Effects of cofD gene knock-out on the methanogenesis of Methanobrevibacter ruminantium

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Abstract
This study aimed to investigate the effects of cofD gene knock-out on the synthesis of coenzyme F₄₂₀ and production of methane in Methanobrevibacter ruminantium (M. ruminantium). The experiment successfully constructed a cofD gene knock-out M. ruminantium via homologous recombination technology. The results showed that the logarithmic phase of mutant M. ruminantium (12 h) was lower than the wild-type (24 h). The maximum biomass and specific growth rate of mutant M. ruminantium were significantly lower (P < 0.05) than those of wild-type, and the maximum biomass of mutant M. ruminantium was approximately half of the wild-type; meanwhile, the proliferation was reduced. The synthesis amount of coenzyme F₄₂₀ of M. ruminantium was significantly decreased (P < 0.05) after the cofD gene knock-out. Moreover, the maximum amount of H₂ consumed and CH₄ produced by mutant were 14 and 2% of wild-type M. ruminantium respectively. In conclusion, cofD gene knock-out induced the decreased growth rate and reproductive ability of M. ruminantium. Subsequently, the synthesis of coenzyme F₄₂₀ was decreased. Ultimately, the production capacity of CH₄ in M. ruminantium was reduced. Our research provides evidence that cofD gene plays an indispensable role in the regulation of coenzyme F₄₂₀ synthesis and CH₄ production in M. ruminantium.

Keywords: cofD gene, Methane, Coenzyme F₄₂₀, Methanobrevibacter ruminantium, Gene knock-out

Introduction
The mitigation of greenhouse gases has become a hot topic in recent years owing to their severe environmental impact. Methane (CH₄) is a strong greenhouse gas, accounting for approximately 16% of the total global greenhouse gas emissions (calculated as CO₂ equivalent). Previously, several studies have been conducted to investigate the reduction of CH₄ emissions to attenuate the greenhouse effect (André-Denis et al. 2011; Huang et al. 2020). In animal husbandry production, ruminants, contributing to 33% of the total CH₄ emissions of human activities, are important sources of CH₄ emissions. On the other hand, approximately 2 ~ 12% of the gross energy from feed can be transformed into CH₄ during the ruminal fermentation of ruminants and exhausted because of unavailability (Johnson et al. 1995). In recent years, various strategies have been researched to inhibit CH₄ production in the rumen, such as artificial regulation of ruminal microbiota structure, using the biological inhibitors and vaccines, chemical inhibitors, and nutritional control measures. Unfortunately, the rumen micro-ecosystems can adapt and recover the original methane-generating level soon after administration (Hook et al. 2010; Martin et al. 2010). Therefore, external regulations can not steadily reduce the CH₄ production and emission from ruminants in the long term.

Methanobrevibacter is the dominant archaea in the gastrointestinal tract of herbivorous animals (Cersosimo...
et al. 2015). In the rumen, the Methanobrevibacter ruminantium (M. ruminantium) has higher methanogenic activity and adaptability of environmental changes (Li et al. 2014). As a kind of hydrogenotrophic methanogen, the M. ruminantium can utilize H2 and CO2 for CH4 production (Danielsson et al. 2012; Benepal 2012). Coenzyme F420 is a key metabolic coenzyme in the process of energy metabolism of hydrogenotrophic methanogens and involved in the critical steps of CH4 generation from CO2 by hydrogen reduction (Eirich et al. 1978). The generation of phosphodiester F420-0 by condensation of L-lactoyl diphosphate guanosine (LPPG) and Fo is a critical process for coenzyme F420 activation (Graupner et al. 2002). Hence, CofD plays an essential role in the process of F420 biosynthesis. However, at present, few researches have addressed the question of how CofD enzyme affects the synthesis of coenzyme F420, thereby regulating the methanogenic activity and growth of methanogens.

In the current study, a cofD activity deficient M. ruminantium strain was constructed by knocking out cofD gene using homologous recombination of the tetracycline resistance gene into the cofD sequence in the chromosome. The polymerase chain reaction and western blotting were performed to verify the success of constructed cofD knock-out strain and disruption of cofD activity. With the purpose of new molecular target towards mitigation of CH4 emission, the effects of a knocking-out cofD activity deficient strain were investigated in anaerobic culture.

