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A functional Wood–Ljungdahl pathway devoid of a formate dehydrogenase in the gut acetogens *Blautia wexlerae*, *Blautia luti* and beyond

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Summary

Species of the genus *Blautia* are typical inhabitants of the human gut and considered as beneficial gut microbes. However, their role in the gut microbiome and their metabolic features are poorly understood. *Blautia schinkii* was described as an acetogenic bacterium, characterized by a functional Wood–Ljungdahl pathway (WLP) of acetogenesis from $H_2 + CO_2$. Here we report that two relatives, *Blautia luti* and *Blautia wexlerae* do not grow on $H_2 + CO_2$. Inspection of the genome sequence revealed all genes of the WLP except genes encoding a formate dehydrogenase and an electron-bifurcating hydrogenase. Enzyme assays confirmed this prediction. Accordingly, resting cells neither converted $H_2 + CO_2$ nor H_2 - $+ HCOOH + CO_2$ to acetate. Carbon monoxide is an intermediate of the WLP and substrate for many acetogens. *Blautia luti* and *B. wexlerae* had an active CO dehydrogenase and resting cells performed acetogenesis from $HCOOH + CO_2 + CO$, demonstrating a functional WLP. Bioinformatic analyses revealed that many *Blautia* strains as well as other gut acetogens lack formate dehydrogenases and hydrogenases. Thus, the use of formate instead of $H_2 + CO_2$ as an interspecies hydrogen and electron carrier seems to be more common in the gut microbiome.

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Introduction

Acetogenic bacteria are a phylogenetically heterogeneous group of strictly anaerobic bacteria that have in common one metabolic feature: The ability to reduce two molecules of carbon dioxide by a specialized pathway, the Wood–Ljungdahl pathway (WLP), to acetyl-CoA and further to acetate (Fig. 1) (Müller, 2003; Müller *et al*., 2004; Drake *et al*., 2008; Ragsdale, 2008). The WLP has two branches. In the carbonyl branch, CO_2 is reduced to enzyme-bound carbon monoxide and in the methyl branch, a second molecule of CO_2 is first reduced to formate by formate dehydrogenases, the formate is then bound in an ATP-dependent reaction to tetrahydrofolic acid (THF) (Himes and Harmony, 1973; Lovell *et al*., 1988), water is split off the formyl-THF to yield methenyl-THF which is further reduced via methylene-THF to methyl-THF (Ragsdale and Ljungdahl, 1984). The key enzyme of this pathway that makes the carbon–carbon bond is the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS); it condenses an enzyme-bound CO, methyl group and coenzyme A (Pezacka and Wood, 1984; Raybuck *et al*., 1988; Seravalli *et al*., 1997). Acetyl-CoA is then converted through acetyl-phosphate to acetate, the name-giving product of acetogenic bacteria (Schaupp and Ljungdahl, 1974). Some acetogenic bacteria can take up the acetate again and reduce it via acetaldehyde to ethanol, thus producing a mixture of acetate and ethanol (Bengelsdorf *et al*., 2018).

Acetate formation from $H_2 + CO_2$ does not go along with net ATP formation by substrate-level phosphorylation: one ATP is gained in the acetate kinase reaction, but one ATP is consumed in the formyl-THF synthetase reaction (Himes and Harmony, 1973; Schaupp and Ljungdahl, 1974). Since acetogens grow on $H_2 + CO_2$, there must be additional, chemiosmotic mechanisms of energy conservation (Schaupp and Ljungdahl, 1974; Drake *et al*., 1981; Müller, 2003). Indeed, every acetogen analysed thus far has either one of the two respiratory enzymes found in acetogens, the ferredoxin:NAD oxidoreductase (Rnf) or the ferredoxin:H^+ oxidoreductase (Ech) complex (Schuchmann and Müller, 2014). Both

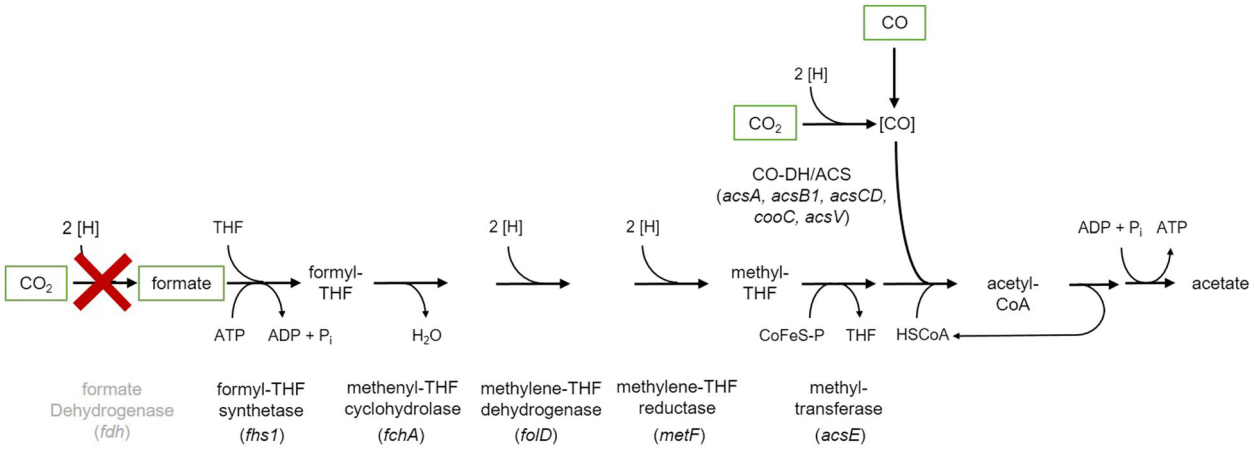

Fig. 1. The Wood–Ljungdahl pathway. Substrates are boxed. [H], reducing equivalents; THF, tetrahydrofolic acid; CoFeSP, corrinoid iron–sulfur protein; CoA, coenzyme A; [CO], enzyme-bound CO.

oxidize reduced ferredoxin as electron donor, and electron acceptor reduction is coupled to the generation of an ion gradient across the cytoplasmic membrane, that in turn drives ATP synthesis by an ATP synthase; the coupling ion can be either H^+ or Na^+ (Biegel and Müller, 2010; Hess *et al*., 2014; Schuchmann and Müller, 2014; Westphal *et al*., 2018; Schölmerich and Müller, 2019). Ferredoxin reduction with molecular hydrogen is highly endergonic but driven by a soluble, NAD and ferredoxin-reducing electron bifurcating hydrogenase (Poehlein *et al*., 2012; Schuchmann and Müller, 2012, 2014).

