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Construction and Characterization of Shuttle Vectors for Succinic Acid-Producing Rumen Bacteria▽†

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Shuttle vectors carrying the origins of replication that function in *Escherichia coli* and two capnophilic rumen bacteria, *Mannheimia succiniciproducens* and *Actinobacillus succinogenes*, were constructed. These vectors were found to be present at ca. 10 copies per cell. They were found to be stably maintained in rumen bacteria during the serial subcultures in the absence of antibiotic pressure for 216 generations. By optimizing the electroporation condition, the transformation efficiencies of 3.0×10^6 and 7.1×10^6 transformants/μg DNA were obtained with *M. succiniciproducens* and *A. succinogenes*, respectively. A 1.7-kb minimal replicon was identified that consists of the *rep* gene, four iterons, A+T-rich regions, and a *dnaA* box. It was found that the shuttle vector replicates via the theta mode, which was confirmed by sequence analysis and Southern hybridization. These shuttle vectors were found to be suitable as expression vectors as the homologous *fumC* gene encoding fumarase and the heterologous genes encoding green fluorescence protein and red fluorescence protein could be expressed successfully. Thus, the shuttle vectors developed in this study should be useful for genetic and metabolic engineering of succinic acid-producing rumen bacteria.

The capnophilic rumen bacteria *Mannheimia succiniciproducens* and *Actinobacillus succinogenes* can produce high levels of succinic acid, which is an industrially important four-carbon dicarboxylic acid (5, 10–12). Although their use in commercial succinic acid production is still limited, mainly due to low productivity and by-product formation (12, 22), recent systematic gene knockout studies based on the complete genome sequence of *M. succiniciproducens* (6) have raised the expectation of increasing succinic acid productivity with reduced by-product formation (13). However, further metabolic engineering of *M. succiniciproducens*, including gene amplification, was not possible due to the lack of a gene expression system. As *M. succiniciproducens* is a poorly studied bacterium with respect to gene cloning, transformation, and gene expression, it is desirable to develop a shuttle vector system. Although the plasmid pMVSCS1 in *Mannheimia varigena* has been reported to be transformable into *Escherichia coli* JM107 (8), it has a limited value as a shuttle vector due to its low transformation efficiency in *E. coli*.

In this paper, we report the development and characterization of *E. coli*-rumen bacteria shuttle vectors. After several shuttle vectors were constructed, their basic characteristics, including transformation efficiency, plasmid copy number, and plasmid stability, were determined. The origin of replication was characterized in terms of its sequence and the mode of replication. Finally, these shuttle vectors were used for the overexpression of homologous and heterologous genes in *M. succiniciproducens*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *M. succiniciproducens* MBEL55E (KCTC 0769BP; Korean Collection for Type Cultures, Daejeon, Korea) and *A. succinogenes* sp. 130Z (ATCC 55618; American Type Culture Collection, Manassas, VA) were cultivated at 39°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) supplemented with 5 g/liter glucose. *M. succiniciproducens* and *A. succinogenes* were always cultured under anaerobic condition in an anaerobic chamber (Forma Scientific, Marjetta, OH) filled with a gas mixture of hydrogen, nitrogen, and carbon dioxide (volume ratio of 1:1:3). *E. coli* JM109 was cultivated at 37°C in Luria-Bertani medium containing 10 g/liter Bacto tryptone, 5 g/liter yeast extract, and 10 g/liter sodium chloride.

DNA manipulation and transformation. Restriction endonucleases, T4 DNA ligase, polymerase, and nuclease were purchased from New England Biolabs (Beverly, MA) and used as described by Sambrook et al. (19). Plasmid DNA was prepared using a GENErALL Plasmid SV miniprep kit (General Biosystem, Seoul, Korea). DNA fragments and PCR products were recovered using a QIAquick gel extraction kit and a QIAquick PCR purification kit (QIAGEN, Valencia, CA), respectively. The primer sequences for gene cloning are listed in Table 2.

E. coli was transformed by electroporation (3) or by the heat-shock method (19). Transformation of rumen bacteria *M. succiniciproducens* and *A. succinogenes* was carried out under anaerobic condition, as follows. Cells grown to the optical density at 600 nm (OD_{600}) of 0.4 to 0.6 at 39°C under an anaerobic condition were harvested by centrifugation at 3,100 × *g* for 10 min (SUPRA22K model; Hanil Science Industrial, Inchon, Korea). The cell pellet was washed three times with cold (4°C) 10% (vol/vol) glycerol solution and finally resuspended in 10% (vol/vol) glycerol solution to give 2.5×10^{10} cells per milliliter.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
M. succiniciproducens		
MBEL55E	Wild-type strain (KCTC 0769BP)	11
A. succinogenes		
130Z	Wild-type strain (ATCC 55618)	4
E. coli		
JM109	Host strain for cloning	Stratagene[a]
Plasmids		
pMVSCS1	Plasmid isolated from *M. varigena*	7
pKK223–3	*E. coli* expression vector	Amersham[b]
pUC18	*E. coli* cloning vector	Amersham
pGFPuv	GFPuv expression vector	Clontech[c]
pDsRed2	DsRed2 expression vector	Clontech
pKKD	pKK223–3 derivative; pBR322 *ori*; Amp^r	This study
pMVD	*E. coli*-rumen bacteria shuttle vector from pMVSCS1 and pKKD; Amp^r Cm^r Sul^r Str^r	This study
pME	pMVD derivative lacking the 2,369-bp NcoI fragment; Amp^r	This study
pMEx	*M. succiniciproducens* expression vector; pME containing the *M. succiniciproducens pckA* promoter; Amp^r	This study
pME18	pUC18 containing the 2.0-kb pMVSCS1 replicon fragment: Rep^+, $iteron^+$, AT $repeat^+$, putative *dnaA* box^+; Amp^r	This study
pME18RIA	pUC18 containing the 1.7-kb pMVSCS1 replicon fragment: Rep^+, $iteron^+$, AT $repeat^+$, putative *dnaA* box^-; Amp^r	This study
pME18RI	pUC18 containing the 1.3-kb pMVSCS1 replicon fragment: Rep^+, $iteron^+$, AT $repeat^-$, putative *dnaA* box^-; Amp^r	This study
pMEFUMC	pME containing the *M. succiniciproducens fumC* gene	This study
pOS1	*E. coli* cloning vector containing pMB1 *ori*; Amp^r	O. Kwon (unpublished)
pMS1	pOS1 derivative containing the *M. succiniciproducens frdA* promoter	This study
pMS3	pMS1 derivative containing the 1.7-kb pMVSCS1 replicon fragment	This study
pMS3-G	pMS3 derivative containing the *gfpuv* gene	This study
pMS3-R	pMS3 derivative containing the *rfp* gene	This study

[a] La Jolla, CA.
[b] Little Chalfont, Buckinghamshire, United Kingdom.
[c] Mountain View, CA.

