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Change in the plasmid copy number in acetic acid Bacteria in response to growth phase and acetic acid Concentration

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Plasmids pGE1 (2.5 kb), pGE2 (7.2 kb), and pGE3 (5.5 kb) were isolated from *Gluconacetobacter europaeus* KGMA0119, and sequence analyses revealed they harbored 3, 8, and 4 genes, respectively. Plasmid copy numbers (PCNs) were determined by real-time quantitative PCR at different stages of bacterial growth. When KGMA0119 was cultured in medium containing 0.4% ethanol and 0.5% acetic acid, PCN of pGE1 increased from 7 copies/genome in the logarithmic phase to a maximum of 12 copies/genome at the beginning of the stationary phase, before decreasing to 4 copies/genome in the late stationary phase. PCNs for pGE2 and pGE3 were maintained at 1–3 copies/genome during all phases of growth. Under a higher concentration of ethanol (3.2%) the PCN for pGE1 was slightly lower in all the growth stages, and those of pGE2 and pGE3 were unchanged. In the presence of 1.0% acetic acid, PCNs were higher for pGE1 (10 copies/genome) and pGE3 (6 copies/genome) during the logarithmic phase. Numbers for pGE2 did not change, indicating that pGE1 and pGE3 increase their PCNs in response to acetic acid. Plasmids pBE2 and pBE3 were constructed by ligating linearized pGE2 and pGE3 into pBR322. Both plasmids were replicable in *Escherichia coli*, *Acetobacter pasteurianus* and *G. europaeus*, highlighting their suitability as vectors for acetic acid bacteria.

[**Key words:** Acetic acid bacteria; *Gluconacetobacter europaeus*; *Acetobacter pasteurianus*; Plasmid copy number; Real-time quantitative PCR]

Bacterial plasmids are small circular strands of extra-chromosomal DNA that can carry and then transfer exogenous genes to a host cell. Plasmid transfer facilitates genetic exchange in bacterial populations and can foster an accelerated evolution of the host cell. Interactions between bacterial cells and plasmids have been widely investigated in an attempt to understand the relationship between these extra-chromosomal elements and the growth of host cells (1). Plasmids generally encode genes with specific functions that provide the host with an advantageous phenotype, such as antibiotic resistance, pathogenicity, and metabolic breakdown pathways activated under certain environmental conditions. Although the presence of extra-chromosomal genes can provide a competitive advantage under particular conditions, they can also be a metabolic burden on the host (2). The extra stress on the cells is attributed to the additional energy expenditure needed for the replication of plasmids and expression of their genes (1), which often results in a decrease in the growth rate of the host cell (3). To minimize the metabolic load and co-exist with their host, plasmids control their copy number (2,4,5), which is fixed and dependent on the growth conditions for the cell (2,4,5). Plasmid copy number (PCN) is one of the most important characteristics, and is defined as the number of copies of a particular plasmid per genome in a cell (5). The PCN is usually controlled by a plasmid-encoded regulatory protein or RNA (2,6). Previous studies have reported that the growth rate of the host cell is also an important factor for the control of PCN (7,8). The PCN in *Bacillus cereus* and *Bacillus thuringiensis* varies according to the growth phase (9,10).

Acetic acid bacteria (AAB) are Gram-negative obligately aerobic microbes. *Gluconacetobacter europaeus* is one of AAB that has been widely used for the industrial production of vinegar because of its ability to oxidize ethanol and tolerance to acetic acid. The draft genome sequence of the type strain *G. europaeus* LMG 18890^T has been determined (11), and a targeted gene disruption system using the endogenous *pyrE* gene has been developed for use in strain KGMA0119 (12,13). However, investigations focusing on the genetic characteristics of extra-chromosomal elements, such as native cryptic plasmids found in *G. europaeus*, are limited (14). Completion of entire genome sequences of some AAB has recently revealed the presence of numerous native cryptic plasmids (15–18). The functions of cryptic plasmids are often not known, however their loss (e.g., *Corynebacterium renale*) can cause a reduction in cell size and decreases in growth and respiratory rates, indicating a co-evolution between the host and plasmids (19). The impact of native plasmids on the growth and metabolism of AAB is unclear.

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TABLE 1. Strains, plasmids and primers used in this study.

Strain, plasmid or primer	Relevant characteristics or sequence (5′−3′)	Source or purpose[a]
Strain		
E. coli		
DH5α	F⁻, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rK⁻, mK⁺), *phoA*, *supE*44, λ⁻, *thi*-1, *gyrA*96, *relA*1	20
G. europaeus		
KGMA0119	Wild-type isolated from rice vinegar	12
KGMA0119 (pBE2)	KGMA0119 harboring pBE2 (Amp^r)	This work
KGMA0119 (pBE3)	KGMA0119 harboring pBE3 (Amp^r)	This work
A. pasteurianus		
KGMA0054	Wild-type isolated from rice vinegar	This work
KGMA0054 (pBE2)	KGMA0054 harboring pBE2 (Amp^r)	This work
KGMA0054 (pBE3)	KGMA0054 harboring pBE3 (Amp^r)	This work
Plasmid		
pBR322	Amp^r	21
pGE1	Native cryptic plasmid possessing 3 ORFs isolated from KGMA0119 (2524 bp)	This work
pGE2	Native cryptic plasmid possessing 8 ORFs isolated from KGMA0119 (7187 bp)	This work
pGE3	Native cryptic plasmid possessing 4 ORFs isolated from KGMA0119 (5537 bp)	This work
pBE2	pGE2-derivative; composed of pGE2 and pBR322 (11548 bp)	This work
pBE3	pGE3-derivative; composed of pGE3 and pBR322 (9898 bp)	This work
Primer		
322-F	TATCAGGGTTATTGTCTCATGAGCG	Genotyping
pGE2-R1	TGGCATCATTCACTCCTGACGTG	Genotyping
pGE3-R1	CAACACGTTCCAAGTTTCCAG	Genotyping
H2-F	CTCCTTGGCCTGTTGCTGAACTTC	Genotyping
H2-R	CAAACTCGCAGAGTTTGCATAAG	Genotyping
H3-F	GTCTCAAGCGAGATATTC	Genotyping
H3-R	GTTCCAAGTTTCCAGCATC	Genotyping
pGE1-q-Fw	TTGCATCTGTCCCTACCCTACC	Measurement of PCN
pGE1-q-Rv	GGGAATACTGGCTCATCGTCTC	Measurement of PCN
pGE2-q-Fw	TGACTGTTTGGCATCACGTAAG	Measurement of PCN
pGE2-q-Rv	CATCACAAGAAGTCGGCAAAAG	Measurement of PCN
pGE3-q-Fw	CCTTGCGGTATCTGCCTTTC	Measurement of PCN
pGE3-q-Rv	ATGACTGGGAGCGGTTTGAG	Measurement of PCN
aldC-Fw	ATGCTATTCGGTAGGCGATG	Measurement of PCN
aldC-Rv	AGTTCGTCAAGCGTGGTTTC	Measurement of PCN

[a] PCN, plasmid copy number.