The WLP is well suited to convert C1 substrates of different reduction states such as CO_2, formate, methyl-group containing substrates such as methanol or glycine betaine or carbon monoxide (Drake *et al*., 1997; Müller, 2019). Electrons for CO_2 reduction can be derived from oxidation of molecular hydrogen or carbon monoxide but even from the oxidation of different organic carbon and energy sources such as hexoses, pentoses, aldehydes, alcohols, diols or carboxylic acids (Balch *et al*., 1977; Bache and Pfennig, 1981; Eichler and Schink, 1984; Buschhorn *et al*., 1989; Drake *et al*., 2008; Dönig and Müller, 2018). Thus, the presence of the WLP gives enormous metabolic flexibility to acetogens. They can grow lithoautotrophically on $H_2 + CO_2$ but also organoheterotrophically (Schuchmann and Müller, 2014). There is also an enormous ecological advantage during organotrophic growth (Schuchmann and Müller, 2016). Since the electrons are disposed in the WLP, fermentation of sugars yield the highest ATP gain, 4 ATP/hexose by SLP plus a fraction of an ATP by the chemiosmotic mechanism in the WLP, thus acetogens overgrow classical fermenters due to the higher ATP yield (Müller, 2003). Another advantage is that they can oxidize alcohols such as ethanol. This reaction is highly endergonic and only possible by coupling alcohol oxidation to the disposal of electrons in the reductive WLP (Bertsch *et al*., 2016). Due to the enormous metabolic flexibility and the energetic advantages of using the WLP, acetogenic bacteria are ubiquitous in nature, they are found in every anoxic ecosystem, and they are part of the anaerobic food web (Drake *et al*., 2008). They are also present in the gut of humans (Smith *et al*., 2019), but their role in the gut microbiome is not well defined. *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 like obesity or gut inflammation (Benitez-Paez *et al*., 2020) but is also correlated to psychological diseases such as autism (Johnson, 2020). Increased amounts of *Blautia* spp., on the other hand, are beneficial for human health and reduce, for example, lethality due to graft-versus-host disease (Jenq *et al*., 2015). A prerequisite to understand their ecological role in the gut is to understand their metabolic properties first. In general, *Blautia* spp. can metabolize various sugars and produce as major end products acetate, ethanol, hydrogen, lactate and succinate. Interestingly, $H_2 + CO_2$ is used by some species as major energy source (Liu *et al*., 2008). Only little is known, about which pathways are used by those bacteria. To this end, we have addressed the question of whether the two gut bacteria *Blautia luti* DSM14534 and *Blautia wexlerae* DSM19850, which were recently reclassified as *Blautia* spp. (Liu *et al*., 2008), are true acetogens as their relative, *Blautia schinkii* DSM10518. Our studies revealed that *B. luti* and *B. wexlerae* have an active WLP but are devoid of a formate dehydrogenase and an electron-bifurcating hydrogenase. Bioinformatic analyses revealed that loss of formate dehydrogenase is found often in gut acetogens and beyond.

Results

Blautia schinkii DSM10518 has been described to grow on $H_2 + CO_2$ and was thus considered as acetogenic bacterium (Rieu-Lesme *et al*., 1996). In contrast, autotrophic growth of *B*. *luti* DSM14534 and *B*. *wexlerae* DSM19850 has not been examined and it is therefore unclear whether they are indeed acetogens. To address this question, we first searched for genes encoding enzymes of the WLP with *B*. *schinkii* as a reference strain.

There are four types of formate dehydrogenases (Fdh) known in acetogens: a two subunit Fdh (Liu and Mortenson, 1984), an electron-bifurcating enzyme that has three subunits in addition (Wang *et al*., 2013b), an electron-bifurcating formate dehydrogenase coupled to an electron-bifurcating hydrogenase (Wang *et al*., 2013a; Dietrich *et al*., 2021) or a hydrogen-dependent CO_2 reductase (HDCR) in which the Fdh subunit is linked by two small [4Fe-4S] cluster containing subunits to a hydrogenase subunit (Schuchmann and Müller, 2013; Schwarz *et al*., 2018), Inspection of the genome sequence of *B*. *schinkii* DSM10518 type strain revealed the presence of a gene encoding a formate dehydrogenase of the HDCR type (Fig. 2). As in other acetogens the *fdh*/*hdcr* genes are not part of the WLP gene cluster (Poehlein *et al*., 2012). The latter contains in *B*. *schinkii* the gene encoding a bifunctional methylene-THF dehydrogenase/methylene THF cyclohydrolase and a type III methylene-THF reductase (*metFVrnfC2*) (Öppinger *et al*., 2022). The genes encoding the condensing machinery, methyltransferase, CODH and ACS are also present in two clusters on the chromosome (Fig. 2). Essential for autotrophic growth is a hydrogenase, a respiratory enzyme and an ATP synthase. *Blautia schinkii* has the genes for an ion-translocating ferredoxin:NAD oxidoreductase (Rnf) (Supplementary Fig. S1A and B), two proton translocating F_1F_0 ATP synthases (Supplementary Figs S2-3) and an electron-bifurcating hydrogenase (Supplementary Fig. S4). While *A*. *woodii* has an RnfB subunit with six ferredoxin domains, *B*. *schinkii* has a much smaller RnfB subunit with only three ferredoxin binding motives (Supplementary Fig. S1B). *Blautia schinkii* has two operons each encoding an F_1F_0-type ATP synthase. None of the *c* subunits does have a conserved Na^+ binding site indicating that both ATP synthases use H^+ as coupling ion (Supplementary Fig. S3). The presence of genes encoding a complete WLP is consistent with the observation that *B*. *schinkii* grows on $H_2 + CO_2$ (Rieu-Lesme *et al*., 1996).

Blautia luti and *B*. *wexlerae* also have the genes encoding the C-C bond forming enzyme complex CODH/ACS and a bifunctional methylene THF-dehydrogenase/cyclohydrolase (Fig. 2). In *B*. *wexlerae* we could find the genes for a type III methylene-THF reductase (MTHFR). These genes were not present in *B*. *luti* but genes encoding a type I MTHFR consisting only of *metF* (Öppinger *et al*., 2022). The respiratory enzymes (Rnf and ATP synthase) are also encoded (Supplementary Fig. S1-3). As in *B*. *schinkii*, RnfB has only three predicted ferredoxin domains and two different F_1F_0-type ATP synthases are encoded but one has a conserved Na^+ binding site, whereas the other is H^+ dependent. The presence of two F_1F_0-type ATP synthases with different ion stoichiometries was recently described for *Pseudobutyrivibrio ruminis* (Schölmerich *et al*., 2020). However, in sharp contrast, the genomes of *B*. *luti* and *B*. *wexlerae* apparently lack genes encoding any type of formate dehydrogenase, ruling out growth of *B*. *luti* and *B*. *wexlerae* on $H_2 + CO_2$. Furthermore, a gene cluster encoding an electron-bifurcating hydrogenase could neither be found in *B*. *luti* nor in *B*. *wexlerae*.

