Metatranscriptomic Evidence for Magnetite Nanoparticle-Stimulated Acetoclastic Methanogenesis under Continuous Agitation

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ABSTRACT Conductive nanomaterials have been reported to accelerate methanogenesis by promoting direct interspecies electron transfer (DIET), while their effects seem to vary depending on operational conditions. The present study examined the effects of magnetite nanoparticles (MNPs) on methanogenesis from acetate by soil-derived anaerobic cultures under continuous agitation. We found that MNPs accelerated methanogenesis in agitated cultures, as has been observed previously for static cultures. Metabarcoding of 16S rRNA gene amplicons showed that *Methanosarcina* substantially increased in the presence of MNPs, while DIET-related *Geobacter* did not occur. Metagenomic and metatranscriptomic analyses confirmed the predominance of *Methanosarcina* in MNP-supplemented agitated cultures. In addition, genes coding for acetoclastic methanogenesis, but not those for hydrogenotrophic methanogenesis, were abundantly expressed in the dominant *Methanosarcina* in the presence of MNPs. These results suggest that MNPs stimulate acetoclastic methanogenesis under continuous agitation.

IMPORTANCE Previous studies have shown that conductive nanoparticles, such as MNPs, accelerate methanogenesis and suggested that MNPs facilitate DIET between exoelectrogenic bacteria and methanogenic archaea. In these methanogens, electrons thus obtained are considered to be used for hydrogenotrophic methanogenesis. However, the present work provides evidence that shows that MNPs accelerate DIET-independent acetoclastic methanogenesis under continuous agitation. Since most of previous studies have examined effects of MNPs in static or weakly agitated methanogenic cultures, results obtained in the present work suggest that hydraulic conditions definitively determine how MNPs accelerate methanogenesis. In addition, the knowledge obtained in this study is useful for engineers operating stirred-tank anaerobic digesters, since we show that MNPs accelerate methanogenesis under continuous agitation.

KEYWORDS anaerobic digestion, methanogen, electrochemically active bacteria, metagenomics, metatranscriptomics, bin genomes, bioelectrochemistry, electron transport, methanogenesis, syntrophy

Methanogenesis is a catabolic process in which methane and carbon dioxide are produced as the end products under anaerobic conditions (1). When complex organic matter is biologically converted to methane, diverse microbes, including fermentative bacteria and methanogenic archaea, establish cooperative metabolic interactions (1). Such methanogenic microbiomes can be used for anaerobic digestion of biomass wastes that generates methane as fuel gas (2). Although anaerobic digestion has been widely used in sustainable energy processes, further studies are necessary to improve its performance and stability (3).

Studies have shown that the bottleneck step in anaerobic digestion is the conversion of volatile fatty acids into methane during syntrophic interaction between fer-

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mentative bacteria and methanogenic archaea, where reducing equivalents, such as hydrogen and formate, are diffusively transferred between these microbes (4, 5). This interaction is termed interspecies electron transfer (IET), and studies have suggested that IET also occurs with electric currents via conductive pili (6) and/or conductive micro/nanomaterials (7) between exoelectrogenic bacteria and methanogenic archaea (termed direct IET [DIET]). Since these studies have shown that the supplementation of methanogenic microbiomes with conductive micro/nanomaterials accelerates methanogenesis (7), DIET is considered to be more efficient than diffusive IET. Based on these results, the use of conductive micro/nanomaterials is currently expected to be a promising technology for accelerating and stabilizing anaerobic-digestion processes.

Despite a number of successful laboratory demonstrations, however, further studies are considered necessary for deepening our understanding of how conductive micro/nanomaterials accelerate methanogenesis. This concern is based on results of recent studies; for instance, a study has found that carbon nanotubes accelerate methane production in pure and syntrophic cultures of methanogens and that this effect is attributable to negative redox potentials established by supplementing with carbon nanotubes (8). In addition, both positive (9) and negative (10) effects of carbon nanotubes on methanogenesis have been reported, while it has not yet been uncovered why such differences occur. We therefore hypothesize that environmental and operational conditions, such as hydraulic conditions, largely influence the effects of conductive micro/nanomaterials on methanogenic microbiomes. In the present study, in order to address this hypothesis, the effects of magnetite nanoparticles (MNPs) on methanogenesis were examined under continuous agitation, and the effects of MNPs on methanogenic microbiomes were compared to those observed previously under static conditions. Methanogenic microbiomes were analyzed by metabarcoding 16S rRNA gene amplicons and metagenomics/metatranscriptomics approaches.