We first attempted to isolate native cryptic plasmids from *G. europaeus* KGMA0119. As a result, three novel cryptic plasmids were obtained. Next, the PCN variations of these plasmids in host cells under different culture conditions and growth phases were estimated using real-time quantitative PCR (RTQ-PCR). Then, we constructed shuttle vectors based on the native plasmids from *G. europaeus* KGMA0119 and investigated their host ranges by introducing them to *Escherichia coli*, *G. europaeus*, and *Acetobacter pasteurianus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media Bacterial strains and plasmids are presented in Table 1. *G. europaeus* KGMA0119 (wild-type) (12) and *A. pasteurianus* KGMA0054 (wild-type) were cultured in yeast peptone dextrose (YPD) broth (12) at 30 °C. Unless otherwise indicated, 0.4% (wt/vol) ethanol and 0.5% (wt/vol) acetic acid were added to the medium. For plate cultures, 0.9% (wt/vol) agar was added to YPD broth. Where appropriate, 50 μg/ml of ampicillin was added to YPD medium to select for ampicillin resistant strains.

E. coli strain DH5α (20) (Takara Bio, Ohtsu, Shiga, Japan) and plasmid pBR322 (21) (Takara Bio) were used to clone restriction fragments of *G. europaeus* plasmids and construct shuttle vectors between *E. coli* and AAB. *E. coli* DH5α was routinely cultured in Lysogeny broth (22) containing 50 μg/ml ampicillin at 37 °C.

DNA manipulation and sequencing General DNA manipulations were performed as described by Green and Sambrook (22). PCR was carried out using KOD plus (Toyobo, Osaka, Japan), KOD FX (Toyobo), or Go Taq Green Master Mix (Promega, Madison, WI, USA) as a DNA polymerase. Restriction and modifying enzymes were purchased from Takara Bio or Nippon Gene (Tokyo, Japan). PCR products and restriction fragments were separated by agarose gel electrophoresis, and DNA fragments were recovered using the NucleoSpin Gel and PCR Clean-up Kit (Takara Bio). DNA sequencing was performed using a BigDye Terminator Cycle Sequencing Kit, ver. 3.1, and a model 3130 capillary sequencer (Applied Biosystems, Foster City, CA, USA).

Plasmid extraction *G. europaeus* KGMA0119 was initially cultured in 5 ml of YPD broth at 30 °C with reciprocal shaking at 150 rpm for 16 h. A 1.0 ml aliquot of the culture was transferred to 30 ml of YPD broth and cells were cultured for a further 24 h. The entire culture was then added to 3 L of YPD broth in a 5 L mini-jar fermenter (MDL-501, B. E. Marubishi, Tokyo, Japan), and cells were cultured at 30 °C with agitation at 500 rpm and aeration at a rate of 1.0 L per min for 24 h. Cells were harvested by centrifugation (4 °C, 10,000 ×*g*, 10 min), and cytoplasmic DNA (including plasmids) was isolated from cells by alkaline lysis as described by Green and Sambrook (22). The resultant DNA pellet was dissolved in $TE_{10\text{-}1}$ (10 mM Tris−HCl and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0). Plasmids were further fractionated and purified by cesium chloride (CsCl) density gradient centrifugation as follows: CsCl and ethidium bromide were added to 6 ml of the plasmids solution at the final concentrations of 6.0 M and 0.5 mg/ml, respectively. The mixture was centrifuged at 268,000 ×*g* for 16 h at 20 °C in an Ultracentrifuge Model CP 100MX using a P65AT rotor (Hitachi Koki, Tokyo, Japan). The fraction containing the plasmids was collected, and ethidium bromide was removed by extraction with *n*-butanol saturated with water, followed by dialysis against $TE_{10\text{-}0.1}$ (10 mM Tris−HCl and 0.1 mM EDTA, pH 8.0). Purity and quality of the plasmids were determined by measuring optical density (OD) at 260 and 280 nm, and agarose gel electrophoresis.

Characterization of plasmids The plasmids isolated from the strain KGMA0119 were digested with HindIII, ClaI, SphI, SmaI, PvuII, EcoRI, PstI, or KpnI (Takara Bio) (Fig. 1). HindIII, ClaI, or SphI digests were then cloned into pBR322 at the corresponding restriction sites, sequenced, and then assembled. The complete sequences of three plasmids were determined. These native cryptic plasmids were designated pGE1 (2524 bp), pGE2 (7187 bp), and pGE3 (5537 bp) (Fig. 2). Open reading frames (ORFs) were predicted using the programs ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and Glimmer (23), and protein-coding

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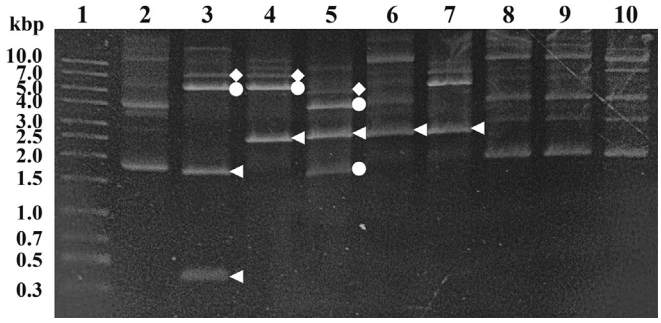

FIG. 1. Digestion of native cryptic plasmids with restriction enzymes. Plasmids extracted from *G. europaeus* KGMA0119 were digested with several restriction enzymes and separated by electrophoresis. Lanes: 1, marker; 2, extracted plasmids; 3, HindIII; 4, ClaI; 5, SphI; 6, SmaI; 7, PvuII; 8, EcoRI; 9, PstI; 10, KpnI. Restriction fragments highlighted by triangles, diamonds, and circles indicate those derived from pGE1, pGE2, and pGE3, respectively.

genes (identified by putative Shine-Dalgarno (SD) sequences upstream of an initiation codon) were manually selected. Annotation for each gene product was done using the BLASTP program (24) against the non-redundant protein database (Table 2).

