + H_2$ (Munoz and Philips, 2023). At the same time, they continuously remove the dissolved gas at high rates due to their high biomass concentrations, maintaining close to zero concentrations of CO and H_2 in the bulk liquid throughout the operating period (C_2). Therefore, gas fermentation systems maintain the maximum flux of the gas into its dissolved form. Second, the microbial medium, the production of products, which could be solvents, and the microbial cells themselves all change the liquid characteristics, including the viscosity, compared to distilled water, reducing the bubble size and increasing a considerably, thus, increasing the gas transfer rate (Puiman et al., 2022).

7.2. Bioreactor configurations

Because the bioreactor scale and mixing intensity affect the k_La considerably, choosing the optimum correct bioreactor configuration at scale is pertinent to maintaining a large k_La without too high energy consumption rates to maintain proper mixing intensities. The mixing intensity also increases the residence time of the bubble, which is advantageous (increase in a) (Puiman et al., 2022). Many bioreactor configurations exist, including continuously-stirred tank reactors, bubble columns, a hybrid bubble column with mixing, and numerous air-lift bioreactors (Munasinghe and Khanal, 2010). For research purposes without energy constraints, continuously-stirred tank reactors with a high mixing intensity of 500–1000 rotations per min are common (Schill et al., 1996). However, such a system would not scale well.

For dissolved and solid products from gas fermentation, industrial-scale bioreactors have been based on an external-loop, air-lift bioreactor configuration with a downcomer for liquid recirculation (Wiebe, 2004). Examples of this reactor configuration are utilized for ethanol production with syngas fermentation at steel mills with the involvement of LanzaTech (Skokie, Illinois) and protein production with single-cell fungi (*Fusarium venenatum*) with O_2 as the electron acceptor (Quorn Foods, Stokesley, UK). This bioreactor type employs the higher hydraulic pressures at the bottom of the tower-like configuration to increase the diffusivity of the gasses, while decreasing the bubble size at the bottom of the bioreactor. The lower density of the bubble-rich broth makes it rise, while it drops spontaneously through the downcomer due to the higher density of the broth at the top of the bioreactor (Wiebe, 2004). This spontaneous drop of the liquid through the downcomer creates a liquid recirculation for extra contact at the bottom of the bioreactor without additional energy input.

7.3. Two-stage systems and the LanzaTech industrial-scale plant technology

Shougang LanzaTech, which is a joint venture between the Shougang Jintang steel factory and LanzaTech, designed and built an industrial off-gas fermentation plant to produce ethanol at a rate of approximately 50,000 tons per year. The plant is located at the steel factory in Caofeidian District and includes: (1) gas cleaning to remove O_2 from the basic oxygen furnace (BOF) gas from steel making; (2) compressors for gas pressurization to 600 kPa; (3) water tanks; (4) tanks for media preparation and supply; (5) a smaller 20-m^3 bioreactor for inoculation events; (6) six external-loop, bubble column bioreactors of 30-m height with a volume of 600 m^3 each; (7) distillation columns to concentrate ethanol; (8) steam generation to heat the distillation columns; (9)

Fig. 5. Six external-loop, airlift bioreactors at the Shougang Jintang steel factory from the Shougang LanzaTech venture in Caofeidian District, China (photograph provided by Shougang LanzaTech).

centrifuges to gain protein from biomass; (10) a wastewater treatment plant, including water recycling and energy recovery by anaerobic digestion; and (11) ethanol storage tanks and distribution systems.

LanzaTech utilizes *"C. autoethanogenum"* as the acetogenic bacterium, which turns high partial pressures of CO into ethanol by reducing acetic acid. The six airlift bioreactors (Fig. 5) are operated in pairs as three two-stage systems instead of six single-stage systems to produce ethanol. A two-stage operation with two bioreactors *in series* has several advantages, specifically for the stability of industrial off-gas fermentation, to promote the stability of ethanol production and prevent a cyclic behavior (Richter et al., 2013). Two bioreactors in series produce relatively high volumes of acetate in the first bioreactor and almost exclusively ethanol in the second bioreactor (Valgepea et al., 2017; Richter et al., 2016). A relatively high CO partial pressure in the second bioreactor in the presence of high acetate concentrations at a lower pH value, steers the biological pathway towards acetate reduction into ethanol (Kwon et al., 2022; Schulz et al., 2023). By reducing acetate in the second bioreactor, a relatively short pathway (only two genes [*aor* and *adh*]) is always available to re-oxidize reduced ferredoxin and NADH that are produced from CO conversion as part of an overflow metabolism (Richter et al., 2016; Allaart et al., 2023). Thus, feeding acetate from the

first bioreactor into the second bioreactor, prevents the inhibition due to high CO partial pressures, and promotes fermentation stability.