RESULTS AND DISCUSSION

MNP-stimulated methanogenesis under agitation. A previous study has shown that MNPs accelerate methanogenesis in anaerobic cultures of rice paddy soil supplemented with acetate and promote the growth of bacteria affiliated with the family *Geobacteraceae* (7). In that study, methanogenic cultures were incubated under static conditions, in which soil and MNPs were settled at the bottom of vials (7). By reference to results of axenic cultures (11), it has been suggested that the accelerated methanogenesis in the presence of MNPs is attributable to the formation of electric conduits that facilitate DIET between *Geobacter* and *Methanosarcina*. Studies have also shown that when *Methanosarcina* is grown on DIET with *Geobacter*, this archaeon upregulates transcription of genes coding for electron transfer components that supply reducing power to the methanogenic pathway, such as $F_{420}H_2$ dehydrogenase and heterodisulfide reductase (12). Since these components are particularly necessary for CO_2 reduction, it has been concluded that methanogens utilize DIET for efficient hydrogenotrophic methanogenesis (12).

Given the wide use of stirred-tank reactors for the anaerobic digestion of organic wastes (13), the present study examined the effects of MNPs on methanogenesis in continuously agitated anaerobic cultures that were inoculated with rice paddy soil and supplemented with acetate (Fig. 1). As has been observed in a previous study of static cultures (7), methanogenesis from acetate under continuous agitation was accelerated by supplementing with MNPs (Fig. 1a and b). Specific growth rates for the methanogenic cultures were estimated from the methane formation curves (Fig. 1b), showing that the average rate for the cultures in the presence of MNPs was 1.4 times that for the cultures in the absence of MNPs (Fig. 1c). Similar levels of acceleration by MNPs have been reported for static cultures (7).

Microbiomes assessed by metabarcoding. Microbiomes established in methanogenic cultures in the presence and absence of MNPs under continuous agitation were analyzed by metabarcoding of 16S rRNA gene amplicons (Fig. 2). It was detected that microbes affiliated with the family *Methanosarcinaceae* were >2-fold more abundant in

FIG 1 Effects of MNPs on methanogenesis under continuous agitation. (a) Acetate concentration. (b) Methane concentration expressed as the mM equivalent, estimated by assuming that all methane was present in culture medium. (c) Specific growth rate as estimated from methane production.

the presence than in the absence of MNPs. In the presence of MNPs, *Methanosarcinaceae* were detected at the highest relative abundance (approximately 26% of the total). Substantial increases in response to the MNP addition were also observed for *Peptostreptococcaceae* (3.8-fold) and *Rhodocyclaceae* (1.9-fold). Unexpectedly, however, *Geobacteraceae* were detected as minor populations even in the cultures supplemented with MNPs, comprising only approximately 0.2% of the total.

Bacterial groups that were detected abundantly, albeit to lesser extents than *Methanosarcinaceae*, in the presence of MNPs included *Peptostreptococcaceae* and *Rhodocyclaceae*. These bacteria have also been detected in microbial fuel cells in previous studies (14, 15), and researchers have considered that these families include exoelectrogens. It is therefore likely that these microbes utilize MNPs as solid electron acceptors that may thereafter transfer electrons to other microbes.

This result is different from microbiome information obtained in the previous study on static cultures (7), in which the addition of MNPs resulted in substantial increases (~10-fold) of *Geobacteraceae*, comprising over 20% of the total in the presence of MNPs. In order to determine whether *Geobacteraceae* also occur abundantly under static conditions in the soil that was used in the above-described methanogenesis experiment under continuous agitation, the same soil was also incubated anaerobically under static conditions in the presence and absence of MNPs (see Fig. S1 in the supplemental material). As expected, supplementation with MNPs accelerated acetate consumption and methane formation under static conditions. In addition, *Geobacteraceae* became abundant (approximately 5.5% of the total) when the cultures were supplemented with MNPs. Taken together, these results suggested the possibility that

FIG 2 Effects of MNPs on microbiomes as assessed by metabarcoding of 16S rRNA gene amplicons. Each bar represents the means of values obtained from three independent cultures.