Preparation of genomic DNA for PCN measurement KGMA0119 was cultured in 5 ml of YPD broth at 30 °C for 16 h. Aliquots of the bacterial suspension were transferred to 30 ml of YPD broth supplemented with either 0.4% (wt/vol) ethanol and 0.5% (wt/vol) acetic acid, or 3.2% (wt/vol) ethanol and 0.5% (wt/vol) acetic acid, or 0.4% (wt/vol) ethanol and 1.0% (wt/vol) acetic acid, such that a cell density (OD_{660}) of 0.03 was achieved in each treatment. Cells were cultured in each medium and collected at the mid-logarithmic (OD_{660} = 0.2), early stationary (OD_{660} = 1.0), and late stationary phase of growth (OD_{660} = 1.3–1.5) by centrifugation. Genomic DNA was extracted and purified as previously described (22,25), and used to determine PCN by RTQ-PCR. The cell-free supernatants were used to determine ethanol and acetic acid concentrations by gas chromatography (see below).

Measurement of PCN by RTQ-PCR PCN was determined by comparing the number of plasmids with genomes in the DNA samples used as templates for RTQ-PCR (5). To determine the number of genomes in samples, the α-acetolactate decarboxylase gene (*aldC*) was selected as a suitable reference gene. This is because *aldC* is a single-copy gene found on the *G. europaeus* genome (12), and was used to infer the number of genomes in a cell. RTQ-PCR was performed using

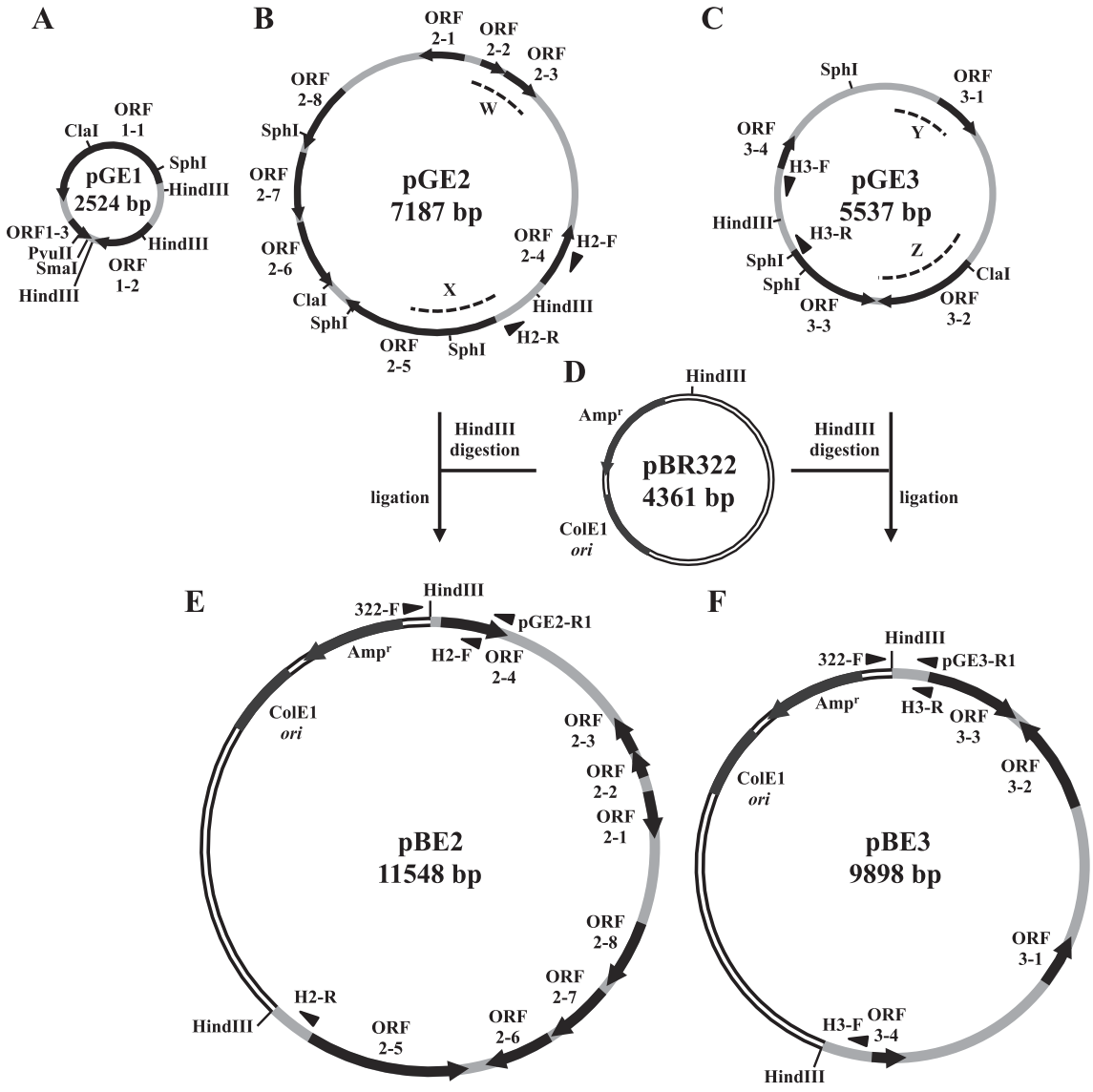

FIG. 2. Schematic representations of native cryptic plasmids isolated from *G. europaeus* KGMA0119 (A–C) and their derivatives (E, F). (A) pGE1, (B) pGE2, (C) pGE3, (D) pBR322, (E) pBE2, and (F) pBE3. Plasmids pGE2 and pGE3 were linearized with HindIII and each fragment was cloned into pBR322 to construct pBE2 and pBE3. The dotted lines W–Z represent regions with high nucleotide sequence identity with plasmid pGX4 of *G. xylinus* E25 (CP004364) (line W), plasmid Apa386Bp6 of *A. pasteurianus* 386B (HF677576) (line X), plasmid pGDIPal5I of *G. diazotrophicus* Pal5 (AM889287) (line Y), and chromosomal DNA of *G. xylinus* E25 (CP004360) (line Z). Thick arrows (A–C, E, F), and arrowheads (B, C, E, F) indicate predicted ORFs (see Table 2 for detailed annotation of each ORF) and primers used for genotyping PCR (see Figs. 4 and 5).