Enzyme activities in cell-free extract of B. luti*,* B. schinkii *and* B. wexlerae

To further investigate the presence of an active WLP in *B*. *luti*, *B*. *schinkii* and *B*. *wexlerae*, we measured activities of WLP enzymes in cell-free extract. Cells were grown on 20 mM glucose to the late exponential phase, harvested and disrupted in the French pressure cell. Cell debris was removed and the cell-free extract was used for enzymatic assays. The cell-free extracts of *B*. *luti*, *B*. *wexlerae* and *B*. *schinkii* reduced methylene-THF with NADH as electron donor with activities of 442.7 $\pm$ 58.1, 537.5 $\pm$ 51.2 and 130 $\pm$ 10 mU mg^{-1} (Table 1), respectively. NADPH was not used as electron donor. These data are in line with the genetic prediction of an NADH-oxidizing type I MTHFR in *B*. *luti* and type III MTHFR in *B*. *wexlerae* and *B*. *schinkii*.

$NADP^+$-dependent methylene-THF oxidation was found in all three species (Table 1). Activities ranged from 1.6 $\pm$ 0.1 U to 4.3 $\pm$ 0.8 U mg^{-1} of protein. NAD^+ was not reduced in any species. As expected from the apparent absence of an *fdh* gene, cell-free extract of *B*. *luti* and *B*. *wexlerae* did not catalyse formate oxidation with methylviologen or with benzylviologen as artificial electron acceptor. In contrast, cell-free extract of *B*. *schinkii* had an active FDH that catalysed formate:benzylviologen oxidoreductase activity with 21.31 $\pm$ 4.06 mU mg^{-1}. CO dehydrogenase and hydrogenase activities were present in cell-free extracts of all three strains (Table 1), albeit with different activities. Activities of hydrogenase and CO dehydrogenase were about 25- and 10-times higher in *B*. *schinkii* respectively, but very similar in *B*. *luti* and *B*. *wexlerae*, which can be explained by the absence of electron-bifurcating hydrogenase in *B*. *luti* and *B*. *wexlerae*. An increase of those activities could be possible through gene regulation in cells grown not only on glucose but mixotrophically on glucose with H_2 or CO.