The competent cells (95 μl) were mixed with 2 μl of plasmid DNA solution (containing 0.1 to 1.6 μg of plasmid DNA). Electroporation was performed using a Gene Pulser II (Bio-Rad, Richmond, CA) and a 0.1-cm electrode gap cuvette (Bio-Rad). The transformed cells were immediately mixed with 900 μl of the TSB (Difco Laboratories) medium supplemented with 5 g/liter glucose and incubated at 39°C for 0.5 h. Recombinant cells harboring the plasmid were selected on a trypticase soy agar (TSA; Difco Laboratories) plate containing suitable antibiotics at 39°C in an anaerobic chamber (Forma Scientific).

Determination of the plasmid copy number. Quantitative real-time PCR (qPCR) amplification was carried out using an iCycler IQ instrument (Bio-Rad). qPCRs were conducted in 200-μl PCR tubes containing 25 μl of reaction mixture. The concentration of template DNA was determined by measuring the absorbance at 260 nm with a UV/Vis spectrophotometer (Ultrospec 3000; Pharmacia Biotech., Uppsala, Sweden). DNA samples showing an $OD_{260/280}$ of 1.8 to 2.0, thus indicating minimum protein contamination, were used. The primer sequences for qPCRs are listed in Table 2. The Sau3AI-digested total DNA extracts were serially diluted (0.01, 0.1, and 1 ng per reaction) and were analyzed using 0.4 μM (final concentration) of the relevant forward and reverse primers. Each reaction mixture contained 8.5 μl of template DNA, 12.5 μl of 2× QuantiTech SYBR green PCR master mix (QIAGEN), and 4 μl of forward and reverse primer mixture. The qPCR reactions were initiated by 15 min of incubation at 95°C (hot-start *Taq* DNA polymerase activation), followed by 40 cycles of 94°C for 15 s (denaturation), 55°C for 30 s (primer annealing), and 72°C for 30 s (elongation). Fluorescence data were recorded after the elongation steps. After the completion of 40 cycles, the temperature was steadily raised from 55 to 94°C for 20 min (dissociation), during which the fluorescence signal was continually monitored for melting curve analysis. At the melting temperature, a rapid decrease of fluorescence could be detected; this results in a peak in the dissociation curve which plots the first derivative of fluorescence signal against temperature. The qPCR primer sequences and the melting temperatures of the amplified products are listed in Table 2. Template-free negative controls were used to estimate nonspecific binding. All experiments were carried out in triplicate. The copy number was calculated from the threshold cycle (C_T). ΔC_T is the difference between the mean C_T value of the single-copy reference and the mean C_T value of the plasmid *ori* amplicon whose copy number is being calculated. The *M. succiniciproducens fumC* (GenBank accession no. NC006300) and *A. succinogenes pckA* (GenBank accession no. AY308832) genes were used as single-copy references. ΔC_T was determined by comparing the *y*-axis intercepts from linear fit in plots of C_T versus template concentration (18, 20).

Determination of plasmid stability and antibiotic sensitivity. Plasmid stability was examined using the cells harboring the plasmid pMVSCS1 or pMEx. A single colony was inoculated into the TSB medium without selection pressure and cultured at 39°C. At the OD_{600} of 1.2, an appropriate volume of culture broth was serially transferred into a fresh medium (400× dilution in every eight generations) and cultivated further. Aliquots were taken during the serial cultures, diluted appropriately, and spread on a TSA plate with and without the corresponding antibiotics. After colony formation, the numbers of colonies on the TSA plates with and without the antibiotics were compared for estimating plasmid stability. Colonies were counted in triplicates for each strain by using Quantity-One software (Bio-Rad). Also, plasmid minipreparation and restriction mapping experiments were performed for several independent colonies to confirm the presence of plasmids and to examine the structural plasmid stability.

Antibiotic susceptibility tests were performed by adopting a broth dilution method suggested by the National Committee for Clinical Laboratory Standards (now Clinical and Laboratory Standards Institute [CLSI]) (16). TSB medium supplemented with antibiotics ranging from 0.03 to 8,192 μg/ml were loaded into each well of a 24-well plate. Then, the cultivated *M. succiniciproducens* and *A. succinogenes* organisms were inoculated into each well. The well plates were incubated in an anaerobic chamber (Forma Scientific) filled with a gas mixture of hydrogen, nitrogen, and carbon dioxide (volume ratio of 1:1:3) at 39°C for 48 h.

TABLE 2. Oligonucleotide primers used in this study

Primer pair	Sequence (5′ to 3′)[a]	Product size (bp) and T_m[b]	Target	Comment(s) and restriction site(s)
Ori-F	GGCCCCAAGCTTTTGCCAAATGTTCTTCTTC	1,991	pMVSCS1	Amplification for pME18; HindIII
Ori-R	GGCCCCAAGCTTGCGGTCGATCAAAAAAC		*ori*	HindIII
Ori-F	GGCCCCAAGCTTTTGCCAAATGTTCTTCTTC	1,714	pMVSCS1	Amplification for pME18RIA; HindIII
Ori-A-R	AAAACTGCAGCGTAATGATACTACTGCTCACC		*ori*	PstI
Ori-F	GGCCCCAAGCTTTTGCCAAATGTTCTTCTTC	1,260	pMVSCS1	Amplification for pME18RI; HindIII
Ori-I-R	GGCCCCAAGCTTGAGATTTTAACGACTACAAATT		*ori*	HindIII
Ppck-F	CAAATTAACCGAAGATCTACATCACCTCATAAAA TAAATTAAAA	348	*pckA*	Amplification for pMEx; BglII
Ppck-R	CGCGGATCC CGGCAATCGAGGTATTTGTATA		Promoter	BamHI
TTpck-F	CCATCGATAGCCTGATAACTTCACCAACCT	260	*pckA* TT[c]	Amplification for pMEx; ClaI
TTpck-R	ATGAGGTGATGTAGATCTTCGGTTAATTTGATTC AATCCT			BglII
PfrdA-F	CCACAGCTGGACGTCTATTCTGTTGGCTAATGC	511	*frdA*	Amplification for pMS1; PvuII, AatII
PfrdA-R	CCCGAATTCCTCCCCAGTAGAGTTGAT		Promoter	EcoRI
MsMU	CTCGACGTCGTTCTTCTTCGGTCACAG	1,693	pMVSCS1	Amplification for pMS3; AatII
MsMD	CTCGACGTCTACTACTGCTCACCGTAG		*ori*	AatII
FumC-F	CGGGATCCTCGGCTTTGCCCACATTAGC	1,772	*fumC*	Amplification of strain MBEL55E *fumC*; BamHI
FumC-R	CGGGATCCCATCCGCTGCGGTTTTGTAA			BamHI
GFPuv-F	GGCGAATTCATGAGTAAAGGAGAAGAAC	652	*gfpuv*	Amplification for pMS3-G; EcoRI
GFPuv-R	GGCTCTAGAGGATCCTTATTTGTAGAGC			XbaI-BamHI
RFP-F	AATGAATTCCATATGGAGGGCACCGTGAACG	741	*rfp*	Amplification for pMS3-R; EcoRI-NdeI
RFP-R	AATTCTAGAGTCGCGGCCGCTACAG			XbaI
qM-fum1	GATATTCGTTTATTGGCATCCGG	170	*fumC*	qPCR primers; single-copy reference for *Mannheimia* chromosome
qM-fum2	GCGAATGAAATGGTGGTATCGT	83.0°C		
qA-pck1	TGCGGAAGCTTATTTGGTGAAC	170	*pckA*	qPCR primers; single-copy reference for *Actinobacillus* chromosome
qA-pck2	CAACACCCGGTAATGCTTTAGG	81.5°C		
qori1	GGATTAAACAACCGTAGGGCGT	170	*ori*	qPCR primers; detection of the copy no. of the pMEx and pMVSCS1
qori2	GTTGGTAGCTATCCCCGACCTT	78.5°C		