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TABLE 2. Characterization of proteins encoded by pGE1, pGE2 and pGE3 by homology search (BLASTP).

Query			Subject				Homology	
Plasmid	ORF	Length (aa)[a]	Accession No.	Species	Annotation[b]	Length (aa)[a]	Identity (%)	Similarity (%)
pGE1	1–1	414	WP_006726261	*Agrobacterium albertimagni*	Mob	382	39	59
	1–2	170	GAJ28506	*Acidomonas methanolica*	Hypo	171	34	51
	1–3	77	WP_018433099	*Burkholderia* sp.	MP	478	39	57
pGE2	2–1	125	WP_019092408	*G. europaeus*	Hypo	125	96	96
	2–2	72	AHI27224	*G. xylinus*	Hypo	72	89	95
	2–3	107	WP_019092698	*G. europaeus*	Kid	107	96	97
	2–4	182	WP_003631006	*Acetobacter pasteurianus*	Rep	162	47	65
	2–5	441	WP_003627465	*Acetobacter pasteurianus*	Mob	338	54	70
	2–6	197	WP_006117066	*Acetobacter pomorum*	Hypo	215	34	46
	2–7	182	WP_010669352	*Acetobacter aceti*	Hypo	185	42	58
	2–8	186	YP_008411027	*Acetobacter aceti*	Rep	181	60	77
pGE3	3–1	135	WP_023942379	*Gluconobacter frateurii*	Hypo	167	74	76
	3–2	280	AHI24732	*G. xylinus*	PhzF	280	94	96
	3–3	265	WP_006115714	*Acetobacter pomorum*	Rep	287	65	80
	3–4	94	WP_003631008	*Acetobacter pasteurianus*	CopG	95	81	92

[a] aa, amino acid.
[b] Mob, mobilization protein; Hypo, hypothetical protein; MP, membrane protein; Kid (killing-determinant), RNase which inhibits growth of plasmid-free cells; Rep, replication protein; PhzF, PhzF family phenazine biosynthesis protein; CopG, transcriptional regulator which represses *rep* expression.

the SuperScript III RT/Platinum Kit (Invitrogen, Carlsbad, CA, USA) and ABI PRISM 7000 sequence detection system (Applied Biosystems). The targeted regions were initially PCR-amplified using the genomic DNA of KGMA0119 as a template and appropriate primers listed in Table 1 as follows: pGE1, pGE1-q-Fw and pGE1-q-Rv; pGE2, pGE2-q-Fw and pGE2-q-Rv; pGE3, pGE3-q-Fw and pGE3-q-Rv; *aldC*, aldC-Fw and aldC-Rv. 100, 10, 1, 0.1, and 0.01 pg of the PCR products were used to construct sequence specific standard curves. Thermal cycling conditions for RTQ-PCR were as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 15 s, 51 °C (*aldC*), or 55 °C (pGE1, pGE2, and pGE3) for 15 s, and 60 °C for 1 min. A melting curve analysis of the PCR products was performed in accordance with the manufacturer's instructions (Applied Biosystems) to confirm the purity and specificity of the PCR products. Quantities (g) of the targeted regions of the plasmids and *aldC* were determined using 1 ng of genomic DNA as a template, and numbers (mol) were calculated using the following equation based on the length of each PCR product (bp) and average molecular weight of a nucleotide pair (660 g/mol/bp):

$$[\text{DNA amount (g)}]/[\text{DNA length (bp)} \times 660\ (\text{g/mol/bp})] \quad (1)$$

PCN was estimated using the following equation:

$$[\text{Plasmid (mol)}]/[\textit{aldC}\ (\text{mol})] \quad (2)$$

Measurement of acetic acid and ethanol by gas chromato graphy Concentrations of acetic acid and ethanol were determined in cell-free supernatants taken from different stages of the bacterial growth using a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a packed column (PEG20M 10%, SHINCARBON A 60/80, 2.1 m × 3.2 mm, Shinwa Chemical, Kyoto, Japan) (12). The column oven temperature was initially held at 60 °C for 3 min, followed by an increase of 10 °C per min up to 200 °C.

Construction of shuttle vectors between *E. coli* and AAB To construct plasmid shuttle vectors between *E. coli* and AAB, pGE2 and pGE3 were linearized with HindIII and ligated into pBR322. Two derivatives from pGE2 and pGE3 were designated pBE2 (11,548 bp) and pBE3 (9898 bp), respectively (Fig. 2).

pBE2 or pBE3 was introduced into competent cells of *G. europaeus* KGMA0119 and *A. pasteurianus* KGMA0054 by electroporation (12). Cells were allowed to recover in 1 ml of YPD broth at 30 °C for 3 h. Cells were then harvested by centrifugation, washed with sterile saline (0.85% [wt/vol] NaCl) and transferred to 5 ml of YPD broth supplemented with ampicillin (50 μg/ml). Cells were cultured at 30 °C for at least 48 h to select for ampicillin resistant cells. An aliquot of the culture was mixed with sterile saline and spread onto YPD agar containing ampicillin and incubated at 30 °C for 2–3 days. A positive clone with ampicillin resistance was randomly selected from the plate. Genomic and plasmid DNA was extracted (as described earlier) from these strains. To investigate genotypes of the positive clones, two types of PCR were performed as follows: The junction of pBR322 and pGE2 in pBE2 (0.9 kb), or pBR322 and pGE3 in pBE3 (0.6 kb) was amplified using total DNA isolated from the ampicillin resistant strains as the target and the respective primer pairs 322-F – pGE2-R1 or 322-F – pGE3-R1 (Fig. 2 and Table 1). Then, a 0.8 kb region including the HindIII site in pGE2 or pGE3, and a 5.2 kb region including the entire pBR322 backbone (4.4 kb) in pBE2 or pBE3 were amplified using total DNA isolated from the ampicillin resistant strains as the target and the respective primer pairs H2-F – H2-R or H3-F – H3-R (Fig. 2 and Table 1). Strains of *G. europaeus* harboring pBE2 or pBE3 were designated as KGMA0119 (pBE2) or KGMA0119 (pBE3), respectively, and similarly, *A. pasteurianus* strains harboring pBE2 or pBE3 were designated KGMA0054 (pBE2) or KGMA0054 (pBE3).