FIG 3 Cross mapping of contigs based on coverage values. Colors for contig dots represent phylogeny, while sizes represent length. Boxes indicate areas in which contigs were collected for assembling bin genomes.

the *Geobacter*-mediated DIET occurs under static conditions but not under agitated conditions.

Microbes detected by metagenomics/metatranscriptomics. In order to further our understanding of the effect of MNPs on methanogenic microbiomes under continuous agitation, comparative metagenomic and metatranscriptomic analyses were conducted for cultures actively producing methane in the presence or absence of MNPs. DNA and RNA were extracted from these methanogenic cultures and subjected to NovaSeq 6000 sequencing (see Table S1 in the supplemental material). Contigs were assembled from the mixture of all DNA reads in the six libraries, followed by the reconstruction of bin genomes by coverage-based cross mapping (Fig. 3) and subsequent selection. Consequently, we were able to reconstruct 27 bin genomes with completeness values of >60% (Table 1). Figure 3 shows the differential enrichment of these bin genomes under the two conditions (no addition versus MNP added). For instance, Mes1 is shown to be specifically enriched under the MNP-added condition.

Phylogenetic assignment using the BLASTP program identified four bin genomes (Mes1, Mes9, Mes13, and Mes16) as archaea affiliated with the order *Methanosarcinales*, while the others were affiliated with the domain *Bacteria* (Table 1). Although many bin genomes were reconstructed for bacteria, none was affiliated with the order *Desulfurimonadales*, which includes *Geobacter*. Phylogenetic analyses based on *mcr* genes (coding for methyl coenzyme M reductases) were conducted for three *Methanosarcinales* archaea, represented by bin genomes Mes1, Mes13, and Mes16 (the Mes9 bin genome contains no *mcr* gene). This analysis shows that the three bin genomes are affiliated with the genus *Methanosarcina* (see Fig. S2 in the supplemental material). These results demonstrate that *Methanosarcina* was the only methanogen that was detected as bin genomes.

The DNA and RNA RPKM (reads per kilobase per million mapped reads) values were used to estimate relative abundances and relative transcription levels for organisms represented by the bin genomes (Fig. 4). The figure shows that Mes1 represents the dominant microbe under the MNP-added condition, comprising approximately 80% of the total microbiomes. In addition, as assessed by the RNA-based analysis, Mes1 actively expressed genes under the MNP-added condition. This organism was, however, minor under the no-addition condition, indicating that MNPs specifically stimulated the growth of Mes1. It is noteworthy that the growth of Mes13 and Mes16, which were

TABLE 1 Bin genomes reconstructed in the present study

Bin ID	Taxonomy	Length (bp)	GC (%)	No. of contigs	No. of ORFs	DNA RPKM		RNA RPKM		Completeness (%)
						No addition	MNP added	No addition	MNP added	
Mes1	<i>Methanosarcinales</i>	3,865,823	41	48	3,516	220	613,717	1,218	160,522	94
Act2	<i>Actinomycetales</i>	2,078,021	68	221	2,079	11	16,289	0	460	91
Des3	<i>Desulfovibrionales</i>	4,138,709	65	296	3,530	74	25,104	936	5,105	76
Bac4	<i>Bacteroidales</i>	4,124,816	40	83	3,144	8	14,794	0	305	92
Clo5	<i>Clostridiales</i>	3,559,733	40	199	3,476	6	5,955	0	8,909	91
Des7	<i>Desulfovibrionales</i>	6,442,764	57	310	4,005	5	3,087	0	2,285	94
Bac8	<i>Bacteroidales</i>	3,865,220	38	3,110	265	41	1,132	17	77	62
Mes9	<i>Methanosarcinales</i>	2,893,516	38	133	2,564	43	245	115	1,834	82
Bac10	<i>Bacteroidales</i>	3,867,786	43	381	3,310	167,456	40,225	16,966	1,303	98
Rho11	<i>Rhodocyclales</i>	3,581,420	64	138	3,386	144,430	22,933	85,918	5,382	99
Ent12	<i>Enterobacteriales</i>	5,077,842	52	61	4,632	33,130	10,913	1,078	125	99
Mes13	<i>Methanosarcinales</i>	2,654,992	40	2,476	275	14,669	223	35,311	807	62
Ver14	<i>Verrucomicrobiales</i>	6,024,558	56	82	4,551	58,755	169	356,400	2,221	93
Bur15	<i>Burkholderiales</i>	4,202,088	68	16	3,868	170,001	99	35,806	0	99
Mes16	<i>Methanosarcinales</i>	4,555,019	41	555	4,364	6,404	1,447	4,397	4,129	86
Bac17	<i>Bacteroidales</i>	3,648,784	37	19	3,051	5,865	633	499	18	95
Clo18	<i>Clostridiales</i>	4,257,955	35	27	3,990	12,234	79	8,799	324	90
Xan19	<i>Xanthomonadales</i>	3,611,376	68	14	3,215	2,838	15	8,135	2	90
Fus20	<i>Fusobacteriales</i>	4,263,363	44	7	3,402	6,835	4	7,112	0	93
Pse21	<i>Pseudomonadales</i>	6,232,806	68	256	2,802	3,539	186	29,229	192	88
Clo22	<i>Clostridiales</i>	3,689,611	29	80	3,378	3,562	238	284	48	98
Des23	<i>Desulfovibrionales</i>	6,124,113	60	861	6,106	4,609	483	20,841	678	94
Clo24	<i>Clostridiales</i>	3,471,192	31	186	3,228	3,850	78	2,846	5	98
Clo25	<i>Clostridiales</i>	2,945,201	37	69	2,952	1,801	72	1,200	272	94
Clo27	<i>Clostridiales</i>	5,650,210	38	318	5,423	5	2,947	0	1,084	91
Act28	<i>Actinomycetales</i>	4,009,912	64	135	3,704	2	2,222	0	353	98
Pse30	<i>Pseudomonadales</i>	3,406,748	39	104	3,301	4,434	206	270	139	100