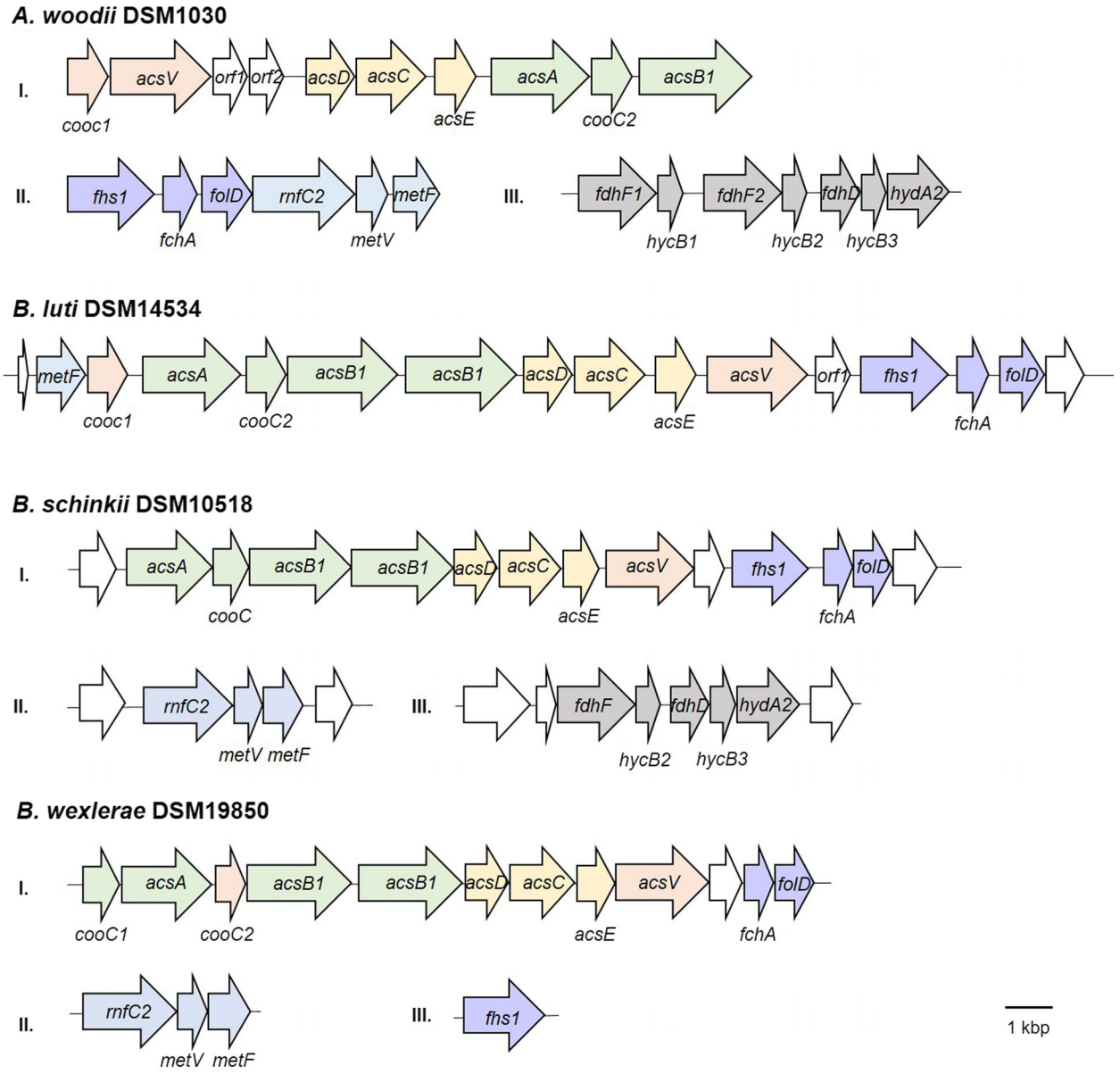

Fig. 2. Wood–Ljungdahl pathway gene cluster in *B. luti* DSM14534, *B. schinkii* DSM10518 and *B. wexlerae* DSM19850 in comparison to *A. woodii* DSM1030. Cluster I contains genes encoding the subunits of the CO dehydrogenase/acetyl CoA synthase complex, which consists of CO dehydrogenase (*acsA*), acetyl-CoA synthase (*acsB1*), corrinoid-iron sulfur protein (*acsCD*), methyltransferase (*acsE*), nickel-insertion accessory protein (*cooC*) and corrinoid activation/regeneration protein (*acsV*). Cluster II harbours the genes encoding the formyl-THF synthetase (*fhs1*), methenyl-THF cyclohydrolase (*fchA*), methylene-THF dehydrogenase (*folD*) and methylene-THF reductase (*metF*, *metV*, *rnfC2*). Cluster III contains HDCR genes (formate dehydrogenase *fdhF*, FeS-containing subunits *hycB*, [FeFe]-hydrogenase *hydA2* and formate dehydrogenase maturation protein *fdhD*). The WLP genes of *B. luti* DSM14534 are present in only one cluster; formate dehydrogenase genes, *rnfC2* and *metV* are not present in the genome. *Blautia wexlerae* DSM19850 does not have formate dehydrogenase genes; *fhs1* is encoded separately from the other WLP genes cluster in the genome.

Acetate formation from C1-compounds by resting cells of B. luti*,* B. schinkii *and* B. wexlerae

To unravel whether *B. luti*, *B. schinkii* and *B. wexlerae* are indeed acetogenic bacteria, we asked whether resting cells would make acetate from $H_2 + CO_2$ or other C1 compounds. For the cell suspension experiments, *B. luti*, *B. schinkii* and *B. wexlerae* were grown on 20 mM glucose, harvested in the late exponential growth phase, washed and re-suspended in imidazole buffer. *Blautia schinkii* is able to grow on $H_2 + CO_2$ (Rieu-Lesme

Table 1. Identification of WLP enzymes in cell-free extract of *B. luti* DSM14534, *B. schinkii* DSM10518 and *B. wexlerae* DSM19850.

Enzyme activity	Substrates	Specific activity (mU mg^{-1})[a]		
		B. luti	*B. schinkii*	*B. wexlerae*
Methylene-THF dehydrogenase	Methylene-THF + $NADP^+$	1743.3 ± 361.2	1600.0 ± 70.0	4353.4 ± 830.0
	Methylene-THF + NAD^+	–	–	–
Methylene-THF reductase	Methylene-THF + NADPH	–	–	–
	Methylene-THF + NADH	442.7 ± 58.1	130.0 ± 10.0	537.7 ± 51.2
Formate dehydrogenase	Formate + BV	–	21.31 ± 4.06	–
Hydrogenase	H_2 + MV	890.9 ± 52.7	23 120 ± 1840.0	690.3 ± 31.1
Carbon monoxide dehydrogenase	CO + MV	127.6 ± 36.3	1200 ± 110.0	127.5 ± 28.8

[a]Enzyme activities were determined as described in 'Experimental procedures'. All values are mean ± SEM; $N = 5$.

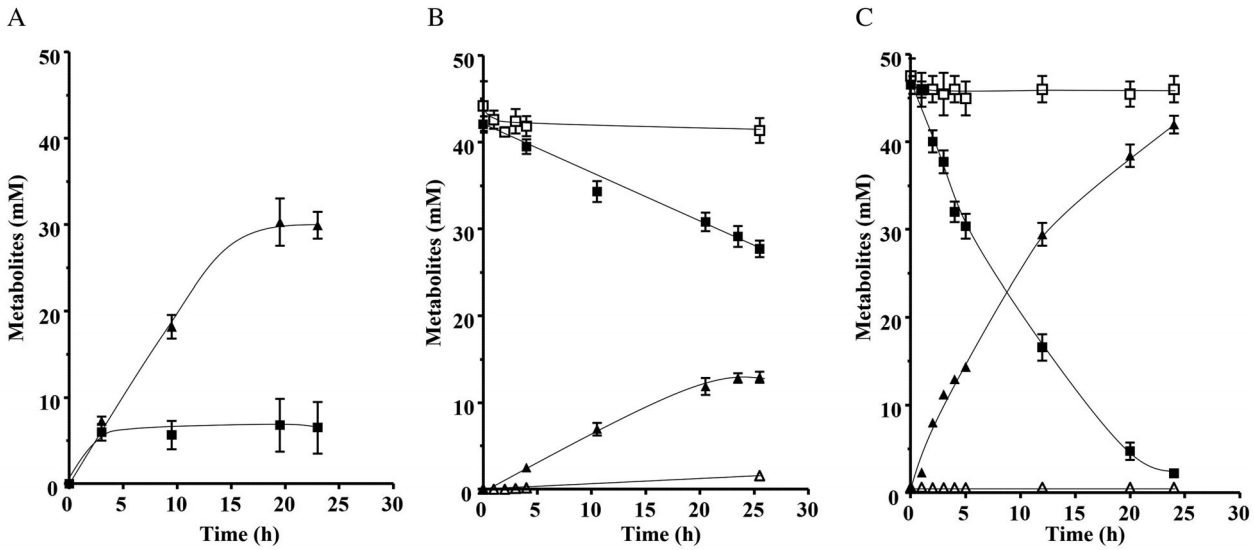

Fig. 3. Acetate formation from C1 compounds by resting cells of *B. schinkii*, *B. luti* and *B. wexlerae*. Cells were grown in CO_2/bicarbonate-buffered media at 37°C with 20 mM glucose as substrate to late exponential growth phase, harvested and washed. The cells were re-suspended in 20 ml imidazole buffer (50 mM imidazole, 60 mM $KHCO_3$, 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 2 mM DTE, 4.4 μM resazurin, pH 7.0) in 115 ml serum flasks with N_2/CO_2 atmosphere [80/20% (vol./vol.)] to a final protein concentration of 1 mg ml^{-1}. Acetate formation from H_2 + CO_2 by resting cells of *B. schinkii* (A). Resting cells were incubated with 1 bar H_2+ CO_2 [100% (vol./vol.)] at 37 °C. The production of formate (■) and acetate (▲) was determined at each time point. Resting cells of *B. luti* (B) and *B. wexlerae* (C) were incubated at 37°C with 40 mM formate and 20% (vol./vol.) CO. The concentrations of formate (■) and acetate (▲) were determined at each time point, as well as the concentrations of formate (□) and acetate (Δ) in an assay that only contained formate but no CO. All data points mean ± SEM; $N = 2$ independent experiments.