[a] Underlined sequences are the restriction sites.
[b] Melting points (T_m) were determined based on melting curve analyses following qPCRs.
[c] TT, transcription terminator.

The MIC was determined as the lowest concentration of the antibiotic that inhibited cell growth.

Determination of the plasmid *ori* and replication mechanism. The GC skew analysis (14, 17) was performed to predict the origin of replication of the plasmid pMVSCS1. The fragment containing the predicted origin of replication was obtained by PCR as follows. DNA polymerase *Pfu* (Solgent, Daejeon, Korea) was used for PCR. The 2-kb length of putative origin was obtained by Ori-F and Ori-R primers (Table 2) using pMVSCS1 as a template. After the PCR product was digested with HindIII, the fragment was cloned into the HindIII site of pUC18. The smaller putative origin fragments of 1.7 and 1.3 kb were amplified by PCR with Ori-F/Ori-A-R and Ori-F/Ori-I-R primer pairs (Table 2), respectively, and subcloned into pUC18 to determine the minimum size of the replicon. These recombinant plasmids were transformed into *M. succiniciproducens*, and the transformants were screened on the TSA plates containing 5 μg/ml ampicillin.

Southern blots were performed to determine the replication mechanism. Cells at exponential phase were harvested, and their total DNA was extracted. DNA samples were subjected to 0.8% (wt/vol) agarose gel electrophoresis and blotted on nitrocellulose membranes (Bio-Rad) with or without denaturation. Probe labeling and fluorescence detection were performed with a Gene Image random prime labeling kit (Amersham Biosciences, Buckinghamshire, United Kingdom) and a Gene Image CDP-Star detection module (Amersham Biosciences), respectively.

Cloning of the *M. succiniciproducens fumC* gene and its expression. The *M. succiniciproducens fumC* gene, including its promoter and transcription terminator, was amplified by PCR with the primers FumC-F and FumC-R (Table 2) using the chromosomal DNA as a template. *Pfu* DNA polymerase (Solgent) was used for PCR. The resulting *fumC* gene and the shuttle vector pME were digested with BamHI and ClaI/BamHI, respectively. After they were made blunt ended by using T4 DNA polymerase, they were ligated to construct pMEFUMC. The sequences of the cloned DNA were determined by using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster, CA). The pMEFUMC construct was transformed into *M. succiniciproducens* and *A. succinogenes* for the expression of the *fumC* gene encoding fumarase.

Fumarase activity assay. Recombinant cells harboring pMEFUMC or pME (as a control) were harvested at the late exponential phase, washed twice with a buffer solution (100 mM Tris-HCl, 20 mM KCl, 5 mM $MnSO_4$, 2 mM dithiothreitol, 0.1 mM EDTA [pH 7.0]), and resuspended in the same buffer solution (5×10^{10} cells per milliliter). Cells were disrupted by sonication (Vibra Cell, Microprobe CV26; Sonic and materials, Newtown, CT) by applying 150 pulses of 1 s each at 20 amplitude for 10 min at 0°C. Cell debris was removed by centrifugation for 10 min at 16,000 × *g* at 4°C, and the supernatant was used for the enzyme assay. The cell extract (10 μl) was added to a 1-cm path cell (Thermo Electron Corporation, Aurora, CO) containing 990 μl of reaction buffer (0.1 M HEPES-KOH, 50 mM L-malate [pH 8.0]). Fumarase activity was measured by observing the appearance of fumarate at 240 nm, using a temperature-controlled spectrophotometer (Spectramax M2; Molecular Devices, San Francisco, CA), with the extinction coefficient of 2.53 $(mM \cdot cm)^{-1}$. Protein concentrations were measured by the Bradford method with bovine serum albumin as a standard (1). The fumarase activity of 1.0 U was defined as the amount of enzyme required for converting 1 nmol of L-malate to fumarate at 37°C per min per milligram of protein.

Cloning of the *Aequorea victoria gfp* gene and the *Discosoma* sp. *rfp* gene. The *Aequorea victoria gfp* gene was PCR amplified with the primers GFPuv-F and GFPuv-R, using pGFPuv (Clontech, Mountain View, CA) as a template. PCR products were purified, digested with EcoRI and XbaI, and inserted into EcoRI-XbaI-digested pMS3 to make pMS3-G. The *rfp* gene was PCR amplified with the primers RFP-F and RFP-R, using pDsRed2 (Clontech) as a template. PCR products were purified, digested with EcoRI and XbaI, and inserted into EcoRI-XbaI-digested pMS3 to make pMS3-R.

Fluorescence microscopy and image processing. The expression of the jellyfish *A. victoria* green fluorescence protein (GFPuv) and the *Discosoma* sp. red fluorescence protein 2 (DsRed2) was monitored by confocal microscopy (model LSM 510 META; Carl Zeiss GmbH, Oberkochen, Germany) equipped with a 103-W mercury lamp and a plan apochromat 63×/1.4-numerical aperture oil differential interference contrast objective lens. Fluorescence filter sets were HFT488 (excitation), NFT490 (beam splitter), and BP505-530 (emission) for GFPuv and HFT543 (excitation), NFT545 (beam splitter), and LP560 (emission) for DsRed2. Images were analyzed using Zeiss LSM Image Browser version 3.2.0.70 (Carl Zeiss).

RESULTS AND DISCUSSION

Construction of shuttle and expression vectors. The first shuttle vector, pMVD, was constructed as follows. Plasmid pKK223-3 was partially digested with BamHI and AccI to obtain a 2.7-kb fragment containing the pBR322 replication *ori* and the *bla* gene. The overhangs of the fragments were removed by treating them with mung bean nuclease to make blunt ends and were self-ligated to make pKKD. Then, pMVSCS1 (5.6 kb) and pKKD (2.7 kb), digested with XhoII and BamHI, were ligated to construct an 8.3-kb shuttle vector, pMVD (Fig. 1). Thus, pMVD has the origins of replication functioning in both *M. succiniciproducens* and *E. coli*, the pBBR332 *ori*, and the four antibiotic resistance genes against ampicillin, chloramphenicol, streptomycin, and sulfonamide.

