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Characterization of unique cryptic plasmids in *Komagataeibacter europaeus*

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[Background] *Komagataeibacter europaeus* is one of acetic acid bacteria (AAB) that is widely used for the industrial production of vinegar. Recently, a targeted gene disruption system using an endogenous *pyrE* gene as a selectable marker has been established[1]. However, despite its significance in vinegar production, various genetic aspects of *K. europaeus*, such as native cryptic plasmids, still remain unclear. Genome analyses of other AAB have revealed the presence of numerous native cryptic plasmids[2], and several recombinant plasmids have been constructed from them and used as shuttle vectors with *Escherichia coli*[3]. Native plasmids from *K. europaeus* and their derivatives are hence expected useful for genetic engineering of the AAB.

[Experimental Method] Native cryptic plasmids were isolated from *K. europaeus* KGMA0119 (wild-type) by alkaline method followed by cesium chloride (CsCl) density gradient centrifugation. The plasmids were digested with several restriction enzymes, and the obtained digests were sequenced and then assembled. The complete sequences of three plasmids were determined and designated pGE1 (2.5 kb), pGE2 (7.2 kb), and pGE3 (5.5 kb). Plasmid copy numbers (PCNs) were measured by real-time quantitative PCR (RTQ-PCR) at various growth phases under several growth conditions. To construct shuttle vectors between *E. coli* and AAB, pGE2 and pGE3 were linearized with HindIII and ligated with pBR322, and the resulting recombinant plasmids were designated as pBE2 and pBE3, respectively. pBE2 and pBE3 were introduced into competent cells of *K. europaeus* KGMA0119, *Acetobacter pasteurianus* KGMA0054 (wild-type), and *Komagataeibacter obediens* KGMA0009 (wild-type) by electroporation[1], and ampicillin resistant cells were screened. To investigate genotypes of positive clones, PCR and Southern blot analyses were carried out. Curing experiment was conducted to obtain pGE3/pBE3-free strains by sub-culturing *K. europaeus* KGMA0119 harboring pBE3 in the absence of ampicillin seven times. To confirm the absence of pGE3/pBE3 in the positive clones, PCR and Southern blot analyses were conducted.

[Result and Discussion] Three native plasmids designated as pGE1, pGE2, and pGE3 were identified from KGMA0119 and predicted to possess 3, 8, and 4 ORFs, respectively. When KGMA0119 was cultured in a medium with 0.4% ethanol and 0.5% acetic acid, PCN of pGE1 increased from 7 copies/genome in the logarithmic growth phase to its maximum (12 copies/genome) at the beginning

of stationary growth phase and decreased to 4 copies/genome at the late stationary phase, whereas those of pGE2 and pGE3 were maintained at low levels (1-3 copies/genome) in all the growth phases. PCNs for pGE1 and pGE3 at the logarithmic phase increased to 10 copies/genome and 6 copies/genome, respectively, in response to higher concentration (1.0%) of acetic acid, suggesting that some genes encoded by pGE1 and pGE3 are involved in the resistance and catabolism of acetic acid. pBE2 and pBE3 were replicable in *E. coli*, *A. pasteurianus*, *K. obediens*, and *K. europaeus*, highlighting their advantage as shuttle vectors for AAB. A pGE3-cured strain was obtained by sub-culturing KGMA0119 harboring pBE3 in the absence of ampicillin, and designated as KGMA0119C3. Combined with pBE3, KGMA0119C3 is a potent platform for genetic engineering in *K. europaeus*.

[References]

(1) Akasaka et al, Appl. Environ. Microbiol., **79**, 7334 (2013)

(2) Azuma et al, Nucleic Acids Res., **37**, 5768 (2009)