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PHYSIOLOGY

Formate-Dependent Acetogenic Utilization of Glucose by the Fecal Acetogen *Clostridium bovifaecis*

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ABSTRACT Acetogenic bacteria are a diverse group of anaerobes that use the reductive acetyl coenzyme A (acetyl-CoA) (Wood-Ljungdahl) pathway for CO_2 fixation and energy conservation. The conversion of 2 mol CO_2 into acetyl-CoA by using the Wood-Ljungdahl pathway as the terminal electron accepting process is the most prominent metabolic feature for these microorganisms. However, here, we describe that the fecal acetogen *Clostridium bovifaecis* strain BXX displayed poor metabolic capabilities of autotrophic acetogenesis, and acetogenic utilization of glucose occurred only with the supplementation of formate. Genome analysis of *Clostridium bovifaecis* revealed that it contains almost the complete genes of the Wood-Ljungdahl pathway but lacks the gene encoding formate dehydrogenase, which catalyzes the reduction of CO_2 to formate as the first step of the methyl branch of the Wood-Ljungdahl pathway. The lack of a gene encoding formate dehydrogenase was verified by PCR, reverse transcription-PCR analysis, enzyme activity assay, and its formate-dependent acetogenic utilization of glucose on DNA, RNA, protein, and phenotype level, respectively. The lack of a formate dehydrogenase gene may be associated with the adaption to a formate-rich intestinal environment, considering the isolating source of strain BXX. The formate-dependent acetogenic growth of *Clostridium bovifaecis* provides insight into a unique metabolic feature of fecal acetogens.

IMPORTANCE The acetyl-CoA pathway is an ancient pathway of CO_2 fixation, which converts 2 mol of CO_2 into acetyl-CoA. Autotrophic growth with H_2 and CO_2 via the acetyl-CoA pathway as the terminal electron accepting process is the most unique feature of acetogenic bacteria. However, the fecal acetogen *Clostridium bovifaecis* strain BXX displayed poor metabolic capabilities of autotrophic acetogenesis, and acetogenic utilization of glucose occurred only with the supplementation of formate. The formate-dependent acetogenic growth of *Clostridium bovifaecis* was associated with its lack of a gene encoding formate dehydrogenase, which may result from adaption to a formate-rich intestinal environment. This study gave insight into a unique metabolic feature of fecal acetogens. Because of the requirement of formate for the acetogenic growth of certain acetogens, the ecological impact of acetogens could be more complex and important in the formate-rich environment due to their trophic interactions with other microbes.

KEYWORDS acetogen, fermentation, formate, formate dehydrogenase, glucose, Wood-Ljungdahl pathway

Acetogens are anaerobes, which use the reductive acetyl coenzyme A (acetyl-CoA) (or Wood-Ljungdahl) pathway for CO_2 fixation and energy conservation (1). As an ancient pathway of CO_2 fixation, the acetyl-CoA pathway converts 2 mol of CO_2 into acetyl-CoA and is coupled to energy conservation. The most unique feature of aceto-

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gens is their autotrophic growth with H_2 and CO_2 by using the Wood-Ljungdahl pathway as the terminal electron accepting process. For heterotrophic growth, 1 mol of glucose is converted to 3 mol of acetate, which is 1 mol more than that of the classic fermentations of glucose. Notably, because reducing electron carriers are derived from exogenous CO_2 rather than the CO_2 derived via the decarboxylation of pyruvate, exogenous CO_2 is required for the acetogenic growth of acetogens (2). Carbon dioxide is the sole carbon substrate for chemolithotrophic acetogenic acetate formation, and glucose is the substrate for fermentatively produced acetate, while glucose and exogenous CO_2 are the substrates for heterotrophic acetogenesis. In addition, acetogens usually do not form acetate as their sole end products, and almost all known acetogens are able to generate other end products, such as ethanol, butyrate, and lactate (2).

Acetogens are widely distributed in diverse habitats such as soils, sediments, sewage sludge, and intestinal tracts (2, 3) and play an important ecological role in the global carbon cycle (4). They have diverse metabolic capabilities. Generally, acetogens can not only grow autotrophically by utilizing inorganic substrates, e.g., H_2, CO_2, and CO (5), but also grow heterotrophically on a variety of organic substrates, e.g., sugars, alcohols, and aromatic compounds (6–10). However, some species of acetogens also display unique metabolic features. Acetogenesis only occurs with H_2-CO_2 as substrates for *Acetonema longum* (11). *Butyribacterium methylotrophicum* only displays acetogenic utilization of substrates in the presence of H_2-CO_2 and formate (12). Even some acetogens cannot grow with H_2-CO_2, such as *Moorella glycerini* (13), *Natroniella acetigena* (14), *Natronincola histidinovorans* (15), and *Clostridium formicoaceticum* (16). Thus, acetogenesis is usually a conditional capability of an acetogen. Among the nutritional conditions required for the acetogenic growth of some acetogens, the requirement of formate is rare. There are only a few reports on formate-dependent acetogenic growth. Formate is required for optimal homoacetogenic conversion of glucose by *Marvinbryantia formatexigens* (formerly known as *Bryantella formatexigens*) (17, 18). Growth of *Syntrophococcus sucromutans* with carbohydrates or pyruvate was only possible in the presence of formate (19, 20). Studies on the formate-dependent acetogenic growth of certain acetogens are crucial to reveal the diverse metabolic features of acetogens. In this case, because of the requirement of formate for acetogenic growth of certain acetogens, the ecological impact of acetogens could be more complex and important in the formate-rich environment due to their trophic interactions with other microbes.

Clostridium bovifaecis strain BXX was isolated from cow manure as a novel acetogen by our laboratory (21). Nowadays, genome information helps further understanding of the metabolism of acetogenic bacteria, and a good understanding of the metabolic capabilities of most isolates is lacking. The genome sequence of *Clostridium bovifaecis* revealed that it contains almost complete genes of the Wood-Ljungdahl pathway but lacks genes encoding formate dehydrogenase (*fdh*). Formate dehydrogenase performs the first step of the methyl branch of the Wood-Ljungdahl pathway, which reduces CO_2 to formate. This prompted us to study the acetogenic growth of the novel acetogen *Clostridium bovifaecis* with supplemental formate. This study on the formate-dependent acetogenesis of *Clostridium bovifaecis* will provide insight into the diverse metabolic features of acetogens.

RESULTS

Genome properties. The complete genome of strain BXX consisted of one circular chromosome of 3,727,984 bp with a GC content of 32.62% (shown in Fig. S1 in the supplemental material). A total of 3,837 genes, including 3,488 protein-coding genes, 40 rRNAs, and 94 tRNAs were identified. The genome properties of strain BXX were summarized in Table 1. The systematic analysis of metabolic pathways and functions of gene products and compounds showed that the top five genes were involved in carbohydrate metabolism, amino acid metabolism, cofactor and vitamin metabolism, energy metabolism, and membrane transport in sequential order (Fig. 1).