In order to assess whether the *bla*, *cat*, and *strAB* genes could be used as selection markers in rumen bacteria, recombinant *M. succiniciproducens* and *A. succinogenes* harboring pMVD were cultivated in TSB medium containing different concentrations of ampicillin, chloramphenicol, and streptomycin. As shown in Table 3, both strains harboring pMVD exhibited increased resistance to these antibiotics. All three antibiotic markers were found to be suitable in both bacteria. Considering the relatively lower MIC, we decided to use the *bla* gene and remove all the other antibiotic resistance genes to reduce the size of the shuttle vector. Plasmid pME (6.0 kb) was constructed by removing the *sulIII*, *catAIII*, and *strA* genes from pMVD by NcoI digestion, followed by self-ligation (Fig. 1).

Then, two expression vectors, pMEx (6.2 kb) and pMS3 (4.3 kb), were constructed. The vector pMEx was derived from pME by cloning the *M. succiniciproducens pckA* promoter and terminator sequences at the BamHI and ClaI sites of pME (Fig. 1). The *M. succiniciproducens pckA* promoter and transcription terminator sequences were PCR amplified from the chromosomal DNA by using the primer pairs Ppck-F and Ppck-R and TTpck-F and TTpck-R, respectively. The second overlapping PCR was performed with the mixture of both PCR products having overlapping sequences by using the primers TTpck-F and Ppck-R. The purified target products were digested with BamHI and ClaI and inserted into the BamHI-ClaI-digested pME. For the expression of homologous or heterologous genes, the shuttle expression vector pMS3 was constructed using the minimal replicon of pMVSCS1, as follows. The *M. succiniciproducens frdA* promoter was PCR amplified from the chromosomal DNA by using the primers PfrdA-F and PfrdA-R. The PCR products were purified, digested with PvuII and EcoRI, and cloned into the PvuII-EcoRI-digested *E. coli* vector pOS1, which contains the pMB1 origin of replication, the *bla* gene, the multiple cloning sites, and the *rrnBT2* transcription terminator, to make pMS1. The minimal pMVSCS1 replicon (1.7 kb) was PCR amplified by using the primers MsMU and MsMD. The PCR products were purified, digested with AatII, and ligated with the AatII-digested pMS1 to construct pMS3 (Fig. 1).

Transformation efficiency. The transformation efficiencies of pMEx were examined for *M. succiniciproducens* and *A. succinogenes* under various electroporation conditions, as follows: electric field strength, 6 to 25 kV; resistance, 100 to 800 Ω; capacitance, 3 to 50 μF; and DNA amount, 0.1 to 1.6 μg. The optimal electroporation conditions were determined to be 25 kV/cm, 400 Ω, 25 μF, and 0.1 μg of DNA for *M. succiniciproducens*; and 25 kV/cm, 400 Ω, 50 μF, and 0.1 μg of DNA for *A. succinogenes*. Using the plasmid pMEx isolated from *E. coli*, transformation efficiencies of 3.0×10^6 and 7.1×10^6 transformants/μg DNA were obtained with *M. succiniciproducens* and *A. succinogenes*, respectively. Using the plasmid pMEx isolated from each rumen bacterium, transformation efficiencies of 2.3×10^6 and 4.2×10^6 transformants/μg DNA were obtained with *M. succiniciproducens* and *A. succinogenes*, respectively. These results, showing similar transformation efficiencies using the plasmid DNA isolated from *E. coli*, *M. succiniciproducens*, and *A. succinogenes*, suggest that *M. succiniciproducens* and *A. succinogenes* possess restriction and modification systems compatible with *E. coli* JM109.

Plasmid copy number. Plasmids can be categorized as low-copy (1 to 10 copies)-, medium-copy (11 to 20 copies)-, or high-copy (>50 copies)-number plasmids. The plasmid copy number is an important factor in metabolic engineering as it affects the expression level of the cloned gene by gene dosage