et al., 1996) and, accordingly, resting cells of *B. schinkii* produced acetate from H_2 + CO_2 with a rate of 25.17 ± 1.94 nmol mg^{-1} min^{-1}. In addition, minor amounts of formate were formed (Fig. 3A). In contrast, resting cells of *B. luti* and *B. wexlerae* did not produce acetate from H_2 + CO_2, in agreement with the lack of formate dehydrogenase (Supplementary Fig. S5). Since formate is an intermediate of the WLP, it should be reduced with H_2 under an H_2 + CO_2 atmosphere. However, acetate production from H_2 + formate + CO_2 was also not observed in any of the two species, in agreement with the lack of the electron-bifurcating hydrogenase. In addition to molecular hydrogen the strong reductant carbon monoxide can also be used as electron donor for the WLP and indeed, acetate formation was observed. 14.87 ± 4.48 mM formate was consumed with a rate of 8.91 ± 0.69 nmol mg^{-1} min^{-1}, and 13.54 mM ± 0.18 mM acetate was produced with a rate 9.18 ± 0.49 nmol mg^{-1} min^{-1} by resting cells of *B. luti* (Fig. 3B). *Blautia wexlerae* produced 41.00 ± 3.50 mM acetate with a rate of 74.3 ± 5.9 nmol mg^{-1} min^{-1} from 45.0 ± 5.47 formate (68.5 ± 1.48 nmol mg^{-1} min^{-1}) even faster than *B. luti* (Fig. 3C). In the absence of CO, only trace amounts of formate were consumed and acetate was produced by *B. luti* and *B. wexlerae*. CO alone was not metabolized.

Formate dehydrogenase free acetogens are common in gut acetogens

We next sought to determine how widespread the implementation of a WLP without formate dehydrogenase was

across the *Blautia* genus. We annotated 286 *Blautia* genomes from NCBI's RefSeq collection, which revealed that *Blautia* encode single or multiple copies of WLP pathway genes, however, surprisingly only 127 isolates (44% of isolates) encode formate dehydrogenase (Fig. 4A). To confirm the annotations, we searched these genomes for genes with similarity to a panel of known *fdh* genes (Supplementary Fig. S6A). As expected, annotated *fdh* genes were stronger hits to the panel of known formate dehydrogenase genes (Supplementary Fig. S6A). *Blautia* that were not annotated as having *fdh* did have weak blastp hit to some known *fdh* gene queries; however, these hits had only ~35% identity and their predicted domains were inconsistent with a function as formate dehydrogenase. Analysis of formate dehydrogenase sequences in *Blautia* revealed that there is variability across *Blautia* forming three groups: a *Blautia hydrogenotrophica*-type *fdh*, which was only found in the corresponding organism, an *fdh* similar to the HDCR of *A. woodii* present in *Blautia caecimuris* and *Blautia schinkii*, and a third group of highly conserved *fdh* genes (Fig. 4, Supplementary Fig. S7). Despite this sequence variability, examining the predicted domains, redox active centres, and predicted co-factor binding sites of the putative *fdh* genes, revealed a high degree of conservation with known formate dehydrogenase genes (Supplementary Fig. S6C). Interestingly, in *Gottschalkia acidurici*, *Eubacterium callanderi* and most *Blautia* isolates, *fdh* genes were ~900aa, compared to ~730aa in *B. schinkii*, *A. woodii* and *B. caecimuris* (both subunits), and contained additional N terminal iron–sulfur clusters (2Fe-2S and 4Fe-4S) (Supplementary Fig. S6C). We next asked if *fdh*-encoding corresponded to isolate phylogeny within *Blautia*. Therefore, we generated a phylogenetic tree based on the full-length 16S rRNA sequences (Supplementary Fig. S6B). Unexpectedly, there was no single monophyletic group of *fdh* positive isolates, and instead isolates encoding *fdh* were grouped in multiple clades across the *Blautia* genus (Supplementary Fig. S6B). In line with our functional analyses, this indicates that there are both *fdh* positive and *fdh* negative *Blautia*, despite the fact that all *Blautia* encode WLP pathway genes. Based on sequence variability and domain architecture, there are several distinct groups of *Blautia* formate dehydrogenase genes; therefore, we next examined the genomic location of *fdh* genes (Fig. 4, Supplementary Fig. S7). This revealed that in contrast to *B. hydrogenotrophica* or *B. ceacimuris* (Fig. 4C) or *B. schinkii* (Fig. 2), in the majority of *fdh* positive *Blautia* isolates, *fdh* genes are located in a large cluster consisting of a single *fdh* gene along with genes homologous to electron bifurcating hydrogenase complex subunits (labelled *hydABC*-like), molybdopterin co-factor biosynthesis genes, and *fdhD* (Fig. 4C, Supplementary Fig. S7) suggests that these encode indeed formate dehydrogenases. Finally, we observed that *Blautia* isolates encoding *fdh* tended to have a single copy of *fhs*, while most *fdh* negative isolates encoded two copies (Fig. 4A). This prompted us to analyse the structure of the WLP gene clusters in these isolates. The WLP gene cluster was highly variable across the *Blautia* genus (Fig. 4D, Supplementary Fig. S8). Grouping isolates on the basis of *fdh* encoding revealed differences in the WLP gene cluster between these groups, even though *fdh* itself is encoded in a separate gene cluster (Fig. 4C, Supplementary Fig. S7). Both *fdh* positive and negative *Blautia* encode a copy of *fhs* outside of the WLP gene cluster (marked by *acsC*/*acsD*/*acsE*). However, in 73% of *fdh* negative isolates (116/158), which are reliant on formate for the methyl-branch of the WLP, an additional copy of *fhs* is encoded adjacent to *fchA* directly in the WLP gene cluster (Fig. 4C, Supplementary Fig. S7). In contrast, in 97% of *fdh* positive isolates, *fhs* is not encoded adjacent to *fchA* in the major WLP gene cluster (Fig. 4D, Supplementary Fig. S8). Altogether, these analyses demonstrate that the use of the WLP without formate dehydrogenase is widespread in *Blautia*, that most *fdh* positive *Blautia* have a *fdh* gene cluster that is distinct from *B. hydrogenotrophica*, *B. schinkii* or *B. ceacimuris*, and that the WLP gene clusters differ in *fdh* negative isolates compared to *fdh* positive, potentially enabling more efficient formate utilization.