more abundant than Mes1 in the no-addition cultures, was shown to be suppressed by MNPs. Although it is unknown how only Mes1 organisms grow abundantly in the presence of MNPs, comparative analyses of the genomes and transcriptomes of the three *Methanosarcina* archaea may provide insights into relevant molecular mechanisms.

Methanosarcina was overrepresented in the metagenome-based abundance analysis (Fig. 4) compared to that detected by the metabarcoding analysis (Fig. 2), while these two analyses provided similar trends; namely, *Methanosarcina* was more abundant in the MNP-added cultures than in the no-addition cultures. A similar bias had also been observed in a previous study (16), in which methanogens were more abundantly detected by metagenomics than by metabarcoding. We deduce that this is due to

FIG 4 Relative abundances (DNA) and transcription levels (RNA) of bin genomes reconstructed from methanogenic microbiomes in the absence and presence of MNPs.

FIG 5 Methanogenic pathways (a) and transcription levels (RNA RPKM/DNA RPKM) of genes coding for the main methanogenic pathways in Mes1 (b).

preferential amplification of bacterial genes compared to archaeal genes in the PCR system with the primers used in these studies.

Some bacteria, including those of bins Bac10, Rho11, Ver14, and Bur15, were abundantly detected in the absence of MNPs, among which Ver14 was particularly active in terms of gene expression (Table 1 and Fig. 4). Among them, Bac10, Rho11, and Ver14 are related to *Porphyromonadaceae*, *Rhodocyclaceae*, and *Verrucomicrobia* subdivision 3, which were detected by metabarcoding (Fig. 2), while no Bur15 relative was detected. It is likely that these bacteria contribute to methanogenesis from acetate in the absence of MNPs, and one would assume that they are homoacetogens converting acetate into hydrogen and carbon dioxide under methanogenic conditions. It should, however, be noted that these bacteria constituted only minor populations in the presence of MNPs. These results suggest that the predominant *Methanosarcina* represented by the Mes1 bin genome singly converts acetate into methane under continuous agitation in the presence of MNPs.