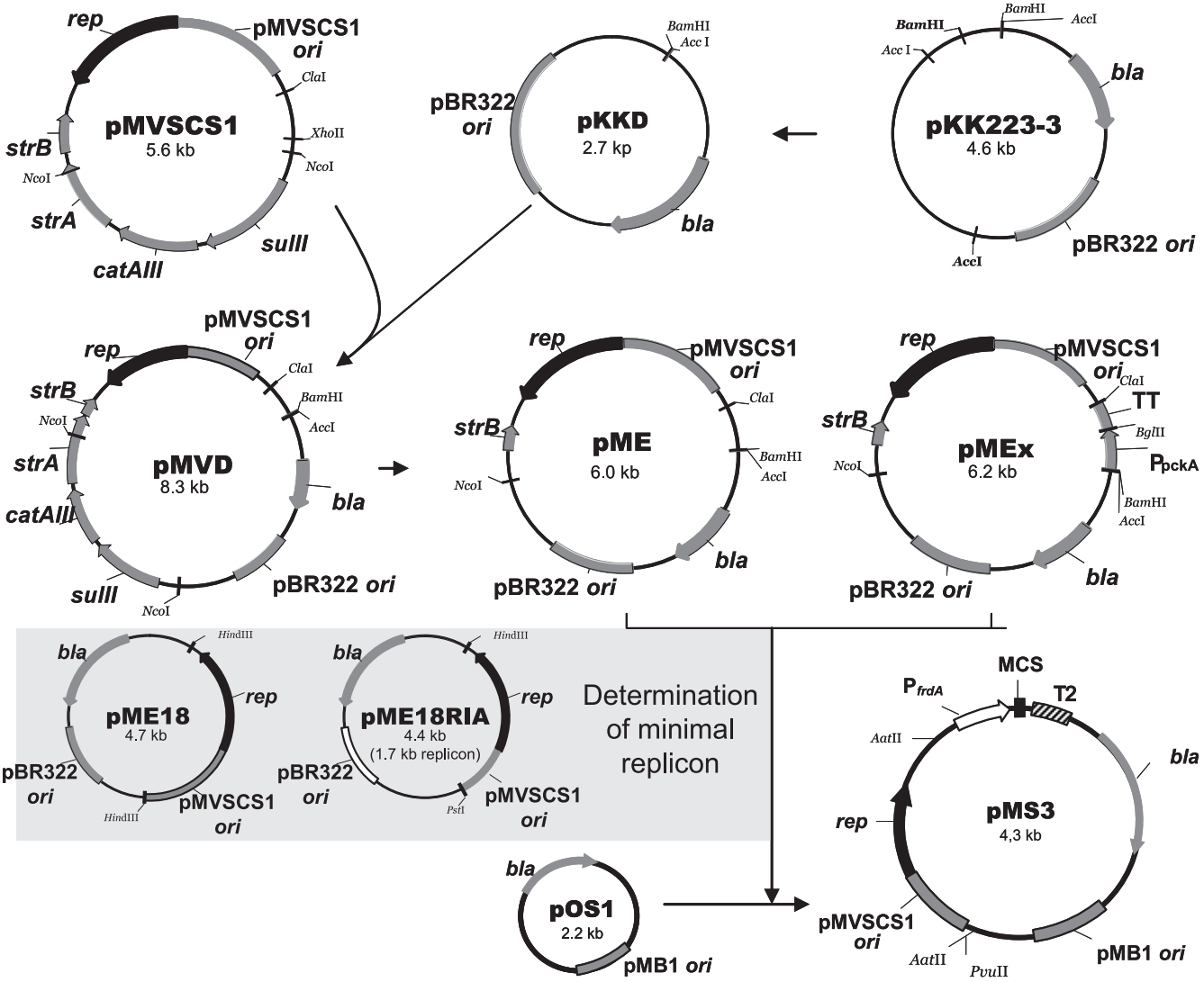


FIG. 1. *E. coli*-rumen bacteria shuttle vectors constructed in this study. The shuttle vectors contain both ColE1 *ori* for *E. coli* and pMVSCS1 *ori* for rumen bacteria. Genes are represented by arrows: *strAB*, streptomycin resistance gene; *catAIII*, chloramphenicol resistance gene; *sulII*, sulfonamide resistance gene; *bla*, ampicillin resistance gene. The *pckA* promoter (P_{pckA}) and transcription terminator (TT) were introduced in pMEx for gene expression. The shuttle vector pMS3 contains the *M. succiniciproducens frdA* promoter and the *rrnBT2* terminator (T2) for gene expression. Multiple cloning sites (MCS) of pMS3 are 5′-EcoRI-SacI-KpnI-SmaI-BamHI-XbaI-SalI-PstI-SphI-HindIII-3′.

effect and exerts a metabolic burden on the cell (21). Internal standard qPCR was used to determine the copy numbers of pMVSCS1 and pMEx in *M. succiniciproducens* and *A. succinogenes*. The relative positions of the targets amplified by qPCR are shown in Fig. 2A. As shown in Fig. 2B, the samples of three different template concentrations were easily distinguishable. For negative controls of the plasmid and genomic DNA, we obtained either a high C_T value (37.2 ± 0.28 for the qM-fum1

TABLE 3. Determination of MICs for *M. succiniciproducens* and *A. succinogenes*

Antibiotics[a]	Gene(s)	Plasmid	MICs determined for[b]:				
			M. succiniciproducens		*A. succinogenes*		*M. varigena* (using plasmid pMVSCS1)[c]
			Wild type	Using plasmid pMVD	Wild type	Using plasmid pMVD	
Amp	*bla*	pKK223-3	0.25	512	0.5	256	ND
Cm	*catAIII*	pMVSCS1	0.5	256	1	128	64
Str	*strAB*	pMVSCS1	8	>8,192	32	4,096	128

[a] Amp, ampicillin; Cm, chloramphenicol; Str, streptomycin.
[b] Wild-type strains were tested without plasmid. ND, not determined.
[c] MIC results reported by Kehrenberg and Schwarz (7).

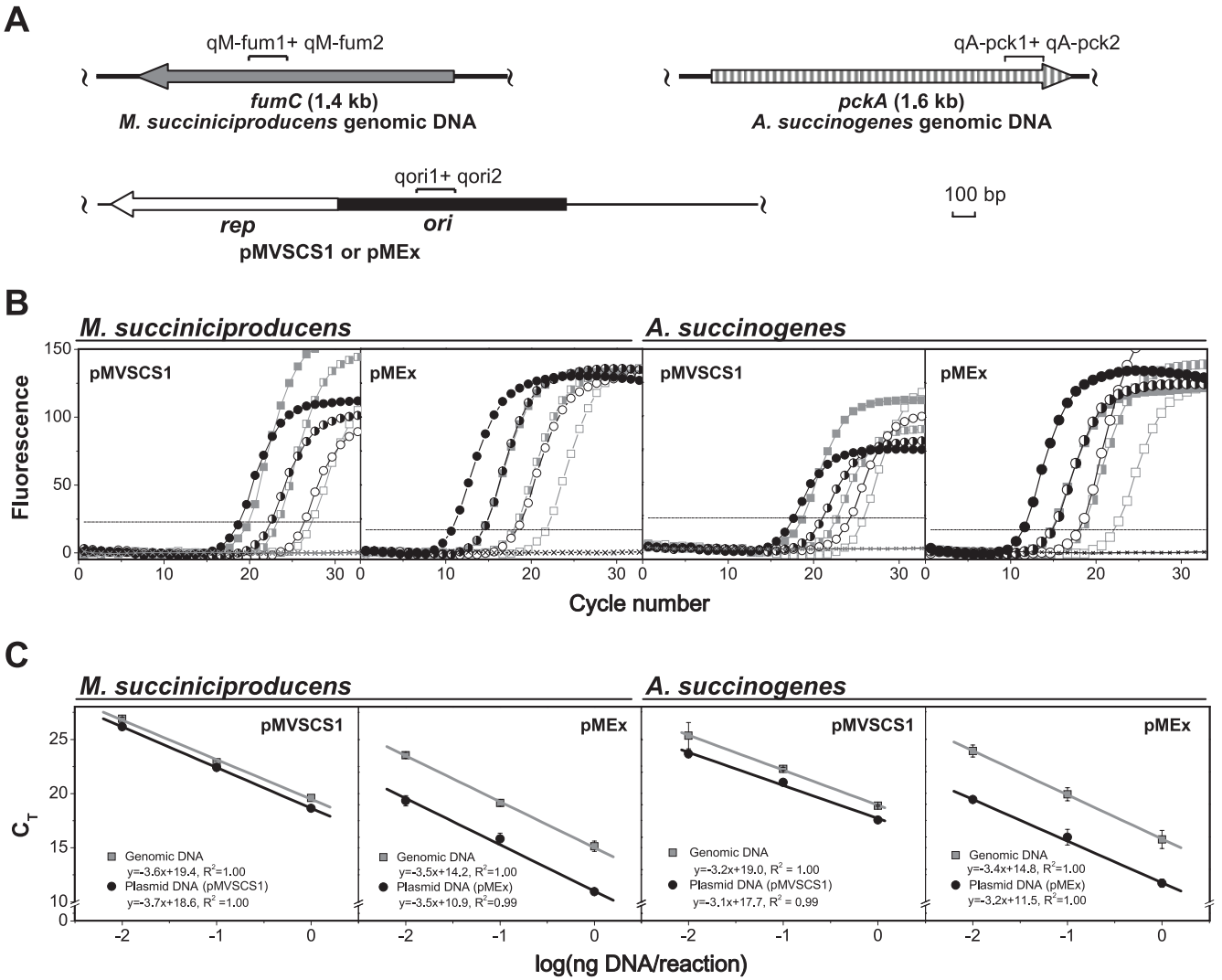


FIG. 2. Determination of plasmid copy numbers by qPCR. (A) Physical maps of the targeted genes for qPCR. The *M. succiniciproducens fumC* (gray arrow) and the *A. succinogenes pckA* (line arrow) genes were used as the single-copy references for *M. succiniciproducens* and *A. succinogenes*, respectively. The replication origin (black) of pMVSCS1 (or pMEx) was the target gene for qPCR. (B) Fluorescence versus cycle number curves for 0.01 (open symbol), 0.1 (semiopen symbol), and 1 ng (closed symbol) of enzyme-digested total template DNA. The lines in the graphs are as follows: black solid, plasmid; gray solid, chromosome of rumen bacteria. (C) C_T versus log concentration curves. Error bars represent the standard deviations of data obtained from the experiments performed in triplicate.

