Journal of Bioscience and Bioengineering
VOL. 119 No. 6, 661–668, 2015

www.elsevier.com/locate/jbiosc

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

Naoki Akasaka,[1] Wiwik Astuti,[2] Yuri Ishii,[2] Ryota Hidese,[3] Hisao Sakoda,[1] and Shinsuke Fujiwara[2,3,*]

Institute of Applied Microbiology, Marukan Vinegar Co. Ltd., 5-6 Koyo-cho West, Higashinada-ku, Kobe, Hyogo 658-0033, Japan,[1] Department of Bioscience, Graduate School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan,[2] and Research Center for Environmental Bioscience, Graduate School of Science and Technology, Kwansei-Gakuin University, Sanda, Hyogo 669-1337, Japan[3]

Received 20 August 2014; accepted 4 November 2014
Available online 6 January 2015

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.

* Corresponding author at: Department of Bioscience, Graduate School of Science and Technology, Kwansei-Gakuin University, Sanda, Hyogo 669-1337, Japan. Tel.: +81 795 65 8300; fax: +81 795 65 9077.
E-mail address: fujiwara-s@kwansei.ac.jp (S. Fujiwara).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.11.003

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