Methanogenic pathways in operation in Mes1. In order to gain insights into methanogenic pathways that work in agitated cultures in the presence of MNPs, methanogenic pathways were reconstructed from the Mes1 bin genome, and the transcription levels of genes in these pathways were estimated from numbers of RNA reads mapped on respective genes. We focused on Mes1 in the presence of MNPs, since Mes1 was predominant and substantially increased in the MNP-added cultures (Fig. 4). We were unable to assess transcription levels of genes in Mes1 in the absence of MNPs, since sufficient numbers of reads were obtained neither from DNA libraries nor from RNA libraries. Transcription levels of genes putatively involved in catabolic processes, including methanogenic and electron transfer pathways, are summarized in Table S2 in the supplemental material, and data for the main methanogenic pathways are presented in Fig. 5. We found that, in addition to *mcr* and *mtr*, genes coding for the acetoclastic methanogenesis pathway (*ack*, *pta*, and *cdh*) were abundantly expressed in Mes1, while genes coding for enzymes in the hydrogenotrophic methanogenesis pathway were not substantially expressed (Fig. 5b). Furthermore, it was also found that most of the genes coding for electron transport components, including those shown to be upregulated in *Geobacter*/*Methanosarcina* cocultures (12), were not substantially transcribed in Mes1 in the presence of MNPs (see Table S2 in the supplemental material). These results, along with the abundance data (Fig. 4), indicate that the acetoclastic pathway mainly works for methanogenesis in the presence of MNPs under continuous agitation.

In previous studies, metatranscriptomic analyses have demonstrated that genes coding for hydrogenotrophic methanogenesis pathways are upregulated in methanogens in association with the occurrence of *Geobacter*, and these data are considered to support the idea that DIET is involved in methanogenesis (17, 18). In contrast, the metatranscriptomic data reported here suggest that MNPs stimulate acetoclastic methanogenesis, and we therefore conclude that MNPs did not promote DIET in agitated cultures. Recent work has demonstrated that MNPs adhere to cell surfaces and are partially incorporated into cells of *Methanosarcina barkeri*, where they facilitate acetoclastic methanogenesis (19). It has been deduced that MNPs serve as electron carriers that compensate for a methanophenazine-mediated step that transfers electrons from hydrogenases to heterodisulfide reductase and is considered to be rate-limiting in acetoclastic methanogenesis (19). Under continuous agitation, while *Methanosarcina* is unable to make close contact with *Geobacter*, MNPs attach to the surface of *Methanosarcina* cells and promote electron transfer for acetoclastic methanogenesis.

Conclusions. Comparative metabarcoding and metatranscriptomic analyses of methanogenic microbiomes established under continuous agitation in the absence or presence of MNPs show that MNPs stimulate acetoclastic methanogenesis. This finding provides novel implications for methanogenesis and anaerobic digestion, since mechanisms behind the effects of MNPs on methanogenesis under continuous agitation are entirely different from those considered for cultures under static conditions; as suggested in previous studies (7, 17), MNPs accelerate DIET-associated hydrogenotrophic methanogenesis under static conditions. Our finding suggests that effects of MNPs on methanogenic microbiomes vary depending on operational conditions, such as hydraulic conditions. It is therefore recommended that researchers should carefully describe experimental conditions in order to deepen our understanding of how conductive nanomaterials affect methanogenesis. In addition, results of the present study suggest that MNPs may be useful for enhancing performances of stirred-tank anaerobic digesters, in particular when methanogenesis stagnates in association with acetate accumulation.

MATERIALS AND METHODS

Methanogenic cultivation. Methanogenic cultivation was conducted in bottles (1 liter) containing 400 ml of acetate medium (pH 7.0) (19). This medium contained 10 mM NH_4Cl , 1 mM KH_2PO_4 , 0.5 mM MgCl_2 , 0.5 mM CaCl_2 , 5 mM NaHCO_3 , 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, and 0.05% (wt/vol) Bacto yeast extract (20) and was supplemented with acetate (30 mM) as a substrate. When initiating cultivation, it was inoculated with soil (30 mg liter⁻¹) and supplemented with MNPs at 1.5 g liter⁻¹. Soil was obtained from a rice paddy field (Noda, Japan) and stored at ambient temperature. MNPs were prepared as described elsewhere (21), and transmission electron microscopy indicated that an average particle size of MNPs was approximately ≤ 10 nm (see Fig. S3 in the supplemental material). Volatile fatty acids were not detected in soil used as the inoculum. A bottle was sealed with a butyl rubber and screw cap, and the headspace was filled with N_2/CO_2 (80/20). Cultivation was conducted at 37°C under continuous agitation at 100 rpm using a magnetic stirrer.

Methane, hydrogen, and carbon dioxide in the headspace were measured using a gas chromatograph as described previously (22), while acetate in the medium was analyzed using a liquid chromatograph as described elsewhere (23). Before analyses, the medium was filtered through a 0.22- μm -pore-size membrane.