and qM-fum2 primer pair) or no amplification (for the qA-pck1 and qA-pck2 and the qori1 and qori2 primer pairs). These values are far from C_T values ranging from 11 to 27 where DNA samples were typically detected (Fig. 2B), which indicated that background amplification was negligible. The amounts of DNA templates were varied from 0.01 to 1 ng to find the linear dynamic range of template that could be detected and quantified (Fig. 2C). Before determining the linear dynamic range, the PCR amplification efficiency was evaluated from the absolute gradient value of C_T versus log(ng of DNA/reaction) curve. Theoretically, PCR efficiency or the slope of the standard curve should be computed as the absolute gradient 1/log2 (3.322). In other words, for a 10-fold difference in template amount, a C_T value of 3.322 cycles is expected. As shown in Fig. 2C, the absolute gradients of the curves for targeted plasmid and genomic DNA PCR products were 3.7 and 3.6 for *M. succiniciproducens* harboring pMVSCS1, 3.5 and 3.5 for *M. succiniciproducens* harboring pMEx, 3.1 and 3.2 for *A. succinogenes* harboring pMVSCS1, and 3.2 and 3.4 for *A. succinogenes* harboring pMEx, showing an average 5% difference from the theoretical value. Then, the plasmid copy numbers of pMVSCS1 and pMEx were calculated from ΔC_T, which was determined by comparing the *y*-axis intercepts from linear fit in plots of C_T versus template concentration (Fig. 2C). The results show that the copy numbers of pMEx in *M. succiniciproducens* and *A. succinogenes* were 9.9 and 9.9, respectively. These values are higher than those of its parental plasmid pMVSCS1, 1.7 copies in *M. succiniciproducens* and 2.5 copies in *A. succinogenes*. These results suggest that the copy number control system in pMVSCS1 might have been altered during partial deletion and fusing with the pKK223-3 fragment, which deserves further study.

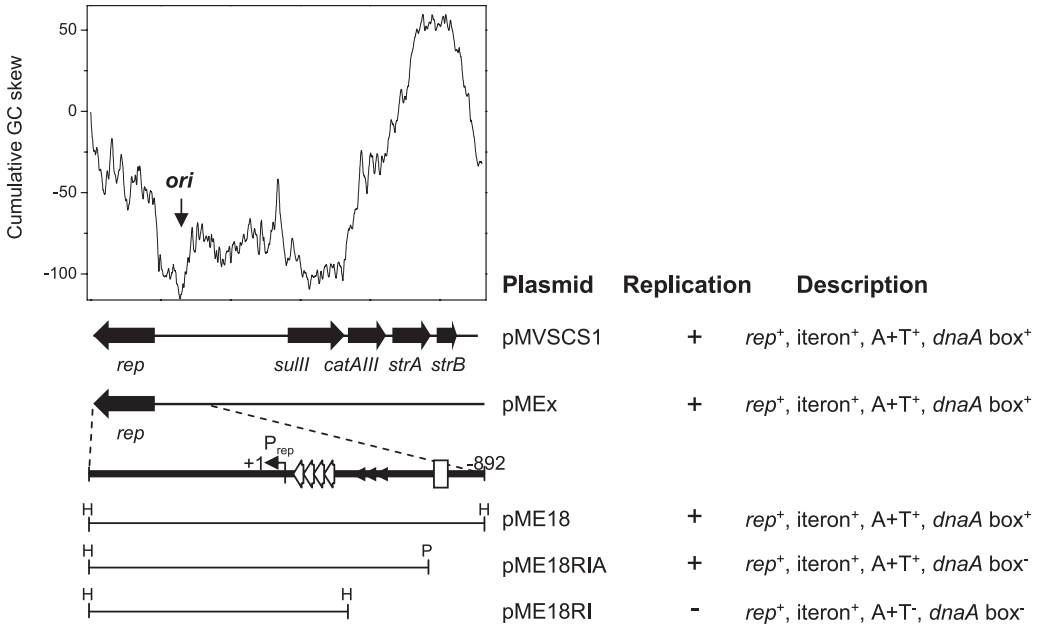
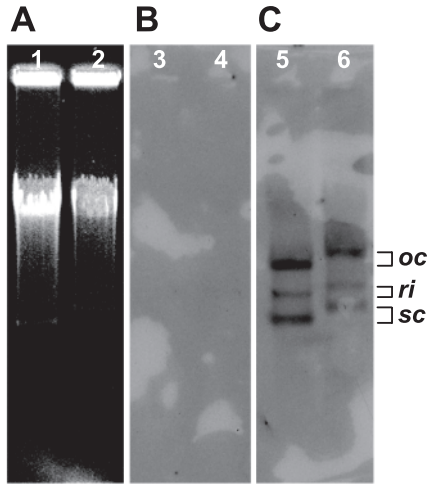


FIG. 3. GC skew analysis and fragment elimination study of the replicon. The symbols are as follows: white arrows, iterons; black arrow heads, repeated sequence in AT-rich regions; white squares, *dnaA* box; H and P, HindIII and PstI sites.

Plasmid stability. The stability of a plasmid vector in the absence of selective pressure is also important for practical applications, since the use of antibiotics is rarely possible in industrial fermentation. *M. succiniciproducens* and *A. succinogenes* harboring pMVSCS1 or pMEx were used to evaluate the plasmid stability. Cells were grown in TSB medium without antibiotics. During the serial subcultures, samples were taken at every 24 generations, up to 216 generations. Appropriately diluted samples were spread on TSA plates containing 32 and 64 μg/ml streptomycin for *M. succiniciproducens* and *A. succinogenes* harboring pMVSCS1 and 5 and 10 μg/ml ampicillin for *M. succiniciproducens* and *A. succinogenes* harboring pMEx. At the same time, samples were also spread on TSA plates without antibiotics as a control. The percentages of cells harboring pMVSCS1 and pMEx in *M. succiniciproducens* and *A. succinogenes* during the serial subcultures were measured. Both pMVSCS1 and pMEx were maintained stably in *M. succiniciproducens* and *A. succinogenes* in the absence of selective pressure for 216 generations (see Fig. S1 in the supplemental material). The structural plasmid stability was confirmed by minipreparation and restriction mapping of plasmids from the samples taken at 104 and 120 generations (data not shown).