After the first successful commercialization of gas fermentation using steel industry tail gas as its primary feedstock in Caofeidian, Hebei, China, Shougang LanzaTech has built three new plants in the last 3 years. In May 2021, a 45,000 ton per year gas fermentation industry facility using ferroalloy tail gas was established in Shizuishan City, Ningxia Hui Autonomous Region. During the same year, large-scale industrial off-gas fermentation bioethanol projects were launched in Guizhou and Ningxia (Table 4). In October 2023, Henan Energy Group partnered with Synata Bio, USA to announce the start of a 50,000-ton-year syngas fermentation project using *C. ljungdahlii* (Shua and Sun, 2023).

China's interest in syngas fermentation may stem from several key factors: climate change mitigation, resource diversification, energy security, as well as industrial upgrading and technological innovation (Zhang et al., 2023b). Ethanol produced from syngas fermentation aligns with this trend, offering a cleaner and greener alternative to conventional transportation fuels. In addition, the crude protein content of the cells harvested after fermentation could reach more than 83 %, and the proportion of 18 amino acids in the protein reached 94 %. The content and structure ratio of 10 essential amino acids were close to fish meal, which was much better than that of soybean meal. Bacterial protein from gas fermentation of Shougang LanzaTech has already been included in the feed catalog in China (Zhang et al., 2023a).

Although syngas fermentation for ethanol production has made considerable progress, it is evident that in the gas fermentation process the high energy consumption of gas compression and forced circulation, which is required to improve the mass transfer efficiency results in relatively low economic returns (Zhang et al., 2023a). Therefore, it is necessary to develop optimized mass transfer technologies to reduce energy consumption (Fan et al., 2021). Additionally, this process primarily uses CO as the source of reducing equivalent, resulting in two-thirds of the CO in the entire fermentation process eventually becoming CO_2 and being emitted, leading to a low carbon atom economy (Zhu et al., 2020). Thus, future research should concentrate on

Table 4
Ethanol production capacities from industrial-scale gas fermentation plants.

Producer	Location	Capacity per year
ArcelorMittal	Ghent, Belgium	64,000 tons
IndianOil Corporation	Haryana, India	33,500 tons
Hebei Shoulang New Energy Technology Co., Ltd	Tangshan, Hebei, China	45,000 tons
Ningxia Shoulang Jiyuan New Energy Technology Co., Ltd	Shizuishan, Ningxia, China	45,000 tons
Guizhou Jinze New Energy Technology Co., Ltd	Zunyi, Guizhou, China	60,000 tons
Ningxia Binze New Energy Technology Co., Ltd	Shizuishan, Ningxia, China	60,000 tons
Henan Sailongtu Bio-technology Co., Ltd	Yongcheng, Henan, China	45,000 tons (Under construction)

using H_2 or other electron donors to reduce CO_2 and improve the carbon atom economy of the entire process. With the increasing popularity of photovoltaic power generation, the cost of H_2 production *via* water electrolysis will decrease, possibly leading the way for H_2-driven CO_2 reduction or direct utilization of green electricity to drive CO_2 reduction in acetogenic bacterial fermentation for energy or chemical production.

8. Research needs and future directions

Acetogens offer a promising new avenue for the production of chemicals from renewable or currently underutilized feedstocks. However, further research is still needed to fully realize their potential for industrial applications. New acetogens are still being isolated from various habitats, and show a remarkable diversity in their individual metabolisms. Continued isolation work may identify novel acetogens that have new variations on the Wood-Ljungdahl Pathway as well as energy conserving mechanisms. Even the acetogens under study still veil several mysteries in their unique metabolisms. Particularly relevant for biotechnology is the means by which exergonic reactions of the WLP pathway (such as that catalyzed by MetFV) are coupled to proton motive force energy conservation, which is still unclear for some species. This involves re-oxidation of electron carriers (NADPH, NADH, and Fd_{red}) by hydrogenases, transhydrogenases, and enzymes of the WLP, where substrate specificities have not been elucidated for these species. Also lacking, but of considerable recent interest, is a clear understanding of the role of the glycine cleavage pathway genes in the WLP gene operon of many species. Is their presence an indication that the glycine cleavage system plays a role in acetogenesis, or are they performing some other function in this context? Unraveling these complexities will enhance our ability to manipulate these pathways for biotechnological applications.