Nucleotide sequence accession numbers The nucleotide sequences of pGE1 (accession number AB972537), pGE2 (AB972538), pGE3 (AB972539), and the partial 16S rRNA gene sequence of *A. pasteurianus* KGMA0054 (AB979194) have been deposited in the DDBJ, EMBL, and GenBank databases.

RESULTS

Characterization of native cryptic plasmids from *G. europaeus* KGMA0119 Extra-chromosomal DNA was isolated from *G. europaeus* KGMA0119 cells by alkaline lysis and fractionated by cesium chloride (CsCl) density gradient centrifugation. The isolated fraction was separated by agarose gel electrophoresis as shown in Fig. 1. The results suggested that the strain harbors several cryptic plasmids (Fig. 1, lane 2). Digestion analyses with various restriction enzymes were then conducted. Digestion of the plasmids with HindIII, ClaI, SphI, SmaI, or PvuII generated several restriction fragments, and different digestion patterns were observed depending on the restriction enzymes used (Fig. 1, lanes 3–7). By contrast, EcoRI, PstI, and KpnI appeared not to cleave the plasmids (Fig. 1, lanes 8–10). HindIII, ClaI, or SphI digests of the plasmids were then cloned into pBR322 at the corresponding restriction sites and sequenced to determine their complete nucleotide sequences. Based on sequences, three kinds of cryptic plasmids were identified and they were designated pGE1 (2524 bp), pGE2 (7187 bp), and pGE3 (5537 bp) (Fig. 2A–C). Several recognition sites of various restriction enzymes were identified on the plasmids (Fig. 2A–C), which were well consistent with the electrophoretic patterns of HindIII, ClaI, and SphI digests of the plasmids (Fig. 1, lanes 3–5). The ClaI digests with the size of 2.5 kb, 5.5 kb, and 7.2 kb were correspond to the linearized pGE1, pGE2, and pGE3, respectively (Fig. 1, lane 4), which was in agreement with the fact that the three plasmids had a single ClaI recognition site (Fig. 2A–C). The G + C content of each plasmid was 52% (pGE1), 51% (pGE2), and 55% (pGE3). pGE1 had no significant nucleotide sequence similarity with plasmids or chromosomal DNA previously characterized from AAB (11,14–18). By contrast, some short regions in pGE2 and pGE3 (Fig. 2B and C, denoted by dotted lines) had high identities with plasmids or chromosomal DNA of other AAB. Regions in pGE2 and pGE3 (Fig. 2) had the following identities: W, 91% with plasmid pGX4 of *Gluconacetobacter xylinus* E25 (CP004364); X, 79% with plasmid Apa386Bp6 of *A. pasteurianus* 386B (HF677576); Y, 81% with

plasmid pGDIPal5I of *Gluconacetobacter diazotrophicus* Pal5 (AM889287); Z, 91% with chromosomal DNA of *G. xylinus* E25 (CP004360). Nucleotide analyses revealed that pGE1, pGE2, and pGE3 possess 3, 8, and 4 ORFs, respectively (Fig. 2A–C), and that each ORF was preceded by a putative Shine-Dalgarno (SD) sequence. Homology searches (BLASTP) using amino acid sequences of putative proteins encoded by these ORFs as queries were performed, and proteins that showed relatively high identity and similarity are listed in Table 2. Many of the ORFs were predicted to encode a hypothetical protein with an unknown function. Some ORFs appeared to encode a protein with specific functions: ORF1-1 and ORF2-5 were predicted to encode a mobilization protein (Mob), which is responsible for plasmid transfer during conjugation (26). Putative polypeptides encoded by three ORFs were predicted to be replication initiator proteins (Rep) involved in plasmid replication and PCN control (6), and had the following identities: ORF2-4, 47% with *A. pasteurianus* (WP_003631006); ORF2-8, 60% with *Acetobacter aceti* (YP_008411027) (27); ORF3-3, 65% with *Acetobacter pomorum* (WP_006115714). The predicted amino acid sequence of ORF3-3 also showed a high identity with a putative RepB of *G. europaeus* (WP_019087597). No *rep*-like sequence was found in pGE1. The putative protein encoded by ORF3-2 showed a significantly high identity (94%) with the PhzF family phenazine biosynthesis protein of *G. xylinus* (AHI24732). PhzF is involved in the biosynthesis of phenazine, which is a broad-spectrum antibiotic produced by *Pseudomonas* sp. (28,29), however, the functions of the PhzF family of proteins in other bacteria are unclear. ORF3-4 encodes a probable CopG transcriptional repressor (WP_003631008). CopG binds to *repB* and its own promoter regions, and represses the synthesis of both proteins. CopG is able to regulate plasmid replication and PCN by controlling the availability of RepB (4). The predicted protein encoded by ORF2-3 showed 96% identity to Kid (killing-determinant) of *G. europaeus* (WP_019092698). Kid is a toxin protein (RNase) and maintains plasmid stability by eliminating plasmid-free cells produced by occasional replication or partition failures (30,31).

Measurement of PCN by RTQ-PCR PCNs in *G. europaeus* were monitored by RTQ-PCR to determine if the native cryptic plasmids were stably maintained. When KGMA0119 was cultured in YPD broth containing 0.4% ethanol and 0.5% acetic acid, the PCN of pGE1 increased from 7 copies/genome during the mid-logarithmic growth phase to a maximum of 12 copies/genome at the start of stationary phase, before falling to a minimum of 4 copies/genome in the late stationary phase of growth (Fig. 3A). PCNs for pGE2 and pGE3 remained at 1–3 copies/genome throughout the growth phases (Fig. 3A). Acetic acid and ethanol can affect the growth of AAB. To determine the effects of ethanol and acetic acid on PCN, KGMA0119 was cultured in higher concentrations of each substrate. In the presence of 3.2% ethanol, the three native plasmids showed similar relative copy number profiles to those in YPD broth with 0.4% ethanol, however, the PCNs for pGE1 were slightly lower at each stage of growth (Fig. 3A and B). Contrastingly, the PCN profile of pGE1 in the presence of 1.0% acetic acid was different to that in YPD containing 0.5% acetic acid: the maximum PCN for pGE1 was in the early part of the stationary phase in 0.5% acetic acid, however, plasmid numbers peaked earlier, during the mid-logarithmic phase, with 1.0% acetic acid (Fig. 3A and C). The PCN of pGE3 was also greater at the mid-logarithmic phase with 1.0% (6 copies/genome) than with 0.5% acetic acid (3 copies/genome) (Fig. 3A and C). The PCN of pGE2 remained low at 1–3 copies/genome throughout the phases of growth for each treatment (Fig. 3).