The genes involved in Wood-Ljungdahl pathway and fermentation via glycolysis. Almost complete genes of the methyl and carbonyl branches of the Wood-

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TABLE 1 Genome statistics of *Clostridium bovifaecis* strain BXX

Attribute[a]	Value (no.)	% of total
Genome size (bp)	3,727,984	100
DNA coding (bp)	3,110,913	83.45
DNA G+C (bp)	1,216,068	32.62
Tandem repeats	13,717	36.79
CRISPR	0	0
DNA scaffolds	1	100
Genomics islands	193,394	5.19
Total genes	3,837	100
Protein-coding genes	3,488	90.90
rRNAs (5S, 16S, 23S)	14, 13, 13	3.65, 3.38, 3.39
tRNAs	94	24.50
Genes assigned to COGs	3,077	80.19
Genes assigned to CAZy	75	1.95

[a]COGs, Clusters of Orthologous Groups; CAZy, Carbohydrate-Active enZYmes database.

Ljungdahl pathway were identified in the genome of strain BXX, but only the gene encoding formate dehydrogenase was lacking (Fig. 2). Formate formation catalyzed by formate dehydrogenase is the first step of the methyl branch of the Wood-Ljungdahl pathway, which reduces CO_2 to formate. In addition, the genome of strain BXX contains complete genes involved in glucose fermentation via glycolysis, which is consistent with its growth with glucose as substrate (21) (Fig. 3). Genes encoding for acetaldehyde dehydrogenase, alcohol dehydrogenase, phosphotransacetylase, phosphate acetyltransferase, and acetate kinase involved in acetate and ethanol synthesis were also

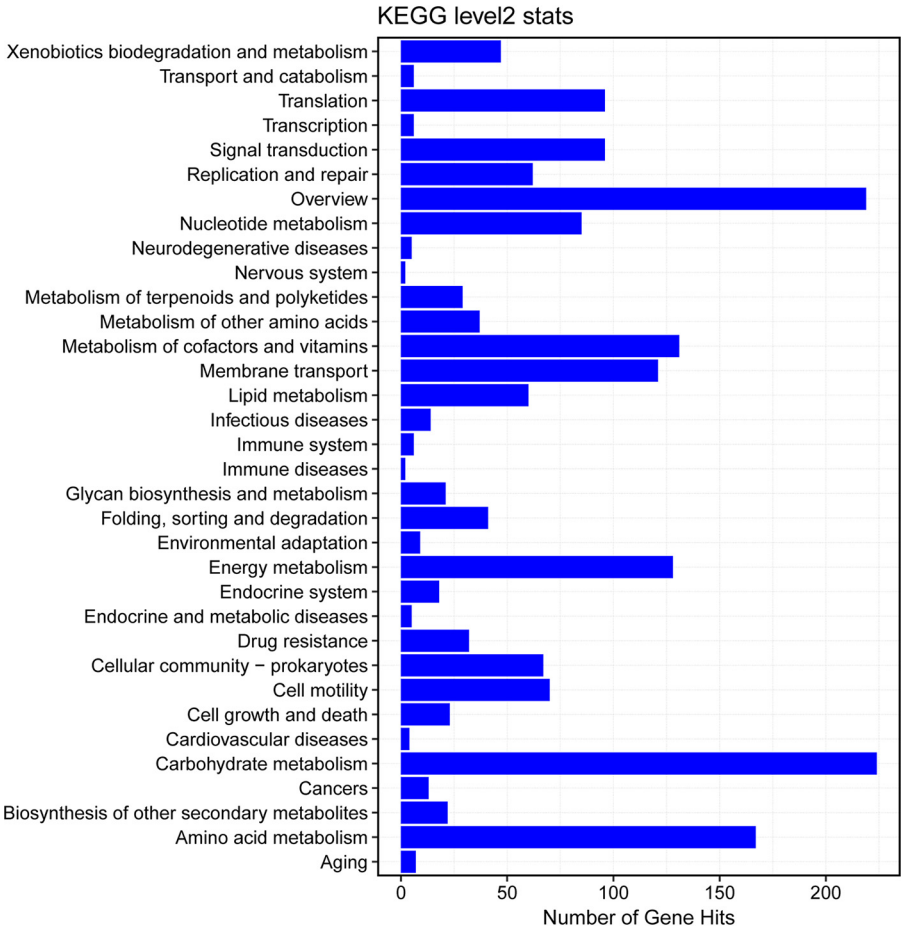

FIG 1 Gene function category of *Clostridium bovifaecis* strain BXX based on KEGG.

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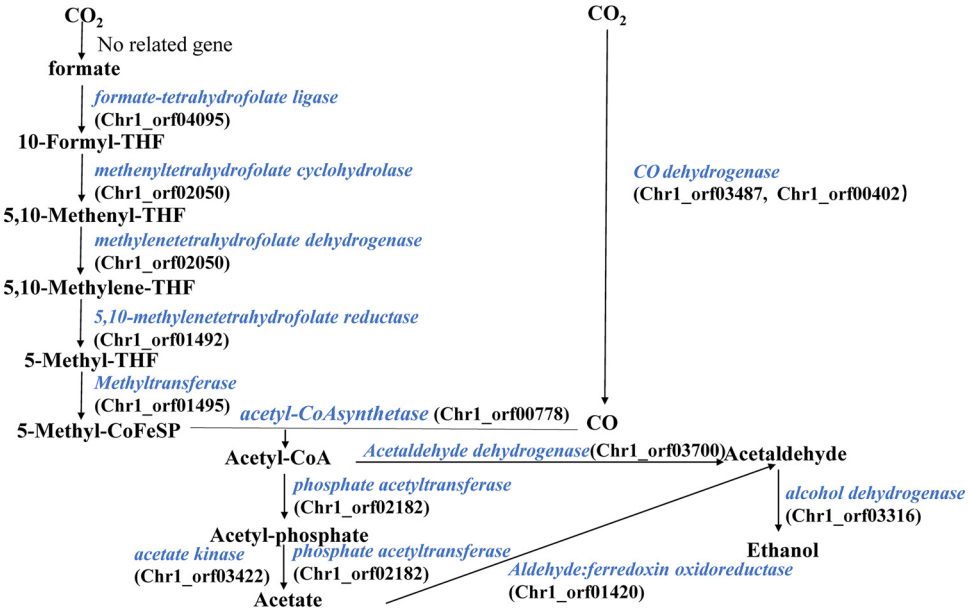

FIG 2 Genes involved in Wood-Ljungdahl pathway in *Clostridium bovifaecis* strain BXX.

contained in the genome of strain BXX (Fig. 2 and 3). Presence of the genes involved in the acetone-butanol-ethanol (ABE) fermentation pathway is a classical characteristic of the genus *Clostridium* (22). According to metabolic characteristics revealed by gene function annotation, we hypothesized that the acetogenic growth of stain BXX may be dependent on the presence of formate.