Materials and methods
Bacterial strains and plasmids
The representative ruminal Methanobrevibacter ruminantium M1 (DSM1093) (this strain have been deposited in a publicly accessible culture collection belonging to the DSMZ) used in this study was obtained from the CSIRO Microbiology Laboratory (Australia) friendly. The plasmid pUC19, pEASY-T1, and pBR322 cloning vector were purchased from JRDUN Biotechnology Co. Ltd (Shanghai, China). The strain E. coli DH5α was used as a plasmid cloning host. The pUC18-cofD-tet vector were constructed in this study.

Preparation of target gene cofD fragment
The M. ruminantium strain was inoculated in deoxygenated sterilized Hungatubets contained with liquid medium for recovery and culture at 39 °C for 30 h. Genomic DNA was extracted from collected thalli cells using the Bacterial Genomic DNA Extraction Kit (Takara, Beijing, China). The cofD gene was amplified by PCR using primers cofDS and cofDS’ (Table 1). Amplification conditions were set at 94 °C for 10 min followed by 30 cycles at 94 °C for 45 s, 60 °C for 45 s for 45 s, and 72 °C for 10 min. Amplified cofD gene segments were excised from agarose gel and purified by QIAquick PCR purification Kit (Qiagen, Hilden, Germany). The target gene cofD was ligated with the vector pEASY-T1 at a molar ratio of 3:1 at 37 °C for 15 min. The ligation products were transformed into E. coli DH5α strain and screened with Blue-White Screenings on the LB plates containing Amp, IPTG, and X-gal.

Chemicals and media
All the chemicals were analytical grade. M. ruminantium strain was cultured in the following liquid medium: yeast extract, 0.2 g/L; peptone, 0.2 g/L; NaHCO3, 6 g/L; L-cysteine·HCl·2H2O, 0.35 g/L; mineral salt solution I, 0.05%; mineral salt solution II, 0.05%; Balch trace element solution, 0.01%, and 0.1% resazurin (W/V) 0.001%, in which solutions were prepared previously and stored at 4 °C. In our research, the modified liquid medium (BJ) was supplemented with 0.1% clarified ruminal fluid (the ruminal fluid was centrifuged at 4 °C and 13,000 r/min for 15 min, and the supernatant was collected). Finally, the deionized water was added to a total volume of 1 L. Mineral salt solution I (values in grams per liter): K2HPO4·3H2O, 7.86; Mineral salt solution II: KH2PO4, 6; (NH4)SO4, 6; NaCl, 12; MgSO4·7H2O, 1.2; MgSO4·7H2O, 2.5; CaCl2·2H2O, 1.2; CaCl2·2H2O, 1.6; Balch trace element solution: nitro acetic acid, 1.5; MgSO4·7H2O, 3.0; NaCl, 1.0; MnSO4·2H2O, 0.5; FeSO4·7H2O, 0.1; CoCl2·6H2O, 0.1; CaCl2·2H2O, 0.1; ZnCl2, 0.1; CuSO4·5H2O, 0.01; AlK(SO4)2, 0.01; H3BO3, 0.01; Na2MoO4·2H2O, 0.01. The liquid medium pH was adjusted and maintained at 6.9 ~ 7.0. The medium was prepared under an 80% nitrogen and 20% carbon dioxide gas phase by the Hungate technique as modified Bryant and Robinson (Balch and Wolfe 1977). The aliquots of the medium were separated under strictly anaerobic condition via autoclaving at 124 °C, and all roller tubes and vials were capped with rubber plugs and aluminum caps. For E. coli incubation, a Luria–Bertani (LB) solid medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl was used and agar was added to the medium to a final concentration of 2% for preparation of solid medium.
Amplification of tetracycline resistance gene
Methanogens belong to the gram positive bacteria, and the tetracycline resistance gene can be expressed in gram positive bacteria according to its characteristics. Thus, in this experiment, it is selected as a marker gene for constructing a vector. The Cla I cleavage site was added to the two specific primers tet-F and tet-R of the tetracycline resistance gene (Table 1). The tetracycline resistance gene was specifically amplified using pBR322 plasmid DNA as a template by YEATaq polymerase (Takara, Beijing, China). The purified PCR product was ligated to the cloning vector pEASY-T1 at 37 °C for 15 min. The resulting plasmid was transformed into E. coli DH5α strain and screened with ampicillin. The extracted plasmid was named pEASY-T1-cofD and pEASY-T1-tet and sequenced.