DNA and RNA extraction. After methanogenic cultivation, cultures were centrifuged at $8,000 \times g$ for 5 min, and pellets were stored at -80°C . DNA was extracted using a FastDNA SPIN kit for soil (MP Biomedicals). DNA quality was assessed by agarose gel electrophoresis, spectrophotometric analysis, and Quant-iT dsDNA BR assay (Invitrogen). RNA was extracted using TRIzol reagent (Invitrogen) and purified using an RNeasy minikit and RNase-free DNase set (Qiagen). The quality of extracted RNA was evaluated using an Agilent 2100 Bioanalyzer with RNA 6000 Pico reagents and RNA Pico chips (Agilent Technologies).

Metabarcoding of 16S rRNA gene amplicons. Fragments of the V4 region in 16S rRNA genes were amplified from the extracted DNA using universal primers (24) according to protocols described elsewhere (16). In the forward primer, the rRNA gene sequence was connected to adaptor and tag sequences as described previously (24). PCR products were purified using a QIAquick PCR purification kit (Qiagen), and after DNA concentrations were determined by measuring the absorbance spectra, the samples were mixed at the same concentration and subjected to pair-end sequencing using a MiSeq sequencer (Illumina) according to a protocol recommended by the manufacturer. Sequence reads greater than 230 bp were collected, and chimeric sequences were detected and removed using USEARCH via the uchime command (25). Sequences were clustered into operational taxonomic units with 97%

similarity using QIIME (24) and taxonomically classified by aligning these values with sequences in the Greengenes database (26).

Metagenomic and metatranscriptomic analyses. Approximately 5 μ g of quality-checked DNA was used to construct paired-end and fragmented libraries and sequenced for 150 cycles using a NovaSeq 6000 sequencing system (Illumina) as described elsewhere (27). For RNA sequencing, approximately 5 μ g of quality-checked RNA was treated using the TruSeq RNA sample preparation kit V2 (Illumina) and sequenced for 101 cycles using the NovaSeq 6000 sequencing system. Prior to cDNA library preparation, rRNA was removed from the total RNA samples using the Ribo-Zero rRNA removal kit for bacteria (Epicentre, Madison, WI) according to the manufacturer's instructions.

DNA reads were trimmed using CLC Genomics Workbench version 6.5.1 (CLC Bio Japan). Quality-trimmed reads obtained from the MNP-added cultures and no-addition cultures (six samples, in total) were mixed and assembled into contigs with scaffolding based on paired-end information with a kmer size of 53 and bubble length of 800 bp (16). Contigs of >1,000 bp were used for subsequent gene prediction and binning analyses. Sequencing and assembly data are summarized in Table S1 in the supplemental material. RPKM values were calculated by mapping DNA or RNA reads to assembled sequences (bin genomes or open reading frames [ORFs]) using CLC Genomics Workbench with default settings, except for the use of 0.7 as the minimum length and 0.97 as the minimum similarity fractions. A normalized gene expression level for each gene (i.e., the mRNA/DNA ratio) was calculated by dividing the mRNA-RPKM for each ORF by the DNA-RPKM for the ORF.

Coding sequences in contigs were predicted using MetaGeneMark (28). Gene identification and annotation were performed by the KEGG automatic annotation server (29) using a single-directional best-hit method and a cutoff bit score of 45. Alignment of *mcrA* gene sequences and construction of neighbor-joining trees were conducted using MEGA program version 6 (30).

Contig clustering and draft genome reconstruction were conducted using a multistep process, including differential coverage binning, G+C content analysis, and tetranucleotide frequency analysis, according to methods described previously (16). The completeness of bin genomes was assessed by core-gene analysis using 107 marker genes for *Bacteria* and 137 marker genes for *Archaea* (16). Bin genomes were taxonomically assigned on the basis of the amino acid sequence homology of single-copy genes (*fsrY*, *rpsO*, *nusA*, *rpsB*, and *rpoB*) using the BLASTP program (31).

Data availability. Nucleotide sequences generated in this study have been deposited in the DDBJ Sequence Read Archive database under accession number [DRA008578](https://doi.org/10.1101/008578).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01733-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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R.I. and M.N. conducted experiments, A.K. designed the study and analyzed data, and K.W. wrote the manuscript and supervised the study. All authors read and approved the final manuscript. We declare no conflict of interest.

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