Verification of the absence of formate dehydrogenase-encoding gene on DNA, RNA, and protein level. To test the presence or absence of the gene encoding formate dehydrogenase, *fdhF*, the gene and enzyme activity of formate dehydrogenase was verified by PCR and reverse transcription-PCR (RT-PCR) analysis as well as enzyme activity assay on DNA, RNA, and protein level, respectively. Both degenerate primers and specific primers targeting the *fdhF* gene were used to check the absence of the formate dehydrogenase gene in strain BXX. As shown in Fig. 4A, B, D, and E, there were no PCR and RT-PCR products of the *fdhF* gene detected, which was consistent with the results of genome annotation. Furthermore, the enzyme activity of formate dehydrogenase was not detected in the crude cell extracts of strain BXX grown on glucose-formate-CO_2 (Fig. 4C and F).

Growth of *Clostridium bovifaecis* on glucose-CO_2-formate. To study the autotrophic growth of *Clostridium bovifaecis* strain BXX on H_2-CO_2, we added formate in the presence of H_2-CO_2. The results showed that strain BXX displayed poor metabolic capabilities of H_2-dependent acetogenesis even with supplemental formate (see Fig. S2 and S3 in the supplemental material). Therefore, a further experiment of glucose-dependent acetogenesis was performed to study the heterotrophic acetogenic growth of *Clostridium bovifaecis*.

For growth on glucose, glucose-CO_2, glucose-formate, or glucose-formate-CO_2, the consumed glucose was about 6.25 to 6.75 mmol/liter with the initial concentration of 23 mmol/liter in all four treatments (Fig. 5A). A larger amount of formate was consumed with a value of 9.53 mmol/liter compared with that of incubations in the absence of exogenous CO_2 (0.98 mmol/liter) (Fig. 5B). As shown in Fig. 5C, the concentrations of CO_2 increased in all of the incubations due to pyruvate decarboxylation. Obviously, the glucose-formate-CO_2 incubations had the smallest CO_2 production, which was much less than that of the glucose-CO_2 incubations, suggesting the consumption of CO_2 during the formate-CO_2 incubation. Accordingly, the highest ethanol concentrations of 15.55 mmol/liter and the highest cell density of 0.55 (optical density at 600 nm [OD_{600}])

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FIG 3 Genes involved in glucose fermentation via glycolysis in *Clostridium bovifaecis* strain BXX.

were observed in the presence of formate and exogeneous CO_2 (Fig. 5D and E), while those of other treatments were 9.92 to 11.38 mmol/liter and 0.45 (OD_{600}), respectively (Fig. 5E). Trace amounts of other volatile fatty acids were detected during the whole incubation, and only 3.06 mmol/liter of acetate was produced with the addition of formate and exogenous CO_2 (see Fig. S4 in the supplemental material). The other two ABE pathway products (acetone and butanol) were not detected. The pH of the glucose-formate-CO_2 incubations was about neutral at 7.0, which was similar to that of the controls, while the pH of the other incubations gradually decreased to 4.41 to 6.51 (Fig. 5F).

Although the amplification of the *fdhF* gene and the enzyme activity of formate dehydrogenase were not detected (Fig. 4), the expression levels of the *fhs* gene encoding formate tetrahydrofolate synthetase and *pyk* gene encoding pyruvate kinase were relatively high. As shown in Fig. S6 in the supplemental material, the copy numbers of the *fhs* gene in the glucose-formate-CO_2 incubations were an order of magnitude greater than those of the other treatments. It is shown that an acetogenic reaction was active with the supplementation of formate and exogenous CO_2. There

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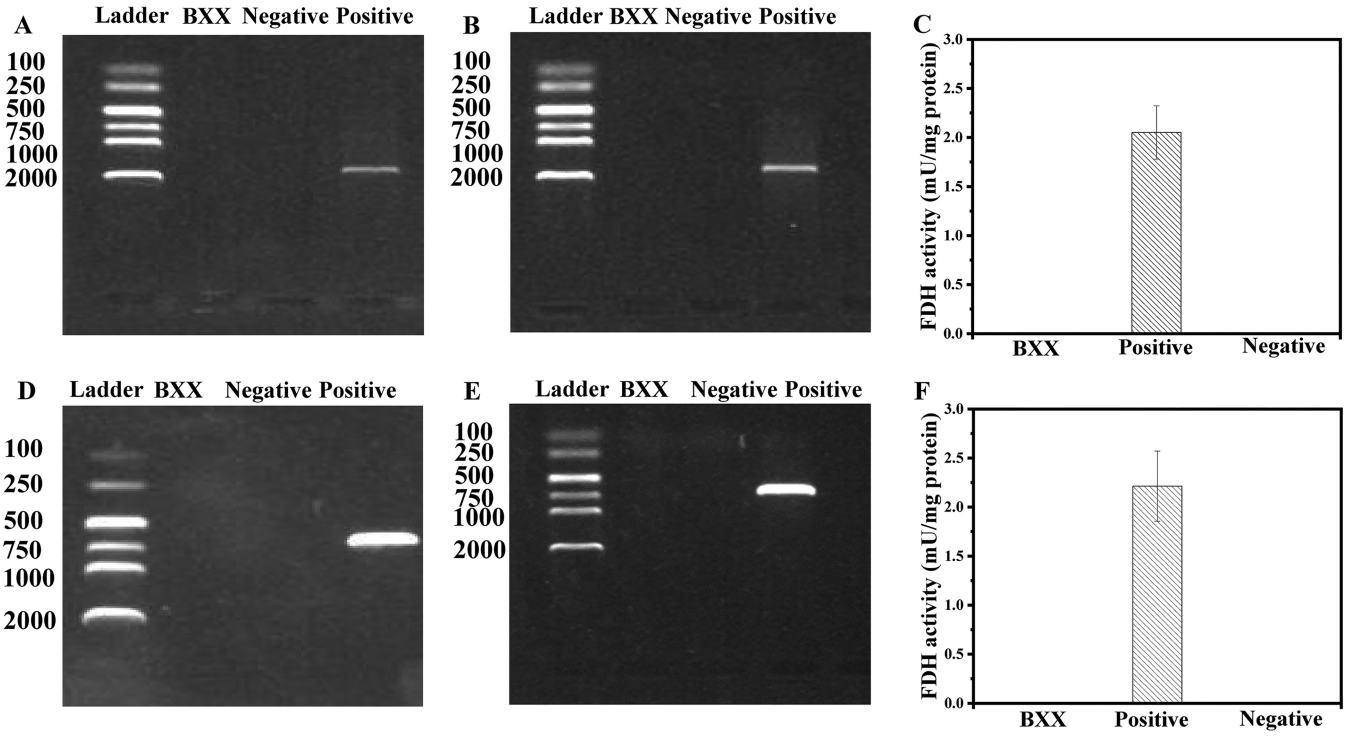

FIG 4 Gel electrophoresis images of PCR (A, D) and RT-PCR (B, E) products of the *fdhF* gene with the *fdhF*1 (A, B) and *fdhF*k12 (D, E) primers and enzyme activity of formate dehydrogenase (C, F) in strain BXX. *Salmonella enterica* serovar Enteritidis and *Escherichia coli* K-12 were used as positive controls of enzyme activity assay and PCR products of the *fdhF*1 and *fdhF*k12 primers, respectively, and deionized water was used as the negative control. The number in the left indicates the band size (bp) using a 2-kb ladder as a marker. Experiments were performed in triplicate, and formate dehydrogenase activity was measured under anaerobic conditions.

was no significant difference in the copy numbers of the *pyk* gene, indicating that glycolysis levels were similar in all of the treatments (Fig. S6).