Construction of recombinant plasmid pUC18-cofD-tet
The constructed pEASY-T1-cofD recombinant plasmid and pUC18 plasmid vector were double digested with EcoRI and HindIII, respectively. After the pEASY-T1-cofD product was detected by gel electrophoresis, the cofD band was recovered and inserted into the pUC18 vector (double enzyme digested) to construct a pUC18-cofD recombinant plasmid. The constructed pEASY-T1-tet and pUC18-cofD plasmid DNAs were cleaved by Cla I respectively. After the detection of pEASY-T1-tet by electrophoresis, the tet band was recovered and inserted into the pUC18-cofD recombination vector, which was digested at 20 °C for 25 min. The resulting plasmid was transformed into E. coli DH5α strain and screened with Blue-White Screenings to select recombinant.

Identification of cofD gene knock-out strains
The constructed pUC18-cofD-tet recombinant plasmid was transformed into the M. ruminantium by electroporation. The mutant strains were screened using tetracycline plates. After anaerobic culture, the knock-out and wild M. ruminantium bacterial fluid were collected and the DNA and RNA were extracted. The cofD gene expression and the CofD enzyme protein expression were determined by qRT-PCR and western blotting. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizing control.

Comparison of cofD gene knock-out and wild-type strains
The mutant and wild-type M. ruminantium were anaerobically inoculated into Hungate tubes which equipped with optimized liquid medium. The inoculation amount of growth strains was 0.6 mL for each serum bottle and cultured at different time points (12, 24, 36, 48, 96 h) with three replicates at each time point. After inoculation, 40 kpa of CO2 was added to the Hungate tube, H2 was added to a pressure of 200 kpa, and the plate was placed in a constant-temperature incubator with a horizontal swing (40 rpm/min). The growth curve of M. ruminantium was measured using a spectrometer at a wavelength of 600 nm. The gas composition was analyzed by gas chromatography, including the respective proportions of the H2, CO2, and CH4. The absorbance of coenzyme F420 was measured by a spectrometer at a wavelength of 420.

Statistical analysis
The data of cofD knock-out and wild-type strains were analyzed using the independent sample t-test of the SPSS statistical software (Version 20.0 for Windows; SPSS, Chicago, USA). The results were expressed as the mean and standard error of the mean (SEM). A significance level was indicated at P < 0.05, and a trend was declared at 0.05 ≤ P < 0.10.

Results
Plasmid construction for knock-out cofD and pEASY-T1-cofD plasmid construction
An 906 bp cofD gene fragment was successfully amplified from chromosome DNA of M. ruminantium using primers cofD designed. Amplification results are shown in Additional file 1: Fig. S1. The target gene cofD was ligated with the vector pEASY-T1 and confirmed by colony PCR. Six white monoclonal colonies grown on LB plates were randomly selected for further verification. DNA fragment sizes were observed in DNA electrophoresis analysis shown in Additional file 1: Fig. S2. The PCR products of positive clones were sequenced and the resulting sequences were subjected to nucleic acid sequence alignment. The results showed that the fragments were...
overlap by 828 bp (Identities = 821/828) and the sequence similarity was 99% (Additional file 1: Fig. S3).

**Plasmid pUC18-cofD-tet construction**

The pCU18-cofD-tet ligation product was cultured in LB solid medium containing tetracycline and then inoculated into tetracycline-containing LB liquid medium. The bacterial liquid was used as a template and the tet-specific primers were used to amplify tet. The results are shown in Additional file 1: Fig. S4. The 9 clones picked out showed clear bands between 1000 and 2000 bp. Compared with the PCR products of the tet gene, the sizes were identical, indicating that the tet and pUC18-cofD vectors were successfully constructed. The pUC18-cofD-tet recombinant plasmid DNA was extracted from the above bacteria solution by an alkaline lysis method, and the extracted plasmid DNA was double digested with EcoRI and HindIII. The results are shown in Additional file 1: Fig. S5.

**Verification of cofD gene knock-out**

After the constructed pUC18-cofD-tet was transformed into the M. ruminantium by electrotransformation, the 100 μL of culture solution was coated on a tetracycline plate, and the mutant strain was selected by static culture at 30°C for 72 h. The expression level of cofD gene of mutant strain was lower (P < 0.05) than that of the wild-type strain (Fig. 1). Moreover, the expressing quantity of CofD enzyme protein in knock-out bacteria was significantly down-regulated (P < 0.05) as compared to the wild-type M. ruminantium (Fig. 2).