Construction of shuttle vectors between *E. coli* and AAB The entire pGE2 and pGE3 linearized with HindIII were

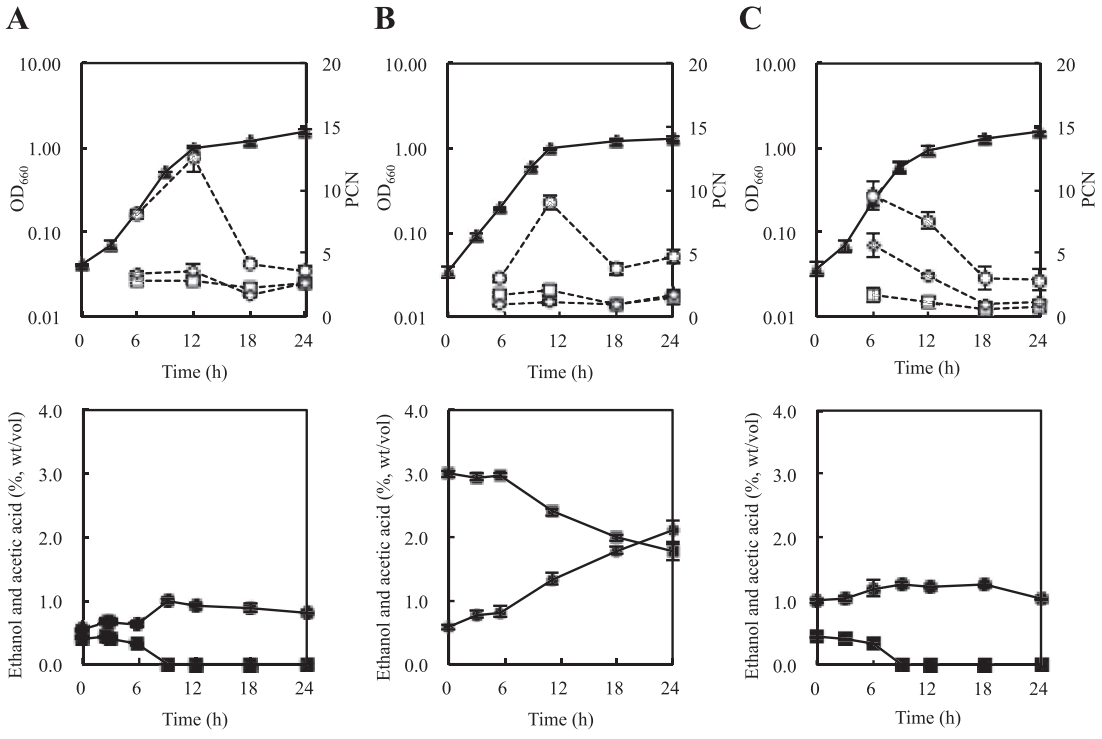

FIG. 3. PCN profiles of the native cryptic plasmids from *G. europaeus* KGMA0119 under different culture conditions. KGMA0119 was cultured in YPD broth supplemented with (A) 0.4% ethanol and 0.5% acetic acid, (B) 3.2% ethanol and 0.5% acetic acid, or (C) 0.4% ethanol and 1.0% acetic acid. Copy numbers of plasmids at the different phases of growth in each culture were determined by RTQ-PCR (upper plots). Concentrations of ethanol and acetic acid in the culture medium were quantified by gas chromatography (lower plots). Upper plots: solid triangles, OD_{660}; open circles, PCN of pGE1; open squares, PCN of pGE2; open diamonds, PCN of pGE3. Lower plots: solid squares, ethanol (%, wt/vol); solid circles, acetic acid (%, wt/vol). All experiments were conducted in triplicate, and error bars indicate standard deviations.

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ligated into HindIII digest of pBR322. Two derivatives from pGE2 and pGE3 were designated pBE2 and pBE3 (Fig. 2E and F), respectively, and were introduced into the strain KGMA0119 by electroporation. A large number of colonies expressing ampicillin resistance were obtained. A candidate clone predicted to harbor pBE2 or pBE3 was randomly selected out of the colonies and designated KGMA0119 (pBE2) or KGMA0119 (pBE3), respectively (Fig. 4A). Confirmatory checks were made on inserts using PCR, and the junctions of pBR322 and the pGE2 or pGE3 in the recombinant plasmids were targeted (Fig. 2E and F). Specific PCR amplifications occurred only when purified pBE2 and pBE3, and the plasmids from KGMA0119 (pBE2) and KGMA0119 (pBE3) were used as templates (Fig. 4B). No PCR products were detected when pBR322 and the plasmids of the parental strain KGMA0119 were used as templates (Fig. 4B). Then, further primer pairs were designed at the upstream and downstream of the HindIII site in pGE2 (H2-F – H2-R) and pGE3 (H3-F – H3-R) (Fig. 2B and C). The linearized pBR322 (4.4 kb) was inserted into the HindIII sites of the plasmids pGE2 and pGE3 to construct pBE2 and pBE3, respectively, as described above (Fig. 2E and F). By using the primer pairs H2-F – H2-R, short (0.8 kb) and long (5.2 kb) fragments are amplified from pGE2 and pBE2, respectively (Fig. 2B and E). As well, by using the primer pairs H3-F – H3-R, short (0.8 kb) and long (5.2 kb) fragments are amplified from pGE3 and pBE3, respectively (Fig. 2C and F). As expected, PCR products amplified from the KGMA0119 (pBE2) (Fig. 4C, upper panel) and KGMA0119 (pBE3) (Fig. 4C, lower panel) were longer than those from the parental strain KGMA0119 that carries plasmids pGE2 and pGE3. These results confirmed that the ampicillin resistant strains contained the recombinant plasmids and that pBE2 and pBE3 were possible shuttle vectors between *E. coli* and *G. europaeus*.