Growth of *Clostridium bovifaecis* on different concentrations of glucose in the presence of formate and CO_2. In order to better understand the acetogenic utilization of glucose by *Clostridium bovifaecis*, the effect of glucose concentrations on the growth and product formation of *Clostridium bovifaecis* was investigated. Glucose was completely consumed at the initial concentration of 11.5 mmol/liter, but less than 50% of glucose was consumed at the initial concentration of 46 mmol/liter, with values of 22.68 and 20.97 mmol/liter for the glucose and glucose-formate-CO_2 incubations, respectively (Fig. 6A). A larger amount of formate (24.93 mmol/liter) was consumed at a glucose of 46 mmol/liter compared with that of 11.5 mmol/liter glucose (4.87 mmol/liter) (Fig. 6B). Similarly, the concentrations of CO_2 increased in all incubations (Fig. 6C), and smaller CO_2 production was observed in the glucose-formate-CO_2 incubations, suggesting that an amount of CO_2 was consumed. Accordingly, 31.64 mmol/liter ethanol was obtained at a glucose of 11.5 mmol/liter, while acetate concentrations of 57.45 mmol/liter were detected at a glucose of 46 mmol/liter (Fig. 6D and E). The highest cell density was obtained in the glucose (11.5 mM)-formate-CO_2 incubations, and that of the glucose (46 mM)-formate-CO_2 incubations was the lowest (Fig. 6F). The pH ranged from 6.13 to 6.16 and 6.06 to 5.90 for the glucose (11.5 mM)-formate-CO_2 and glucose (46 mM)-formate-CO_2 incubations, respectively, and the pH of the other two incubations gradually decreased to about 5.0 (see Fig. S5 in the supplemental material).

Chemical balance of glucose-CO_2-formate incubations. The amounts of consumed glucose and formate as well as produced CO_2, ethanol, and acetate are summarized in Table 2. Theoretically, 1 mol glucose is converted to 2 mol ethanol or acetate during the oxidation of glucose via glycolysis (equations 1 and 2) (23). Ethanol and acetate are formed from formate and CO_2 via equation 3 (17).

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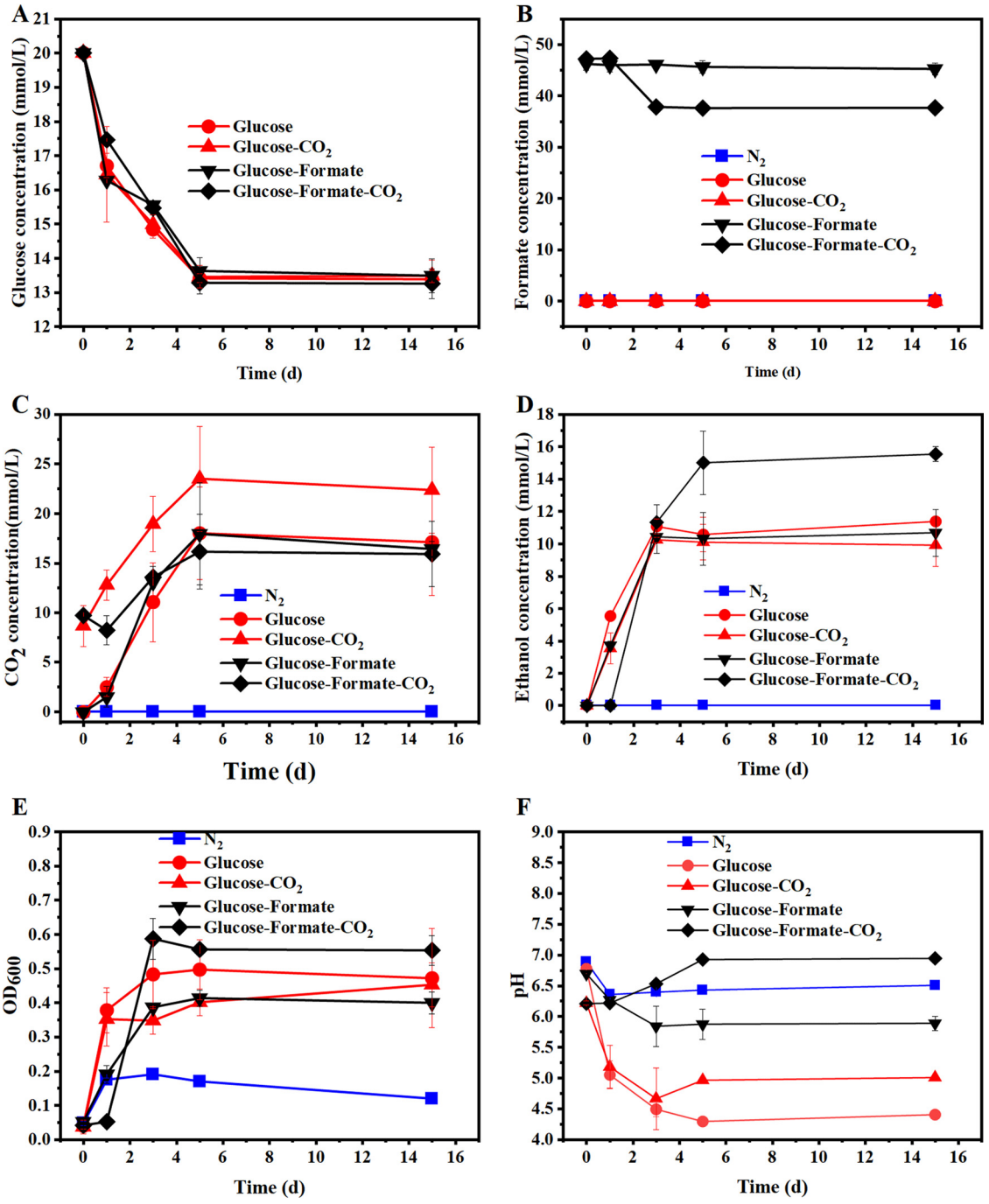

FIG 5 Consumption of glucose (A) and formate (B) and production of CO_2 (C), ethanol (D), cell density (E), and pH value (F) of *Clostridium bovifaecis* strain BXX using 23 mM glucose as the substrate with a supplemental 46 mM of formate and/or with an atmosphere of N_2-CO_2 (80/20 [vol/vol], 10^5 pascals overpressure). Experiments were performed in triplicate at 30°C in defined medium. Mean ± standard deviation, $n = 3$.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (2)$$

$$CO_2 + HCOOH + 10[H] \rightarrow C_2H_5OH + 3H_2O \text{ and } CO_2 + HCOOH + 6[H] \rightarrow CH_3COOH + 2H_2O \quad (3)$$