Discussion

Acetogens with a complete WLP are able to grow on C1 compounds such as $H_2 + CO_2$ or formate (Balch *et al*., 1977; Eichler and Schink, 1984; Genthner and Bryant, 1987; Tanner *et al*., 1993; Moon *et al*., 2021). To reduce formate in the methyl branch and one CO_2 in the carbonyl branch, electrons are required that derive from the oxidation of 3 mol formate to CO_2 by a formate dehydrogenase (Moon *et al*., 2021). Without FDH, formate cannot be oxidized and, therefore, electrons cannot be gained. As result, acetogens without FDH should not be able to grow on formate alone. Furthermore, without FDH acetogens should no longer be able to grow on $H_2 + CO_2$. Electrons for CO_2 reduction can be gained from hydrogen oxidation by the electron-bifurcating hydrogenase, but without FDH, CO_2 cannot be used as electron acceptor in the methyl branch. This was recently confirmed by deletion of the HDCR gene cluster in the acetogen *T. kivui*: Growth on $H_2 + CO_2$ or formate was no longer possible (Jain *et al*., 2020). However, growth of the *T. kivui* mutant on $H_2 + CO_2$ was restored by addition of formate. Even glucose fermentation was only possible in presence of external formate. *Clostridium bovifaecis*, an acetogenic bacterium isolated from cow manure (Zhu

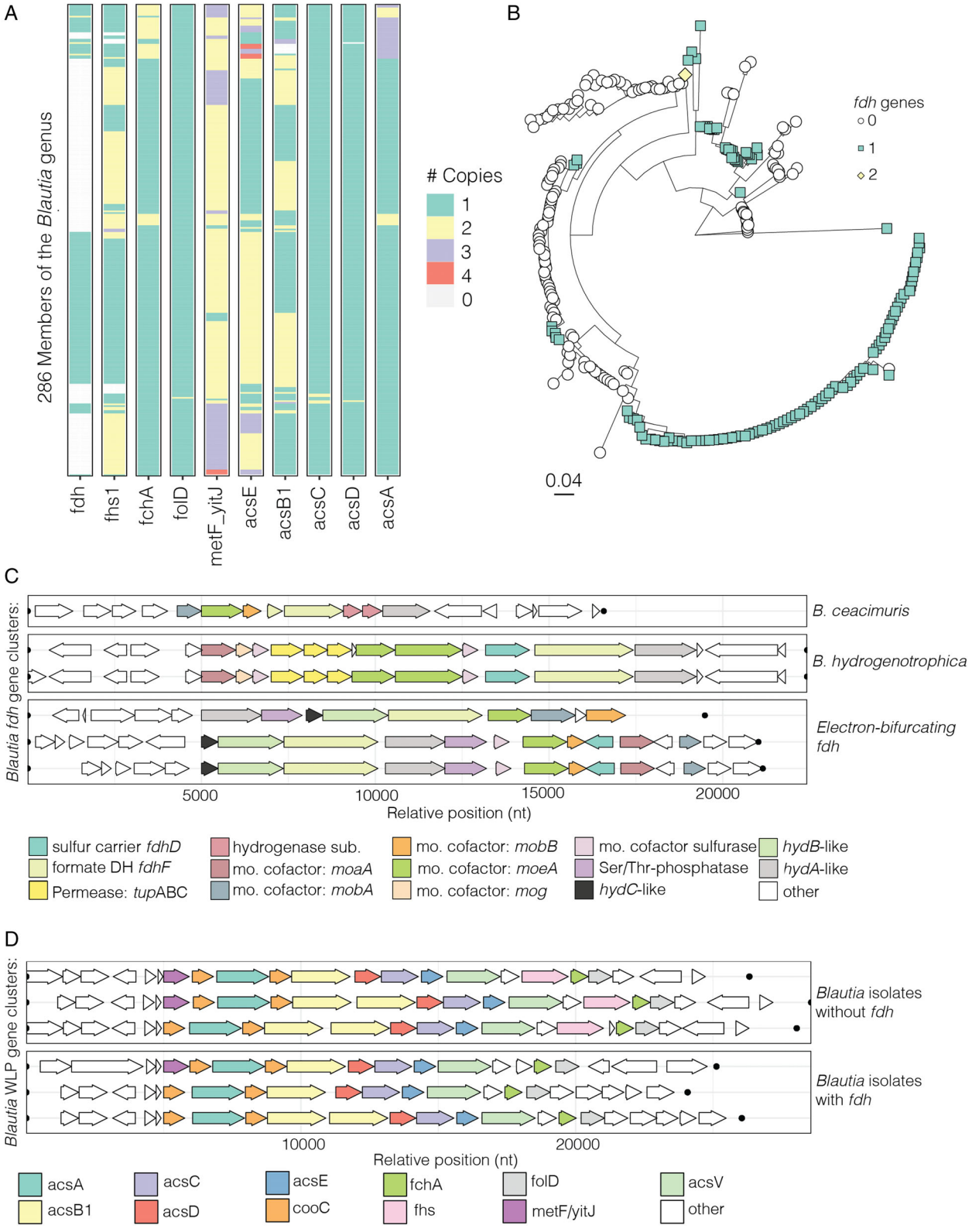

Fig. 4. Legend on next page.

et al., 2018), naturally lacks FDH genes (Yao *et al*., 2020). In contrast to *T. kivui*, growth on glucose was still possible demonstrating that the WLP is not essential for glucose fermentation and electrons derived from glycolysis are re-routed into different pathways.