Determination of the origin of replication and replication mechanism. GC skew analysis was conducted to predict the origin of replication. An asymmetric distribution of bases was observed for the pMVSCS1 sequence, which is visualized as the GC skew (Fig. 3). The switch in C-G deviation was statistically significant at ca. −300 bp upstream of the *rep* gene, which encodes a nucleic acid-binding protein. To determine the minimal region required for replication, DNA fragments of different lengths, including the *rep* gene and the −300-bp upstream region, were cloned from pMVSCS1 into pUC18. The resulting plasmids, pME18 (2.0-kb replicon fragment), pME18RIA (1.7-kb replicon fragment), and pME18RI (1.3-kb replicon fragment), as shown in Fig. 3, were transformed into *M. succiniciproducens*, followed by selection on TSA plates containing 5 μg/ml ampicillin. While pME18 and pME18RIA could be isolated from transformants, pME18RI could not. In other words, pME18RIA lacking the *dnaA* box was able to replicate in *M. succiniciproducens*, but pME18RI lacking both the *dnaA* box and the A+T-rich region was not. Thus, there seems to be another *dnaA* box(es) in the 1.7-kb *ori* fragment of the pME18RIA sequence. The basic characteristics of pME18RIA were almost identical to those of pMEx (data not shown). Therefore, we concluded that the 1.7-kb *ori* fragment of pME18RIA can be used as the minimal origin of replication for *M. succiniciproducens*.

In general, there are three types of plasmid replication control mechanism, such as the theta, the rolling circle, and the strand displacement modes. In order to determine the replication control mechanism of pMEx and pMVSCS1, Southern hybridization and sequence analysis were performed. For the Southern hybridization, total DNA was isolated from *M. succiniciproducens* harboring pMEx or pMVSCS1 and resolved on a 0.8% agarose gel by electrophoresis (Fig. 4A). The gels were blotted directly onto nitrocellulose membranes with and without a priori denaturation process. Without the denaturation process, double-stranded DNA cannot bind to nitrocellulose membrane. The gel denatured before membrane transfer served as a positive control. The membrane was then probed with a fluorescein-labeled pMVSCS1 plasmid (Fig. 4B and C). As shown in Fig. 4B, no band was observed without the denaturing step, which means that no single-stranded DNA existed in the cell. However, with the denaturing step, three distinct bands were observed on the gel (Fig. 4C). These results suggest that pMEx and its parental plasmid, pMVSCS1, did not generate single-stranded DNA; in other words, pMEx and pMVSCS1 do not replicate by the rolling circle mode in *M. succiniciproducens*.



D

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  1  ACATTCAGGCGTTATCAGATGAAGTGGATAAGGCTTTAACACATAGCTATAAGCGGAAAT
 61  TATAAAAAAACAAAACCGCCCTGTTAGGCGGTTATGTGATAAAATCTTCCTTGTGCTATC
121  GGCAAACGGTGTAACGTGTCCTTAGCACGTTGCCCGATTGCCTTAGGCTTATAGCACTAA
181  GGGGGTGATCTATGTGAACGCATTACAATACTTCGTAATGATACTACTGCTCACCGTAGT
241  AGTTGCGGTATTAGGTTTTCGAGTTCTTACCATTCTCGACCTAATTACTTACATCTTGAA
301  TAGATTGTCCTAAGACAATCGGGAAGGGGGTAGTTGGTAGCTATCCCCGACCTTTTTCCC
361  TTGTATGCCCTATCCCCTGATTTTTCAAGGTTTGTATTCAATCAAGCATAGCCACTCTAA
421  AACGCATTTAGAGCGTTTTTCTTTATAACCCTTGCTTTAATATGTATTTTGTCTTTTCTA
481  ACGCCCTACGGTTGTTTAATCCGCATTAAACGTTAAAAGCAAGTTTTAACGATAATGCGG
541  ATTTACGTTTATTCTGAATAAAAATAAAAATAGGGCTGAAAGCCCAAAAAGAGCCGATAG

601  GTCTATTTTTATATTTTATATTTTATACTTGTTTTCGGCTATCCCAGAGCCTTGATTTGC

661  AATGCTTGAGAGATTTTAACGACTACAAATTTGTCGTTTACGACTACAAATTTGTCGTTT

                                                          -35
721  GACGACTACAAATTTGTCGTTTGACGACTACAAATTTGTTGTTTTGAACTACTATTGATT

                    -10
781  ATTTTGTAGTTCAATGTATAATTAAACTACATTTTATTTTTTGTGTAGGTTATATATATG
                                                            M