The yields of ethanol or acetate in the glucose-CO_2-formate incubations were all greater than those in the according glucose incubations (Table 2). Ethanol was mainly

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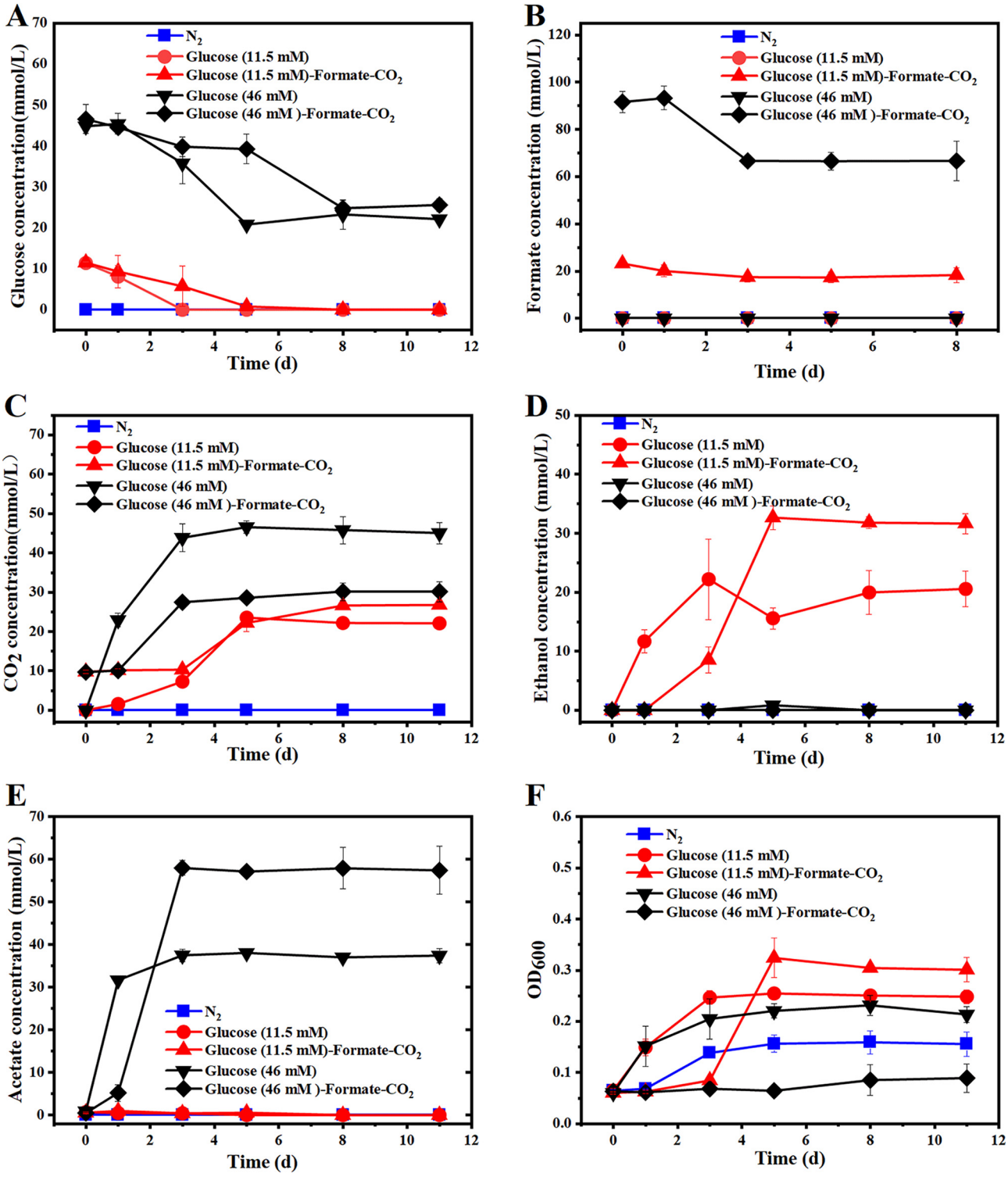

FIG 6 Consumption of glucose (A), formate (B), production of CO_2 (C), ethanol (D), acetate (E), and cell density (F) of *Clostridium bovifaecis* strain BXX using 11.5 mM and 46 mM glucose as substrates with supplemental formate and with an atmosphere of N_2-CO_2 (80/20 [vol/vol], 10^5 pascals overpressure). Experiments were performed in triplicate at 30°C in defined medium.

produced under the glucose concentrations of 11.5 and 23 mmol/liter, and acetate was the major product under the glucose concentrations of 46 mmol/liter. For 11.5 mmol/liter glucose, the ethanol production in the presence of formate and CO_2 was 11.05 mmol/liter greater, and the consumed glucose was about 11.47 mmol/liter. For

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TABLE 2 Accumulated or consumed metabolites in different incubations

Group	Consumed (mM)		Accumulated (mM)			Carbon recovery (%)
	Glucose	Formate	CO_2	Acetate	Ethanol	
Glucose (23 mM)	6.52	0	17.12	0	11.38	101.94
Glucose (23 mM)-CO_2	6.25	0	13.68	0	9.93	89.44
Glucose (23 mM)-formate	6.25	0.98	16.44	0	10.70	98.34
Glucose (23 mM)-formate-CO_2	6.75	9.53	5.77	3.06	15.55	85.47
Glucose (11.5 mM)	11.39	0	22.10	0	20.59	92.60
Glucose (11.5 mM)-formate-CO_2	11.47	4.87	17.01	0	31.64	108.96
Glucose (46 mM)	22.68	0	45.04	37.40	0	88.07
Glucose (46 mM)-formate-CO_2	20.97	24.93	20.44	57.45	0	89.77

23 mmol/liter glucose, ethanol production and acetate production in the presence of formate and CO_2 were 4.85 and 3.06 mmol/liter greater, respectively, and 6.75 mmol/liter of glucose was consumed. However, for 46 mmol/liter glucose, the acetate production in the presence of formate and CO_2 was 20.05 mmol/liter greater, and 20.97 mmol/liter of glucose was consumed. Thus, the molar ratio of consumed glucose to produced ethanol/acetate was about 1:2.75. Additionally, the molar ratios of consumed formate and CO_2 were 1:0.83 to 1:1.01, which also fits a stoichiometry of 1:1 as expected for acetogenesis. Consistently, the highest copy numbers of the *fhs* gene encoding a key enzyme in the Wood-Ljungdahl pathway was found (Fig. S6).