In this study we demonstrate that the absence of formate dehydrogenases is common in gut acetogens, especially in bacteria of the Firmicutes phylum. For the gut bacteria *B. luti* and *B. wexlerae*, the absence of FDH but presence of all other WLP enzymes could be confirmed not only by genomic data but also by biochemical and physiological data. Formate was reduced to acetate by *B. luti* and *B. wexlerae* with CO as electron donor. Two mol of CO are oxidized by CODH/ACS in the carbonyl branch of WLP and used for reduction of 2 mol of ferredoxin, these electrons are used for formate reduction to a methyl group. A third mol of CO is then combined with the generated methyl group by CODH/ACS complex to produce acetate. To generate NADH and NADPH, which are required for formate reduction in the methyl branch, 1.5 ferredoxin needs to be oxidized by Rnf to generate 1.5 NADH. NADPH is then produced from 0.5 ferredoxin and 0.5 NADH by electron-bifurcating transhydrogenase. CO is an intermediate in WLP and could therefore be used as electron donor in cell suspension experiments with *B. luti* and *B. wexlerae*. Because of its high toxicity to the host, CO is most likely not the physiological electron donor used by gut acetogens. Which compound is used as electron donor for formate reduction *in situ* has to be confirmed in future studies, but most likely these are sugars, hexoses or pentoses. In case of the *T. kivui* HDCR mutant, H_2 could also be used as electron donor for formate reduction (Jain *et al*., 2020), which was not observed in *B. luti* and *B. wexlerae* and also not in *C. bovifaecis*. This can be explained by the absence of an electron-bifurcating hydrogenase in *B. luti*, *B. wexlerae* and *C. bovifaecis* (Yao *et al*., 2020). Why molecular hydrogen is not used as electron donor by these FDH lacking acetogens is still puzzling. One explanation could be the presence of methanogens and sulphate-reducing bacteria, which have a higher affinity to H_2 than acetogens (Hylemon *et al*., 2018; Smith *et al*., 2019). Under standard conditions, reduction of sulfate to H_2S is the energetically most favourable reaction with $\Delta G^{\circ\prime} = -152.2$ kJ mol^{-1} ($SO_4^{2-} + 4\ H_2 + H^+ \rightarrow HS^- + 4\ H_2O$), production of methane from CO_2 results in free energy of $\Delta G^{\circ\prime} = -131$ kJ mol^{-1} ($CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$), the least energetic favourable reaction is acetogenesis from CO_2 ($\Delta G^{\circ\prime} = -95$ kJ mol^{-1}; $2\ CO_2 + 4\ H_2 \rightarrow CH_3COO^- + H^+ + 2\ H_2O$) (Thauer *et al*., 1977). Therefore, mathematical models suggested out-competition of acetogens in a homogenous culture with sulfate-reducing bacteria or methanogens when grown together on $H_2 + CO_2$ or $H_2 + CO_2$ + sulphate (Smith *et al*., 2020). Gut acetogens might play an important role in H_2 utilization in humans, which harbour only a small number of methanogens and sulfate-reducing bacteria (Hylemon *et al*., 2018). In fact, in methanogen-free sheep acetogenic bacteria captured 21%–25% of the hydrogen present in the sheep rumen. In sheeps with methanogens and acetogens, more than 90% of the H_2 was captured by methanogens; the amount of H_2 captured by acetogens was insignificant (Fonty *et al*., 2007). One strategy for gut acetogens to avoid competition for hydrogen could be specialization in diverse heterotrophic metabolism, which, as already mentioned before, gives acetogens an enormous ecological advantage and flexibility (Schuchmann and Müller, 2016). The gut acetogens *Blautia hydrogenotrophica* (Bernalier *et al*., 1996) and *B. schinkii* (Rieu-Lesme *et al*., 1996), for example, can grow heterotrophically on glucose but can also grow autotrophically on $H_2 + CO_2$. Interestingly, *B. hydrogenotrophica* can also cross-feed on formate, which is generated by many other gut microbes (Laverde Gomez *et al*., 2019). Due to its ability to diffuse across membranes (Falke *et al*., 2010), formate cross-feeding could even be possible without formate transporters or channels. The absence of FDH in many gut acetogens could be an adaption to the gut. Although many gut bacteria produce formate, the average formate concentration may be low. The bovine rumen has only 12 μmol formate g^{-1} total rumen content, showing the efficient uptake of formate by rumen bacteria (Hungate *et al*., 1970). For many gut acetogens, due to the abundance of formate in the human gut, the utilization of FDH was therefore no longer necessary. Apart from *Clostridium bovifaecis* also *Marvinbryantia formatexigens* (Wolin *et al*., 2003; Wolin *et al*., 2008) and *Syntrophococcus*

Fig. 4. *Blautia* frequently encode a Wood–Ljungdahl pathway (WLP) without formate dehydrogenase (*fdh*) and have variable WLP gene clusters. The copy number of the indicated WLP genes in the whole-genome sequence is plotted for 286 *Blautia* isolates (A). A full-length 16S rRNA derived maximum-likelihood phylogenetic dendrogram of 286 isolates of *Blautia* is plotted with the number of *fdh* genes in each isolate indicated (B). Scale bar indicates substitutions per site. Three types of formate dehydrogenase gene clusters in *Blautia* are plotted, showing the arrangement and presence of genes implicated in electron bifurcation (*hydABC*-like), molybdopterin cofactor biosynthesis (*moaA*/*mobA*/*mobB*/*moeA*/*mog*), the sulfur carrier protein fdhD, formate dehydrogenase (*fdhF*) and permease genes (*tupABC*) (the *fdhF* gene clusters for all isolates are plotted in Supplementary Fig. S7) (C). Circular points show the end of the scanned genomic region or end of the contig. The WLP gene clusters, marked by *acsC*/*acsD* and flanking regions for two representatives of *fdh* positive or *fdh* negative *Blautia* are plotted showing variable presence of *fhs* (D). The WLP gene clusters for all 286 *Blautia* are shown in Supplementary Fig. S6. Circular points indicate the limit of the scanned genomic region or end of the contig.

sucromutans (Krumholz and Bryant, 1986; Dore and Bryant, 1990) were reported to naturally lack formate dehydrogenases. It is speculated that the first acetogens also did not have formate dehydrogenases due to high formate concentrations in the Early Earth environment (Jain *et al*., 2020). While todays *Blautia* spp. most likely lost *fdh* genes in a secondary event, the fact, that acetogenic bacteria naturally live without formate dehydrogenase in formate rich conditions, can help to understand the origin of acetogenic bacteria.

Experimental procedures

Organisms and cultivation

Blautia luti DSMZ 14534, *B. schinkii* DSM10518 and *B. wexlerae* DSM19850 were cultivated at 37°C in CO_2/$KHCO_3$-buffered complex medium (Heise *et al*., 1989). 20 mM glucose was used as carbon and energy source. Growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Gene analysis and comparison

Gene analyses and sequence comparisons were carried out using the Basic Local Alignment Search Tools (BLAST) from the National Center of Biotechnology Information (NCBI, Bethesda, MD, USA (Wheeler *et al*., 2005). Genetic information were also received from the Integral Microbial Genomes and Microbiomes (IMG/M) data management and analysis system (Chen *et al*., 2021).

Assembled genomes from published isolate collections (Poyet *et al*., 2019; Zou *et al*., 2019; Bisanz *et al*., 2020; Sorbara *et al*., 2020) were downloaded from NCBI (Project accessions: PRJNA596270, PRJNA544527, PRJNA412637, PRJNA482748). Assembled genomes were annotated using Prokka (version 1.13) (Seemann, 2014). The presence of WLP pathway components in the Prokka annotation of the isolates was evaluated as following: *acsC*: (gene = *acsC*, product contains Corrinoid), *acsD*: (gene = *acsD*, product contains 'Corrinoid'), *acsE*: (gene = *acsE*, product contains 'corrinoid'), *metF* (gene = *metF* or *yitJ*, product contains 'methylenetetrahydrofolate'), *folD*: (gene = *folD*, product contains 'bifunctional'), *fchA*: (gene = *fchA*, product contains 'Methenyltetrahydrofolate'), *fhs*: (gene = *fhs*, product contains 'Formate'), *acsA* (EC number = 1.2.7.4), *acsB1* (EC number = 2.3.1.169, product contains 'carbon monoxide'), *fdh* (product contains 'formate dehydrogenase'). The copy number of WLP genes was plotted in R Studio (v. 1.4.1717 *R* version: 4.1.1) using ggplot2, with isolates separated by reported phylum and sorted based on hierarchical clustering of the presence/absence of WLP genes.