**Effects of cofD knock-out on the growth of M. ruminantium**

The growth curves of wild-type and knock-out of M. ruminantium were respectively examined at 30°C with equal inoculation amount (Fig. 3). After the cofD gene knock-out, the delay period of the mutant M. ruminantium was shorter than that of wild-type strains; besides, the M. ruminantium entered the logarithmic phase earlier. The logarithmic growth phase (12 h) of mutant M. ruminantium was lower in comparison with wild-type strains (24 h). As can be seen from Table 2, the maximal bacterial mass of cofD knock-out M. ruminantium was only approximately half of the wild-type strains, and the maximum specific growth rate was also lower (P < 0.05) than that of the wild-type strains.
Effects of cofD knock-out on the coenzyme F$_{420}$ synthesis of M. ruminantium

As shown in Fig. 4, the fluorescence value of F$_{420}$ was used to express the content of coenzyme F$_{420}$ in this experiment. From the point view of growth of M. ruminantium, the coenzyme F$_{420}$ content was increased gradually with the growth of the strain. From the total point of view (Fig. 4A), the content of coenzyme F$_{420}$ in the cofD knock-out strains did not change significantly at 12 h (P > 0.05). However, at 24, 36, 48, and 96 h, the content of coenzyme F$_{420}$ was significantly lower (P < 0.05) than that of wild-type M. ruminantium. Furthermore, the content of unit cell volume of coenzyme F$_{420}$ between the cofD gene knock-out type and wild-type M. ruminantium at the same time point were analyzed (Fig. 4B). The content of coenzyme F$_{420}$ per unit of biomass was decreased by 29%, 59%, and 30% at 36, 48, and 96 h respectively. All of them were significantly lower (P < 0.05) in knock-out than those in wild-type strains.

Effects of cofD knock-out on the methane production of M. ruminantium

From the CH$_4$ production and H$_2$ consumption curve of the wild-type M. ruminantium, it can be seen that as the incubation time increases, the concentration of H$_2$ in the culture flask was continuously decreased, and the CH$_4$ concentration was continuously increased (Fig. 5). The concentration of CH$_4$ in the flasks reached a plateau at 48 h. Comparing with CH$_4$ production and H$_2$ consumption of wild-type M. ruminantium, it was found that the cofD knockout strains only consumed a markedly lower amount of H$_2$ in the logarithmic growth phase to generate trace amounts of CH$_4$, and the maximum consumption of H$_2$ and the maximum production of CH$_4$ for cofD knock-out strains were only 14% and 2%, respectively, of the wild-type strains.

Discussion

In recent years, CH$_4$ emissions from ruminants have caused not only adverse effects on the environment, but also loss of energy intake by animals. Therefore, regulation of methanogens in the rumen has become a research

| Table 2 | Comparison in the methane production and growth indexes of wild-type and mutant M. ruminantium |
|---------|-----------------------------------------------------------------------------------------------|
|          | **Indexes** | **Treatments** | **SEM** | **P-Value** |
|          | WT | KO | WT | KO |
| The max amount of bacteria (OD value) | 0.200 | 0.090 | 0.003 | < 0.001 |
| The maximum specific growth rate | 0.017 | 0.008 | 0.001 | < 0.001 |

WT wild-type, KO knock-out, SEM standard error of the mean

Fig. 4 The content (mean and SD) of coenzyme F$_{420}$ of WT and KO M. ruminantium in different culture time. A the content of coenzyme F$_{420}$ of liquid; B the content of coenzyme F$_{420}$ per OD$_{600}$. SD standard deviation, WT wild-type, KO knock-out

Fig. 5 The concentration changes of methane production and hydrogen consumption in WT and KO cofD type M. ruminantium at 5 time points (12, 24, 36, 48, 96 h) (n = 3). WT wild-type, KO knock-out
hotspot. A previous study has compared the 16S rRNA gene sequences of ruminal archaea derived from different ruminants and dietary compositions in 14 studies, and found that 92.3% of archaea in the rumen were originated from 3 genera, among which the Methanobrevibacter accounted for 61.6% and was the dominant methanogen in the gastrointestinal tracts of most herbivorous animals (Janssen and Kirs 2008).