For growth on glucose, glucose-CO_2, and glucose-formate, the molar ratios of consumed glucose to produced ethanol were about 1:1.58 to 1:1.74, indicating a stoichiometry of 1 to 2 as expected for fermentation via glycolysis (Table 2). Collectively, acetogenic utilization of glucose only occurred in the presence of formate and exogenous CO_2 in strain BXX. The theoretically assumed stoichiometry based on glucose oxidation to CO_2 and ethanol/acetate and supplemental formate and CO_2 reduction to ethanol/acetate are depicted in equations 4 to 6 for glucose values of 11.5, 23, and 46 mmol/liter, respectively.

$$1\ C_6H_{12}O_6 + 0.75\ HCOOH + 7.50\ [H] \rightarrow 2.75\ C_2H_5OH + 1.25\ CO_2 + 2.25\ H_2O \quad (4)$$

$$1\ C_6H_{12}O_6 + 0.75\ HCOOH + 5.70\ [H] \rightarrow 2.30\ C_2H_5OH + 0.45\ CH_3COOH + 1.25\ CO_2 + 1.8\ H_2O \quad (5)$$

$$1\ C_6H_{12}O_6 + 0.74\ HCOOH + 0.52\ H_2O \rightarrow 2.74\ CH_3COOH + 1.26\ CO_2 + 1.78\ H_2 \quad (6)$$

DISCUSSION

Exogenous CO_2 is required for the acetogenic growth of acetogens on glucose due to its importance for the recycling of reducing electron carriers (24, 25). The importance of CO_2 to acetogens is also indicated in studies of other acetogenic bacteria. In the presence of exogenous CO_2, *Clostridium thermoaceticum* (reclassified as *Moorella thermoacetica*) can acetogenically convert 1 mol of glucose to 2.5 mol of acetate (26). The acetogenic fermentation of fructose was significantly impaired for the growth of *Clostridium formicoaceticum* in the absence of supplemental CO_2 (27, 28). Similarly, no acetogenic potentials were found for the growth of *Clostridium bovifaecis* on glucose in the absence of exogenous CO_2. The conversion of 2 mol CO_2 into acetyl-CoA by using the Wood-Ljungdahl pathway as the terminal electron accepting process is the most prominent metabolic feature of acetogens. Although *Clostridium bovifaecis* strain BXX did not autotrophically grow on H_2-CO_2, the substrate/product stoichiometry was indicative of acetogenesis during growth on glucose-CO_2-formate (Table 2). The molar ratio of consumed formate and CO_2 fitting a stoichiometry of 1 to 1 suggested that 1 mol of formate and 1 mol of CO_2 instead of 2 mol of CO_2 were electron acceptors for this incomplete Wood-Ljungdahl pathway. Considering that the glucose-dependent acetogenic growth of *Clostridium bovifaecis* strain BXX fulfills the abovementioned requirements of the term acetogen, we defined it as an acetogenic bacterium.

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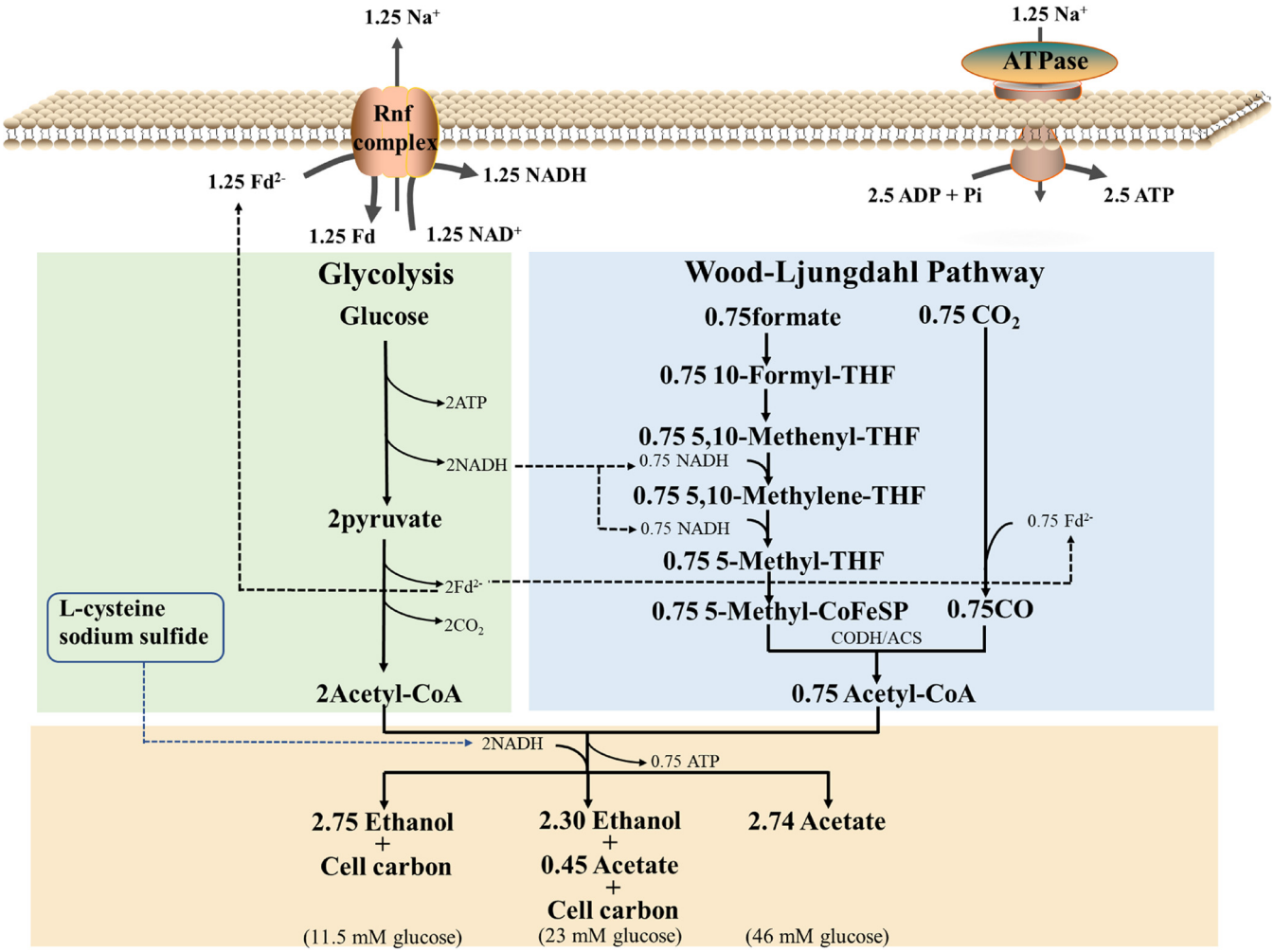

FIG 7 Model for acetogenesis from glucose in the presence of formate and CO_2 in *Clostridium bovifaecis* strain BXX. Fd^{2-}, reduced ferredoxin; CoFeSP, corronoid iron-sulfur protein; CODH, carbon monoxide dehydrogenase; ACS, acetyl-CoA synthase.