For the analysis of *Blautia* isolates, all available '*Blautia*' genomes ($n = 292$) were downloaded from NCBI RefSeq through the *ftp* service (accessed 02/2022). Accession numbers are provided in Supplementary Table S1. To generate the *Blautia* phylogenetic tree, the longest 16S rRNA sequence from each isolate (in most cases, a full-length 16S rRNA gene), was aligned using MUSCLE v3.8.1551 (Edgar, 2004). A maximum-likelihood tree was generated using RAxML v8.2.12 with GTRCAT substitution model (Stamatakis, 2014). The tree was visualized in R studio using the ggtree package (Yu, 2020). After a first analysis, five *Blautia* strains had a 16S rRNA inconsistent with placement in the *Blautia* genus and were excluded from further analyses (accessions: GCF_003435775.1, GCF_00988 1485.1, GCF_015669305.1, GCF_020564165.1, GCF_02 0708755.1). A phylogenetic tree of the remaining 286 was generated, and these isolates were used in subset analyses.

The presence of WLP pathway components was determined using the prokka annotations and the copy number of WLP genes was plotted in R Studio (v. 1.4.1717 *R* version: 4.1.1) using ggplot2. To search for formate dehydrogenase genes based on sequence similarity, all protein-coding sequences were written in fasta format, and a blast database was constructed and a local protein–protein blast was performed (v.2.12.0). The top hit from each isolate (based on the percent identity) was identified. Uniprot Accession numbers of characterized formate dehydrogenase genes were: (*B. hydrogenotrophica* – A0A173R0X0, *A. woodii* – H6LB59_ACEWD, H6LB61_ACEWD, *E. callanderi* – E3GKH8_9FIRM, *C. autoethanogenum* – CAETHG_2790, *C. acidurici* – K0B3A3_GOTA9, K0B1Q4_GOTA9, *B. schinkii* – Patric ID: 1410649.3.peg.977). Domains were predicted using InterProScan (PMID: 33156333).

Preparation of resting cells

Blautia luti DSMZ 14534, *B. schinkii* DSM10518 and *B. wexlerae* DSM19850 were grown with 20 mM glucose as substrate in CO_2/$KHCO_3$-buffered complex medium to late exponential growth phase ($OD_{600} = 3$–4), harvested by centrifugation (8000*g* and 4°C for 10 min, Avanti™J-25 and JA-10 Fixed-Angle Rotor, Beckman Coulter, Brea, CA, USA), washed and re-suspended in 5 ml imidazole buffer (50 mM imidazole-HCl, 20 mM $MgSO_4$, 20 mM KCl, 2 mM DTE, 4.4 μM resazurin, pH 7.0). The final protein concentration was determined as described (Schmidt *et al*., 1963). The preparation of resting cells was performed under strictly anoxic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA), filled with N_2/H_2 [96%–98%:2%–4% (vol./vol.)].

Cell suspension experiments

Resting cell experiments were performed in 115-ml serum flasks, which contained 20 ml imidazole buffer (50 mM imidazole, 60 mM $KHCO_3$, 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 2 mM DTE, 4.4 μM resazurin, pH 7.0) and a N_2/CO_2 [80:20 (vol./vol.)] atmosphere. In all experiments with resting cells a total protein concentration of 1 mg ml^{-1} was used. As substrates 1 bar 100% H_2 + CO_2 [80:20 (vol./vol.)], 50 mM formate or 50 mM formate + 20% CO were used. All experiments with resting cells were performed shaking at 37°C and started by the addition of the substrate. At each time point 0.6 ml sample was taken for the determination of metabolites. Gas chromatography was used to determine H_2 concentrations as described (Weghoff and Müller, 2016). Concentrations of acetate and formate were determined by high-performance liquid chromatography as described (Moon *et al*., 2019).

Preparation of cell-free extract

Cells of *B. luti* DSMZ 14534, *B. schinkii* DSM10518 and *B. wexlerae* DSM19850 were grown on 20 mM glucose in $CO_2/KHCO_3$-buffered complex medium to late exponential growth phase, harvested by centrifugation (8000*g* and 4°C for 10 min, AvantiJ-25 and JA-10 Fixed-Angle Rotor, Beckman Coulter), washed and re-suspended in 7 ml lysis buffer (50 mM Tris, 20 mM $MgSO_4$, 2 mM DTE, 4.4 μM resazurin, pH 7.5). After addition of 0.5 mM PMSF and 0.1 mg ml^{-1} DNaseI, the cells were disrupted by passing them twice through a French pressure cell at 100 MPa. Cell debris was removed by centrifugation at 25 000*g* for 20 min (Centrifuge 5417R; Eppendorf, Hamburg-Eppendorf, Germany). Protein concentration was measured as described (Bradford, 1976). The cell-free extract was prepared under strictly anoxic conditions in an anaerobic chamber (Coy Laboratory Products), filled with an N_2/H_2 atmosphere [96%–98%:2%–4% (vol./vol.)].

Determination of enzyme activities in cell-free extract

All enzyme assays were performed at 37°C under anoxic conditions in glass cuvettes (d = 0.2 cm; Glasgerätebau Ochs, Germany). Each enzyme assay was performed in triplicates. Methylene-THF reductase activity was measured in 50 mM $KHPO_4$ buffer (pH 7.0) that also contained 2 mM DTE, and 4.4 μM resazurin by reduction of methylene-THF with 0.25 mM NADH or NADPH as electron donor. Methylene-THF was synthesized from 1.5 mM formaldehyde and 0.5 mM THF. The oxidation of NADH or NADPH was recorded at 340 nm as described (Wang *et al*., 2013c). MTHF dehydrogenase activity was measured in the reverse direction by reduction of 1 mM NAD^+ or $NADP^+$ at 340 nm with methylene-THF as electron donor. To determine formate dehydrogenase activity, the reduction of 10 mM methylviologen or benzylviologen was measured with 10 mM formate as electron donor in HEPES buffer (100 mM HEPES, 20 mM $MgSO_4$, 2 mM DTE, 4.4 μM resazurin, pH 7) at 604 nm. For the determination of hydrogenase activity and CO dehydrogenase activity, 10 mM methylviologen was used as electron acceptor in Tris buffer (Tris 100 mM, 2 mM DTE, 4.4 μM resazurin, pH 7). The reduction of methylviologen was monitored at 604 nm as described (Schwarz *et al*., 2018). H_2 (1 bar, 100%) or CO (1 bar, 100%) was used as electron donor.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.