841  GTAAATGATTTAACAGTTGTAAAGGCAAATAGTTTAATTGAAGCTAGTTACCGCCTAACT
     V  N  D  L  T  V  V  K  A  N  S  L  I  E  A  S  Y  R  L  T
```

E

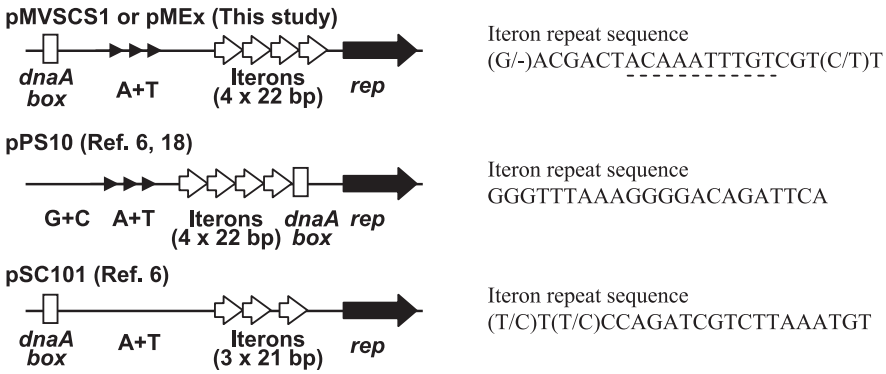

FIG. 4. Replication mechanisms of pMVSCS1 and pMEx. (A) Agarose gel electrophoresis. Southern blotting results without (B) and with (C) denaturation of the gels. Lanes 1, 3 and 5, total DNA from *M. succiniciproducens* harboring pMVSCS1; lanes 2, 4, and 6, total DNA form *M. succiniciproducens* harboring pMEx. Abbreviations: *oc*, open circle double-stranded DNA; *ri*, replication intermediates; *sc*, supercoiled double-stranded DNA. (D) DNA sequence of the partial replication origin of pMVSCS1 (or pMEx). The four 22-bp iteron repeats, three A+T repeats, and a *dnaA* box are indicated with block arrows, arrows, and a box, respectively. Dotted underlines represent the complete palindrome sequences in the iterons. The −35 and −10 boxes of the *rep* promoter are solid underlined. (E) The origin of replication of pMVSCS1 (or pMEx) and other theta mode plasmids in gram-negative bacteria. Symbols: white arrows, iterons; black arrow heads, repeated sequence in A+T-rich regions; white squares, *dnaA* box; H and P, HindIII and PstI sites.

Examination of the pMVSCS1 (or pMEx) replicon revealed four distinct regions: the iteron, the *dnaA* box, the A+T-rich region, and the *rep* gene. The pMVSCS1 sequence has 4 iterons of directly repeated sequences, 5′-(G/-)ACGACTACAAATTT GTCGT(C/T)T-3′ in tandem. In each iteron, a perfect palindrome structure (5′-ACAAATTTGT-3′) was observed (Fig. 4D). The *dnaA* box, 5′-TTTAACACA-3′, which is a binding sequence of the bacterial chromosomal initiator DnaA, is located 679 bp upstream of the iterons (Fig. 4D). It was found that the fourth nucleotide, A, in this box is different from the consensus *dnaA* box in *E. coli*, T (15). The A+T-rich regions contain three 7-mer repeats (5′-TTTTATA-3′), which were also found in the origin site (Fig. 4D). pMVSCS1 has one *rep* gene. All the features mentioned above indicate that the replication origin of pMVSCS1 is most similar to that of pPS10 (4, 7), which is a theta-mode replicating plasmid of *Pseudomonas savastanoi* (Fig. 4E). Plasmids pMVSCS1 and pMEx have only one protein for the initiation of DNA replication (Fig. 4D). It should be mentioned that three *rep* genes are required for replication by the strand displacement mode. Additionally, the origins of pMVSCS1 and pMEx are similar to those of theta-mode replicating plasmids which carry direct repeat iterons, one or more *dnaA* boxes, and adjacent A+T-rich regions containing sequence repeats (2). Thus, it was concluded that pMVSCS1 and pMEx replicate via the theta mode.

Homologous gene expression in *M. succiniciproducens*. We next examined whether the shuttle vector can be used for gene expression in *M. succiniciproducens* and *A. succinogenes*. The *fumC* gene encodes a fumarase which catalyzes the conversion of malic acid to fumaric acid (6). Plasmid pMEFUMC (Fig. 5A) containing the *M. succiniciproducens fumC* gene was constructed and introduced into *M. succiniciproducens* and *A. succinogenes*. As shown in Fig. 5B, a prominent band at 50 kDa, which corresponds to the molecular mass of the fumarase, was clearly visible. Recombinant cells expressing the *fumC* gene were harvested at the late exponential phase, and the fumarase activity was measured for both bacteria. The fumarase activities in *M. succiniciproducens* were 186 and 525 U in the wild-type and recombinant strains, respectively. The fumarase activity in *A. succinogenes* increased from 214 U in the wild-type to 259 U in the recombinant strain (see Fig. S2 in the supplemental material). Thus, the fumarase activities in recombinant *M. succiniciproducens* and *A. succinogenes* increased by 2.8 and 1.2 times, respectively, compared to those of wild-type strains. It should be noted that the *fumC* gene is heterologous to *A. succinogenes*. These results suggest that the shuttle vector can be used for the functional expression of genes in *M. succiniciproducens* and *A. succinogenes*.

Heterologous protein expression in *M. succiniciproducens*. After observing successful homologous gene expression, we next examined heterologous gene expression in *M. succiniciproducens* by using GFPuv and DSRed2 as model proteins. The pMS3-G and pMS3-R expression vectors (Table 1) were transformed into *M. succiniciproducens*, and the reporter genes were expressed from the *frdA* promoter. As shown in Fig. S3 in the supplemental material, both reporter proteins, GFPuv (green) and DsRed2 (red), were successfully expressed in *M. succiniciproducens*. In addition, the reporter fusions were expressed in *E. coli*, suggesting that the *M. succiniciproducens frdA* promoter is also functional in *E. coli* (data not shown).

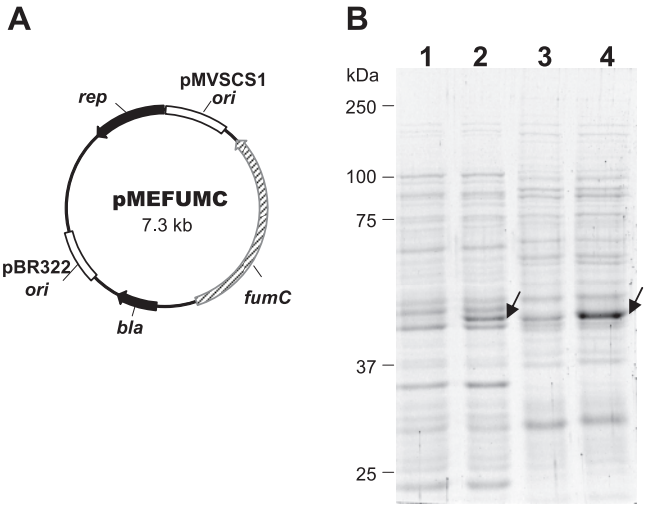

FIG. 5. Expression of the *M. succiniciproducens fumC* gene by use of the shuttle vector pME. (A) Recombinant plasmid pMEFUMC. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels showing the expression of the *fumC* gene in *M. succiniciproducens* (lanes 1 and 2) and *A. succinogenes* (lanes 3 and 4). Lanes 1 and 3, cells harboring pME; lanes 2 and 4, cells harboring pMEFUMC.

Interestingly, the *M. succiniciproducens frdA* gene was originally annotated as the *sdhA* gene which encodes the SdhA protein, a subunit of the succinate dehydrogenase complex converting succinate to fumarate. However, this gene is localized right next to the *frdBCD* genes on the *M. succiniciproducens* chromosome, forming an operon structure (6). It has been suggested recently that this gene encodes FrdA, based on comparative proteome analysis supported by physiological studies (9). Since the fumarate reductase catalyzes the last step in the succinate formation in *M. succiniciproducens*, the expression vector pMS3 employing the *frdA* promoter would be very useful to express heterologous proteins under succinate-producing conditions.

Conclusions. In this paper, we reported the development of *E. coli-M. succiniciproducens*/*A. succinogenes* shuttle vectors that exist at ca. 10 copies per cell and can be stably maintained without selection pressure. The homologous *fumC* gene and heterologous genes encoding fluorescent proteins could be successfully expressed in *M. succiniciproducens*. So far, metabolic engineering of *M. succiniciproducens* and other rumen bacteria has been hampered due to the lack of a suitable plasmid vector system. The shuttle vectors developed in this study should be useful for genetic and metabolic engineering of succinic acid-producing rumen bacteria.

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