To investigate the host ranges of the recombinant plasmids, pBE2 and pBE3 were introduced into *A. pasteurianus* KGMA0054 (sensitive to ampicillin) by electroporation. A candidate clone theoretically harboring pBE2 or pBE3 was randomly selected from colonies growing on YPD agar containing ampicillin, and designated KGMA0054 (pBE2) or KGMA0054 (pBE3), respectively (Fig 5A). Total DNA was extracted from the clones and used as a template for the PCR amplification of the junction between pBR322 and pGE2 or pGE3 as described above. When purified plasmids pBE2 and pBE3, and total DNA from KGMA0054 (pBE2) and KGMA0054 (pBE3), were used as templates, specific PCR amplifications were detected (Fig. 5B). No PCR product was amplified when using pBR322 and total DNA of the parental strain KGMA0054 as targets (Fig. 5B). This confirmed that KGMA0054 (pBE2) and KGMA0054 (pBE3) harbored pBE2 and pBE3, respectively, and that these recombinant plasmids replicated in *A. pasteurianus*. pBR322 was introduced into *G. europaeus* KGMA0119 and *A. pasteurianus* KGMA0054. No colonies with ampicillin resistance were obtained, indicating that pBR322 was not replicable in these AAB.

pGE1 was also linearized with ClaI or SphI and cloned into pBR322, and the two chimeric plasmids were introduced into *G. europaeus* KGMA0119. No ampicillin resistant cells were detected.

DISCUSSION

Three cryptic plasmids (pGE1, pGE2, and pGE3) were isolated from *G. europaeus* KGMA0119. Plasmid pGE1 was the smallest and showed no significant identity with nucleotide sequences previously characterized from AAB, including other strains of *G. europaeus* (14). Only limited regions of nucleotides in pGE2 and pGE3 were identical to partial sequences of plasmids or chromosomal DNA of related species (16,17,32), suggesting that the three plasmids are unique to KGMA0119. Some of the predicted ORFs appeared to encode proteins with specific functions, however, others suggested a hypothetical protein whose function is unclear (Table 2). Plasmids usually provide the host cell with a phenotypic advantage under specific conditions (2). The phenotypic traits of

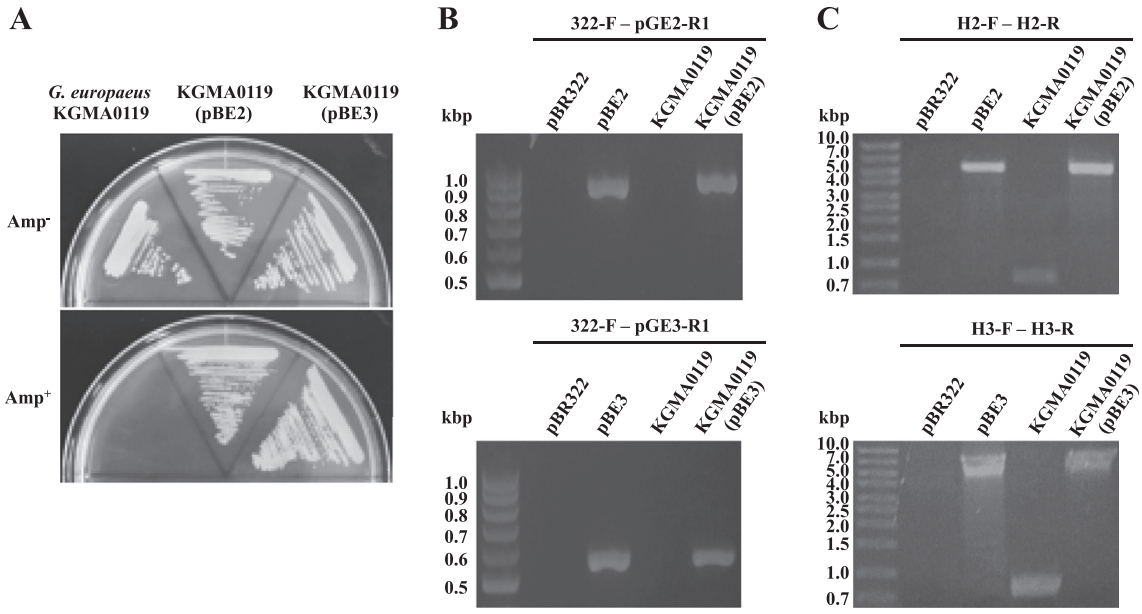

FIG. 4. Ampicillin resistance and genotypes of *G. europaeus* strains harboring pBE2 or pBE3. (A) Ampicillin resistance of KGMA0119 (pBE2) and KGMA0119 (pBE3). KGMA0119 (wild-type), KGMA0119 (pBE2) and KGMA0119 (pBE3) were streaked onto YPD agar plate lacking (upper plot) or containing (lower plot) ampicillin. (B, C) Genotyping demonstrations of KGMA0119 (pBE2) and KGMA0119 (pBE3). (B) The junction of pBR322 and pGE2 in pBE2 (0.9 kb), or that of pBR322 and pGE3 in pBE3 (0.6 kb) was PCR-amplified from the plasmids isolated from KGMA0119 and its derivatives (KGMA0119 (pBE2) and KGMA0119 (pBE3)) using primer pairs 322-F – pGE2-R1 (upper panel) or 322-F – pGE3-R1 (lower panel), respectively. (C) A region including the HindIII site in pGE2 or pGE3 (0.8 kb), and pBR322 backbone in pBE2 or pBE3 (5.2 kb) were PCR-amplified from the plasmids isolated from KGMA0119 and its derivatives (KGMA0119 (pBE2) and KGMA0119 (pBE3)) using respective primer pairs H2-F – H2-R (upper panel) or H3-F – H3-R (lower panel). As references, purified pBR322, pBE2 and pBE3 were also used as templates. See Fig. 2 for the annealing position of each primer.