As a strictly anaerobic bacteria, anaerobic condition is the key point to the successful cultivation of M. ruminantium. In the current experiment, the medium was boiled and deoxygenated. The anaerobic operation station was used for subpackage, inoculation, and incubation; besides, oxygen concentration was monitored in real-time using an oxygen controller to ensure strictly anaerobic conditions. The methanogens bacteria which is classified to Methanobacillus is mostly distributed in the two clades, including Smithii-gottschalkii-millerae-thaurei clusters and Ruminantium-olleyae clusters, and The M. ruminantium belongs to Ruminantium-olleyae clusters (St-Pierre et al. 2015). The M. ruminantium, Methanobrevibacter thaueri, Methanobrevibacter smithii, and Methanospaera stadtmanae of the Methanobacillus have been confirmed to be the main methanogens in the bovine rumen (Jarvis et al. 2000; Zhou et al. 2009; Wright et al. 2007). Due to the short reproductive cycle of the fast-growing methanogens and residence time of ruminal chyme, it is more conducive to the establishment of fast-growing methanogens. In this study, the M. ruminantium entered a platform period at approximately 24 ~ 36 h. During the logarithmic growth phase, it consumed a large amount of H2, contributing to a lower level of hydrogen partial pressure in the rumen. Methanobacillus, tetrahydromethanopterin, coenzyme $F_{420}$, coenzyme M, HS-Coenzyme B, and phenazine are closely related to the CH4 production of the methanogenic bacteria (Garcia et al. 2000). Among them, coenzyme $F_{420}$ is a special low-potential electron carrier in high G+C gram-positive bacteria such as archaea and mycobacteria. In the process of CH4 production of methanogenic bacteria, coenzyme $F_{420}$ is involved in the hydrogen transfer reaction as a two-electron transporter. Its oxidation-reduction potential is $F_{420}H_2/F_{420} + 2e^- (-360 mV)$, lower than NAD(P)H/NAD(P) + 2$e^- (-320 mV)$ and FADH$2/FAD2e^- (-219 mV)$ (Purwantini and Mukhopadhyay 2009). The amino acid sequence of CoF enzyme is highly conserved among the archaea and bacterial organisms that produce coenzyme $F_{420}$, but only weak sequence homologues appear in the thallus that do not produce coenzyme $F_{420}$ (such as Bacillus) (Gorke et al. 2005). However, there are no other obligate anaerobic bacteria that contain coenzyme $F_{420}$ and other substances that emit fluorescence at 480 nm and excitation wavelength is 420 nm. Therefore, fluorescence microscopy to detect the fluorescence produced by colonies has become an important technique for the identification of methanogenic bacteria.

Gene knock-out is an essential biological method for studying gene function and has been widely used. Sendai et al. (2006) constructed a gene knock-out model of $\alpha$-1,3-galactosyltransferase on cattle by Cre/loxP method, which laid a foundation for application of multiple gene knock-outs in agricultural animals. Manabe et al. (2009) found that homologous knock-out of rinderpest virus could prevent bovine spontaneous prion encephalopathy and reduce the risk of bovine spontaneous encephalopathy. At the same time, study has also demonstrated that gene knock-out can block the formation of byproducts in microorganisms, thereby improving the yield and purity of the target product (Kim and Timmusk 2013). Gene knock-out provides new idea for biological research. However, few reports were carried out on the application of gene knock-out technology in the methanone-related studies. In this research, homologous recombination technology was successfully used to screen the mutant strain of M. ruminantium in which cofD gene was knocked out, and the knock-out efficiency of screening was verified by PCR and enzyme digestion. In the process of gene knock-out vector construction, the pEASY-T1 vector was selected as the cloning vector of the cofD gene. The pEASY-T1 vector contains the $\beta$-galactosidase gene (lacZ) and the ampicillin resistance gene. If an exogenous fragment is inserted into a multi-cloning site, the lacZ gene and $\beta$-galactosidase would be inactivated. Since white colonies were produced, blue-white screening of positive clones could be performed using Amp/IPTG/X-gal selection medium. The pUC18 was used as a plasmid backbone, and a tetracycline gene was used as a resistance gene to construct a knock-out vector for cofD gene. This vector that was transformed into the M. ruminantium can not be replicated but has the opportunity to recombine with homologous genes homologously and express the tetracycline resistance, which can be selected. In this study, the cofD knock-out strains were selected, and the expression of cofD gene and CoF enzyme were significantly lower than those of wild-type strains.