Although acetate formation is the classic feature of acetogens, the formation of acetate is not a part of acetogens per the definition of the term acetogen. Actually, acetogens also form ethanol, butyrate, and lactate under certain conditions (26). In this study, ethanol was the main end product of *Clostridium bovifaecis* during growth on 11.5 or 23 mmol/liter of glucose as the carbon source, but acetate was the major product at 46 mmol/liter glucose. It is indicated that product synthesis pathways of strain BXX were regulated with the changes of glucose concentration, which is also suggestive of genes involved in acetate and ethanol synthesis of the Wood-Ljungdahl pathway in strain BXX (Fig. 2). Interestingly, 46 mmol/liter glucose in the presence of formate and CO_2 did not support cell growth (Fig. 6F) but led to acetate production via the homoacetogenic utilization of glucose. Similarly, there was no increase in cell density in the glucose (11.5 mM)-formate-CO_2 incubations during the first 3 days, but these incubations were accompanied with ethanol production. The reason may be that carbon mainly flowed into acetate or ethanol production rather than cell carbon assimilation in this case. Collectively, our results suggested the model for acetogenesis from glucose in the presence of formate and CO_2 in strain BXX in Fig. 7.

Additionally, it has been reported that the acetate and ethanol yields of acetogenic fermentation of glucose with CO_2 derived from glycolysis could be theoretically increased by 51% and 2%, respectively, compared with the yields of those only using glucose (6). In this study, acetate yield under growth on 46 mmol/liter glucose in-

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creased by 53.61%, which was close to 51%. However, the ethanol yield in this study increased by 53.67% and 36.64% for growth on 11.5 and 23 mmol/liter of glucose, respectively, which was much greater than that in the abovementioned study. Conversely, both exogenous CO_2 and formate were required in this study. One reason for the larger ethanol generation may be that two reducing equivalents, which are required for CO_2 reduction to formate, are saved via the methyl branch of the Wood-Ljungdahl pathway using formate rather than CO_2 as substrates. Given that the reducing power from formate or glucose would result in greater production of CO_2, the other reason may be that sodium sulfide or L-cysteine in the medium provides additional reducing equivalents.

Clostridium bovifaecis strain BXX did not autotrophically grow on H_2-CO_2 and acetogenically utilized glucose and CO_2 only with the supplementation of formate. This formate-dependent acetogenic capability may be due to its lack of formate dehydrogenase, which catalyzed the conversion of CO_2 into formate via the first step of the methyl branch of the Wood-Ljungdahl pathway (6). Interestingly, it was reported that ruminal acetogen *Syntrophococcus sucromutans* grows with carbohydrates or pyruvate only in the presence of formate via an incomplete Wood-Ljungdahl pathway that lacks formate dehydrogenase (19, 20) and cannot utilize H_2 either. In addition, another fecal acetogen *Marvinbryantia formatexigens* (formerly known as *Bryantella formatexigens*) cannot grow on H_2-CO_2, and formate is required for its optimal homoacetogenic conversion of glucose (17, 18). As expected, we analyzed the genome of *Marvinbryantia formatexigens* and found that known genes encoding formate dehydrogenase are also lacking. For *Thermoanaerobacter kivui* with a deletion of genes encoding hydrogen-dependent carbon dioxide reductase (HDCR), which contains a formate dehydrogenase and a hydrogenase, formate is also required for growth (29). The above studies suggested that the *fdh* gene should be essential to acetogenic capability.

H_2-oxidizing hydrogenase in acetogenic metabolism includes electron-bifurcating hydrogenase and HDCR, which contain hydrogenase subunits HydA1 and HydA2, respectively (30, 31). The genome annotation of strain BXX shows that it also lacks genes encoding hydrogenase subunit HydA1 or HydA2, which have been purified and functionally characterized in *Acetobacterium woodii* and *Thermoanaerobacter kivui* (30, 32). Thus, the absence of electron-bifurcating hydrogenase and HDCR due to the lack of both *hydA1* and *hydA2* may be the reason for the poor H_2-CO_2 utilization in strain BXX. Similarly, the HDCR deletion mutant of *Thermoanaerobacter kivui* did not grow with H_2-CO_2 (29).

The lack of a formate dehydrogenase gene may be the result of gene loss during adaptive evolution. Given that the intestinal environment is rich in formate, converting CO_2 into formate seems to be dispensable for the fecal acetogen *Clostridium bovifaecis*. There are many examples of adaptive gene loss associated with changes of environmental metabolic supplies (33). Another hypothesis is that the FDH-lacking type may be an ancestral type of acetogenesis for acetogens, and genes encoding formate dehydrogenase may be latterly acquired to be a part of the genome of the Wood-Ljungdahl pathway due to evolutionary pressure (29). Therefore, future studies of the molecular evolution of fecal acetogens and their adaption to the intestinal environment would be interesting. Additionally, another interest in the acetogenic capability of *Clostridium bovifaecis* may be why it has only the ability of heterotrophic CO_2 fixation.

MATERIALS AND METHODS

Source of organisms and medium. *Clostridium bovifaecis* strain BXX was isolated and stored by our laboratory (21). The composition of the basic growth medium was as follows (in grams per liter): 0.50 g NH_4Cl, 0.50 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 10 g NaCl, 2.0 mg $FeSO_4 \cdot 7H_2O$, 1.00 ml trace element solution SL-10 (DSMZ medium 320), 1.00 ml selenite-tungstate solution (see DSMZ medium 385), 0.50 g yeast extract, 2.00 g Casitone, 0.50 ml Na-resazurin solution (0.1%, wt/vol), 0.35 g K_2HPO_4, 0.23 g KH_2PO_4, 10.00 ml vitamin solution (see DSMZ medium 141), 0.30 g L-cysteine-$HCl \cdot H_2O$, and 0.30 g $Na_2S \cdot 9H_2O$. The final pH was adjusted to 7.0.

Formate-dependent acetogenic growth. Incubation experiments were performed in 120-ml serum flasks containing 50 ml of medium at 30°C. For the acetogenic growth on H_2-CO_2-formate, the serum bottles were closed with black rubber stoppers and pressurized to 10^5 pascals overpressure with either

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TABLE 3 Primer sequences used for PCR and RT-qPCR analysis

Target	Primer	DNA sequence (bp)
fhs	*fhs*-f	5′-AGACAGCCAGGATGAAGTGC-3′
	fhs-r	5′-TCAATTCCACCTTCTCCGCC-3′
pyk	*pyk*-f	5′-AACTGGGGCGTATTCCCAAT-3′
	pyk-r	5′-ACCGGAATTCCTGCTGCTAC-3′
*fdhF*k12	*fdhF*k12-f	5′-CTGAATTACGTTGCCGAGCG-3′
	*fdhF*k12-f	5′-TTTCCACGCCCTGGTAGAAC-3′
*fdhF*1	*fdhF*1-f	5′-TGGTAYGGITGGGAYT-3′
	*fdhF*1-r	5′-CCACCAYTGRTAIGTCAT-3′

H_2-CO_2 (80/20) or N_2-CO_2 (80/20). The incubations under N_2 headspace were set as the controls. Sodium formate (2 mM) was added in each treatment and control to test the formate-dependent chemolithotrophically acetogenic growth.