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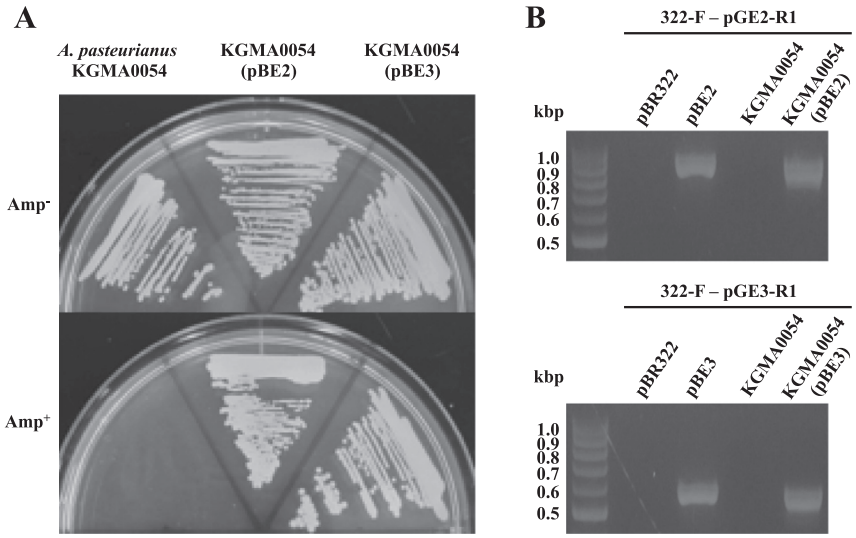

FIG. 5. Ampicillin resistance and genotypes of *A. pasteurianus* strains harboring pBE2 or pBE3. (A) Ampicillin resistance of KGMA0054 (pBE2) and KGMA0054 (pBE3). KGMA0054 (wild-type), KGMA0054 (pBE2) and KGMA0054 (pBE3) were streaked onto YPD agar plate lacking (upper plot) or containing (lower plot) ampicillin. (B) Genotyping demonstration of KGMA0054 (pBE2) and KGMA0054 (pBE3). The junction of pBR322 and pGE2 in pBE2 (0.9 kb), or that of pBR322 and pGE3 in pBE3 (0.6 kb) was PCR-amplified from the total DNA isolated from KGMA0054 and its derivatives using primer pairs 322-F – pGE2-R1 or 322-F – pGE3-R1, respectively. As references, purified pBR322, pBE2 and pBE3 were also used as templates. See Fig. 2 for the annealing position of each primer.

native cryptic plasmids are not known, however their loss from the cell can also induce changes in some bacteria (19). Specific gene disruption or plasmid-curing experiments would provide further information about the effects of plasmid genes on cell growth (33).

Sequencing analyses revealed that three native plasmids have recognition sites of EcoRI, and that pGE1 and pGE2 further have those of PstI and KpnI, respectively. However, these enzymes appeared not to digest the plasmids (Fig. 1). Draft genome analysis of the type strain LMG 18890^T (11) has predicted that *G. europaeus* possesses several restriction-modification (RM) systems, suggesting that the recognition sites of these restriction enzymes on the plasmids from the strain KGMA0119 are methylated by the intrinsic RM systems.

Several recombinant plasmids have previously been constructed from those isolated from AAB and used as shuttle vectors with *E. coli* (14,34,35). Some of those constructs had relatively broad host ranges and are replicable in *Acetobacter* and *Gluconacetobacter* (14,36). The present study indicated that the recombinant plasmids pBE2 and pBE3 (Fig. 2E and F) are useful as shuttle vectors between *E. coli* and *G. europaeus* (Fig. 4). The plasmids were replicable in *A. pasteurianus* (Fig. 5), and our results demonstrated their suitability as genetic tools to transform this bacterium, which has been widely used in the traditional production of vinegar. Natural and recombinant plasmids can have relatively broad host ranges (14,34–36). Thus, two recombinant plasmids constructed in this study may be replicable in other AAB such as *Gluconobacter* species (18). Our results indicated that pGE2 and pGE3 co-exist in the host cell (Fig. 1). Their derivatives pBE2 and pBE3 would be also compatible since their replication origins are independently functional as well as the corresponding parental plasmids. In the present work, β-lactamase gene was used as a selection marker to construct the shuttle vectors (Fig. 2E and F). By introducing another selection marker to pGE2 or pGE3 to confer different antibiotics resistance, such derivatives of pGE2 and pGE3 would be a powerful compatible tool to archive genetic modification in AAB. A recombinant plasmid consisting of the linearized pGE1 and pBR322 was constructed and introduced into strain KGMA0119, however, no ampicillin resistant cells were obtained. This may be attributed to the disruption of ORF1-1 (encoding a probable mobilization protein) by ClaI or SphI digestion (Fig. 2A), resulting in the failure of plasmid transfer to a recipient cell. Further trials using other restriction sites besides ClaI and SphI would be required to construct a shuttle plasmid.

ORF2-3 in pGE2 was predicted to encode for Kid (RNase), which is one of the components involved in maintaining plasmid stability within a cell. Plasmids possess a post-segregational killing (PSK) system (30,31) that encodes a stable toxin (e.g., Kid) and an unstable antitoxin (e.g., Kis, a killing-suppressor of Kid). Loss of the toxin encoding plasmid causes a lack of Kis and subsequent Kid activation, resulting in the selective elimination of plasmid-free cells (30,31). A recent study reported that Kid is activated in cells that are about to lose their plasmid. The Kid toxin uncouples plasmid replication from cell division by degrading mRNAs encoding proteins for the latter process to promote plasmid retention (37). We consider that pGE2 and its derivatives would be stably maintained in cells, and able to undergo *in trans* expression and genetic complementation.

Many plasmids maintain their copy number at low levels to minimize the metabolic load on their hosts (2,4,5). Fluctuations in PCN during the life cycle of *Bacillus* species have been reported, and support the findings of this study. The PCNs varied during the different stages of the growth phase for *B. cereus* and *B. thuringiensis*, and reached their maximum at the mid- or late-logarithmic stage (9,10). In those *Bacillus* strains, the elevated copy numbers of plasmids (gene dosage) at the specific growth phases might be a prerequisite for the transfer of sufficient copies to a metabolically inactive spore during sporulation (9), or for the enhanced expression of an insecticidal crystal protein (10). Studies on PCN variation and fluctuation in other species of bacteria are limited, and the mechanisms that regulate the abundance of plasmids in cells remain unclear. In this study, PCNs of pGE1 and pGE3 appeared to be influenced by the concentration of acetic acid in the medium and the stage of the growth phase (Fig. 3). The findings suggest that a higher concentration of acetic acid (more than 1.0%) can trigger an increase in PCN during the logarithmic phase of growth, and that some of the genes present in pGE1 and pGE3 might be involved in acetic acid metabolism or resistance. Specific pathways to catabolize acetic acid are induced as a response to the presence of this substrate (38,39), and resistance to this organic acid is closely related to ethanol oxidization (40,41). Further investigations are needed to resolve the significance of variation in the copy number of cryptic plasmids in *G. europaeus*.

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