Consistent with Liu and Whitman (2008) study, the results of current research displayed that in the logarithmic growth phase of wild-type M. ruminantium, it consumed greater amount of H2, which benefited to maintain a relatively lower level of H2 partial pressure in the rumen. The growth of strains was slower after the cofD gene knock-out. Moreover, the reproductive ability of strains was significantly reduced and it entered the logarithmic growth phase earlier. After entering the logarithmic growth phase, its coenzyme $F_{420}$ content...
was significantly reduced, and only a small amount of H\textsubscript{2} was consumed to produce CH\textsubscript{4}. The F\textsubscript{420} is utilized in methanogens growth to oxidize their substrates. Thus, when the substrate is H\textsubscript{2}, the H\textsubscript{2} can be coupled to reduce F\textsubscript{420} by reducing F\textsubscript{420} dehydrogenase (Frh) (Vitt et al. 2014). With knock-out of cofD, the M. ruminantium cell’s redox driveability is reduced and lost partial ability to use H\textsubscript{2}, which may lead to a reduction in redox reaction of coenzyme F\textsubscript{420} pool. Coenzyme F\textsubscript{420} exists commonly in methanogens and its concentration in hydrogenotrophic methanogenic strain ranges from 100 to 400 mg/kg (Dolfing and Mulder 1985). The content of coenzyme F\textsubscript{420} is different in varying species of methanogens. In addition, coenzyme F\textsubscript{420} has been proved to be significantly higher in hydrogenotrophic methanogens than that in acetate-producing methanogens (Zábranská et al. 1985). In the study of M. machel\textsubscript{lae} ΔFrh mutants, it was found that Frh was necessary for the growth of methanogens under H\textsubscript{2}/CO\textsubscript{2} culture conditions (Kulkarni et al. 2009). The character of blue-green or green fluorescence under the 420 nm UV laser generated by coenzyme F\textsubscript{420} and methyl thio- phene compounds is used to identify the presence of methanogens. However, Dong et al. (2010) concluded that the fluorescence value of coenzyme F\textsubscript{420} can be used as an index to measure the activity of methanogens when upflow anaerobic sludge blanket reactor is used to treat waste water in soybean production. Some other studies on anaerobic fermentation found that the changes in the content of coenzyme F\textsubscript{420} can be used to determine the activity of methanogens (Dolfing and Mulder 1985; Wang et al. 2011). In the current study, the CofD enzyme expression that was the corresponding protein product of F\textsubscript{420} was significantly reduced after knock-out of cofD gene. Meanwhile, the synthesis of methanogenic coenzyme F\textsubscript{420} was significantly reduced, indicating that the knock-out of the cofD gene resulted in a slower reaction of catalyzing the condensation of LPFG and Fo to produce F\textsubscript{420} (Graupner et al. 2002). The results further demonstrated that the CofD enzyme was a key enzyme for the biosynthesis of coenzyme F\textsubscript{420}.

In summary, our results showed that the growth and proliferation ability of M. ruminantium was decreased after cofD knock-out, subsequently, the production capacity of CH\textsubscript{4} by utilizing H\textsubscript{2} in the M. ruminantium was reduced. The cofD gene knock-out reduced the expression of CofD enzyme and the synthesis of coenzyme F\textsubscript{420} in the M. ruminantium. Our result may provide new insights to clarify coenzyme F\textsubscript{420} and cofD gene involved in the methanogenic mechanisms that can affect CH\textsubscript{4} production by methanogen, helping to develop strategies to reduce CH\textsubscript{4} emissions from ruminants.

Supplementary Information
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Additional file 1 Fig. S1. Amplification of the cofD gene of M. ruminantium by using cofD primers. Fig. S2. The recombinant plasmid pEASY-T1-cofD was amplified using cofD-F and cofD-R as primers, and the transformants were identified by PCR. Fig. S3. PCR product sequencing of positive clones and comparison of similarities of cofD by Blast. Fig. S4. Identification of 9 monoclonal recombinant plasmids pUC8-cofD-tet selected by PCR using tet specific primers. Fig. S5. Double digestion of pUC8-cofD-tet recombinant plasmid DNA using EcoRI and HindIII, detection by 1% agarose gel electrophoresis. (DOCX 437 KB)

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Authors’ contributions
JM, XW, and ZW conceived and designed the research. JM, XW, TZ, CT, XZ, QF, BX, and LW performed the experiment. JM, XW, RH, and HZ analyzed the data. JM and XW wrote the original manuscript. JM, XW, and ZW reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data presented in this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with animals or human participants performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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