For the mixotrophically acetogenic growth on glucose-CO_2-formate, this investigation consisted of five parallel incubations with different substrates as follows: (i) 23 mmol/liter of glucose, (ii) 23 mmol/liter of glucose with exogenous CO_2, (iii) 23 mmol/liter of glucose with 46 mmol/liter supplemented formate, (iv) 23 mmol/liter of glucose with exogenous CO_2 and 46 mmol/liter of supplemented formate, and (v) controls under N_2 headspace.

For acetogenic growth on different concentrations of glucose, this investigation consisted of five parallel incubations with different substrates as follows: (i) 11.5 mmol/liter of glucose, (ii) 11.5 mmol/liter of glucose with 23 mmol/liter formate and exogenous CO_2, (iii) 46 mmol/liter of glucose, (iv) 46 mM glucose with 92 mmol/liter of supplemented formate and exogenous CO_2, and (v) controls under N_2 headspace. The exogenous CO_2 was added by flushing with N_2-CO_2 (80/20) gas 10^5 pascals overpressure.

Bacterial growth was determined by measuring optical density at 600 nm (OD_{600}). The experiments were carried out in triplicate.

Chemical analysis. Liquid samples of 2.5 ml were collected and filtered with a 0.22-μm membrane. Samples were collected at regular intervals, initially after 1 to 2 days and subsequently at 3- to 6-day intervals. The concentration of formate was determined using high-performance liquid chromatography (U3000; Thermo Fisher Scientific, China). A volume of 20 μl of sample was injected at 30°C and separated on a Thermo Scientific C_{18} column (4.6 by 250 mm). A mixed solution of 0.01 mol/liter KH_2PO_4 and methanol (95:5, vol/vol) was used as the mobile phase with a flow rate of 1.0 ml/min. The detection wavelength was 206 nm. The liquid samples were mixed with 3 mmol/liter phosphoric acid (1:1, vol/vol) and centrifuged with 10,000 $\times$ *g* for 10 min; then, supernatant was used to measure ethanol and acetate concentrations by gas chromatograph (34).

The glucose concentration was determined via the phenol-sulfuric acid method (35). Liquid samples were mixed with 5% phenol and sulfuric acid in the proportion of 2:1:5 (vol/vol/vol). The OD_{490} was measured after cooling in cold water.

The concentration of CO_2 was determined by using gas chromatograph (Fuli, China). A 1-ml gas sample was injected and separated on a stainless-steel packed column (TDX-01, 2 m by 3 mm) with helium as the carrier gas. The samples were analyzed with a thermal conductivity detector at 150°C. The column and injection port temperatures were 100°C and 150°C, respectively. The current of the thermal conductivity detector was 80 mA for CO_2 and 35 mA for H_2, respectively.

Genome sequencing and annotation. Total genomic DNA was extracted with a MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The extracted genomic DNA was segmented by a 26 gauge (G) needle, and the fragments above 20 kb were selected by a BluePippin size selection system (Sage Science, USA). After end repairing and adding an A tail, two ends of the fragments were ligated separately to prepare the DNA library. The PacBio Sequel platform at Magigene (Guangzhou, China) was used for sequencing according to the effective concentration of the library and the needs of data output. SMRT Link 5.0 (PacBio, USA) was used to filter out low-quality reads and retain high-quality reads. After sample quality controls, the assembly of pure third-generation sequences was performed with SMRT Link 5.1.0 software, and the assembly of second/third-generation data was performed with Unicycler (36). The reads were compared with the assembled genome sequences, and the sequencing depth distribution of the assembly results was counted. The quality of splicing results was evaluated. Then, original data was compared to the sequence of assembly results. The assembly results were optimized by Arrow software, and error assembly area were corrected. Obtained sequences were compared and analyzed to distinguish chromosome and plasmid sequences.

Basic Local Alignment Search Tool and Diamond software were used for general function annotation. The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used for systematic analysis of the metabolic pathways and functions of gene products and compounds in this study (37).

DNA, RNA extraction, and PCR. Genomic DNA in *Clostridium bovifaecis* strain BXX was extracted with a DNeasy PowerSoil kit (Qiagen GmbH, Germany). Total RNA was extracted with an RNAprep Pure cell/bacteria kit (Tiangen Biotech [Beijing] Co., Ltd, China). Reverse transcription used PrimeScript RT reagent kit with genomic DNA (gDNA) Eraser (TaKaRa, Japan). The quality and concentration of the RNA and cDNA were detected by UV spectrophotometer (NanoDrop ND 2000). Acetogenesis and glycolysis expression levels were quantified by amplification of *fhs* and *pyk* genes, respectively, using the primers listed in Table 3. A degenerate primer *fdhF*1 (38) and a primer *fdhF*k12, which were designed based on the *fdhF* gene sequence of *Escherichia coli* K-12, were used to amplify the *fdhF* gene of strain BXX (Table

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3). The primers of the *fhs*, *pyk*, and *fdhF*k12 genes were designed using Primer-BLAST of the National Center for Biotechnology Information (39). Genomic DNA from *Salmonella enterica* serovar Enteritidis and *Escherichia coli* K-12 were used as positive controls of the *fdhF*1 and *fdhF*k12 primers, respectively, and double-distilled water (ddH_2O) was used as a negative control. All of the oligonucleotide primers were synthesized by Shanghai Bio-Engineering Co., Ltd. (China).

The reverse transcription-quantitative PCR (RT-qPCR) conditions for the *fhs* and *pyk* genes were initialized from 94°C for 2 min, followed by 40 cycles at 94°C for 15 s, 60°C for 15 s, and 72°C for 60 s. The PCR and reverse transcription-PCR (RT-PCR) conditions for the *fdhF* gene were initialized from 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 48°C (*fdhF*k12) and 57.8°C (*fdhF*1) for 30 s, and 72°C for 45 s, and extended at 72°C for 10 min.

Enzyme activity essay of formate dehydrogenase. Cells were harvested by centrifugation at 8,000 × *g* for 10 min, and cell extracts were obtained by bacterial protein extraction kit (Cowin Biosciences, China). The concentrations of protein were measured by bicinchoninic acid (BCA) protein assay kit (Beyotime, China). The enzyme activity of formate dehydrogenase in cell extracts was determined from oxidation of formate coupled with reduction of NAD^+ to NADH (40). An increase in the absorbance of NADH at 340 nm was used to indicate the activity of the formate dehydrogenase catalyst. One unit (U) of enzyme activity was defined as the quantity of enzyme catalyzing the formation of 1 μmol NADH/min. Specific activity was recorded as milliunits per milligrams protein (40). In order to test the feasibility of the activity assay method, *Escherichia coli* K-12 and *Salmonella enterica* serovar Enteritidis, which contain formate dehydrogenase genes, were used as positive controls. H_2O was used as a negative control. All of the operations were carried out under strictly anaerobic conditions. NAD in oxidized form (NAD^+) and reduced form (NADH) were purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Data availability. The genome sequence of *Clostridium bovifaecis* strain BXX was deposited at GenBank under accession number CP046522.1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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