In the last decade, the number of molecular engineering tools available for acetogens has increased, with a growing number of acetogens becoming genetically accessible. However, engineering approaches are still lagging behind those used for biotechnological model microbes already used in established industrial fermentation processes. The currently available genomic engineering techniques have already enabled the recombinant conversion of C_1 carbon sources, including harmful greenhouse gases, into valuable biofuels, biochemicals, and biopolymers. Although these progresses are highly promising for achieving a decarbonized future, industrial-scale applications of recombinant acetogens remain unrealized. Encouragingly, recent studies demonstrated that scaling up recombinant production is possible, offering a hopeful glimpse for the future. To bridge this gap, further development of efficient genetic tools and optimization of new metabolic pathways are crucial.

Even though gas fermentation with native acetogens has been fully scaled up at the industrial scale, research work on further optimization of bioreactor configurations seems warranted. Because the k_La is affected by so many parameters, a full optimization of the parameters, to, for example, understand the interactions is necessary. The factors to regulate a reduction in the bubble size, which would increase the surface area of the gas–liquid interface, are not completely known, and interactions can be pertinent. Here, characteristics of the acetogens themselves can play a role. Advanced bioreactor designs that account for these microbial characteristics could significantly improve gas–liquid mass transfer efficiency and overall process performance.

A further approach are applications of mixed and co-cultures for gas fermentation, but currently such concepts are in laboratory testing and require much further development before they reach industrial-scale implementation. Key areas for future research include exploring synergistic combinations of natural and engineered microbial strains to expand the range of producible chemicals, enhance product selectivity, and improve process efficiency. Metabolic, genome-scale, and ecological or community modeling approaches, along with artificial intelligence tools, could be of aid in the selection of microbial compositions for co-cultures and in predicting and optimizing their performance. On a more fundamental level, deeper insights into microbial interactions and ecological dynamics in mixed and co-cultures will be crucial for enhancing process predictability and stability. Integrating these insights into bioprocess design will be key to moving from laboratory success to commercial viability.

9. Conclusion

Gas fermentation offers several promising applications for industries to reduce their carbon emissions. Companies such as LanzaTech, Electrochaea Synata Bio, Jupeng Bio, Again, and Arkeon are developing or have already developed gas fermentation technologies using various biocatalytic acetogens or methanogens, combining knowledge and expertise from different fields. These technologies are becoming increasingly mature and have the potential to be a disruptive technology for certain products currently derived from crude oil. Given the urgent need to protect our planet, decarbonizing industrial processes is essential. We urge all stakeholders to reduce carbon emissions using available technologies and to consider gas fermentation for emissions that are unavoidable. Additionally, private investments in gas fermentation technologies are crucial to fully realise their potential.

CRediT authorship contribution statement

Anja Poehlein: Writing – review & editing, Writing – original draft, Validation, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Benjamin Zeldes:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Maximilian Flaiz:** Writing – review & editing, Writing – original draft, Data curation. **Tim Böer:** Writing – review & editing, Writing – original draft. **Alina Lüschen:** Writing – review & editing, Writing – original draft, Visualization. **Franziska Höfele:** Writing – review & editing, Writing – original draft. **Kira S. Baur:** Writing – review & editing, Writing – original draft. **Bastian Molitor:** Writing – review & editing, Writing – original draft. **Christian Kröly:** Writing – review & editing, Writing – original draft. **Meng Wang:** Writing – review & editing, Writing – original draft. **Quan Zhang:** Writing – review & editing. **Yixuan Fan:** Writing – review & editing. **Wei Chao:** Writing – review & editing. **Rolf Daniel:** Writing – review & editing, Writing – original draft, Investigation. **Fuli Li:** Writing – review & editing. **Mirko Basen:** Writing – review & editing, Writing – original draft. **Volker Müller:** Writing – review & editing, Writing – original draft, Investigation. **Largus T. Angenent:** Writing – review & editing, Writing – original draft, Investigation. **Diana Z. Sousa:** Writing – review & editing, Writing – original draft. **Frank R. Bengelsdorf:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Formal analysis, Conceptualization.

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.

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Data availability

All data used are already available in indicated repositories.

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