Development of an interspecies genome recombination method using cell fusion and CRISPR/Cas9 techniques

Tomoya MAEDA

RIKEN Center for Biosystems Dynamics Research

Report: A *Corynebacterium glutamicum* strain carrying a single-copy plasmid sequence from *Escherichia coli* was constructed. This strain was then fused with an *Escherichia coli* strain using the heterologous protoplast fusion technique. The obtained fusant strain was confirmed to carry both *E. coli* and *C. glutamicum* genomes; however, the introduced *C. glutamicum* genome was unstable in the fusant strain.

Research aims

Acquisition of heterologous genes and genome segments i.e. horizontal gene transfer (HGT) accelerates evolution in the natural world. However, construction of recombinant strains carrying functional large insertions from foreign genome segments is still challenging. In this study, I aimed to develop a methodology for constructing recombinant strains carrying large insertion sequences derived from a heterologous genome.

Methods

Heterologous protoplast fusion

A protoplast is a cell whose cell wall has been removed, and in which the cytoplasmic membrane is the outermost layer. Protoplasts can be obtained by treating cells with lytic enzymes such as lysozyme to remove the cell wall. When two or more protoplast cells come into contact, they adhere to one another spontaneously to form hybrid protoplasts with heteroplasmic cytoplasm and two fused parent nuclei [1]. *E. coli* protoplast cells were generated by treatment with 30 mg/ml lysozyme, followed by incubation in GP medium containing 500 μg/ml ampicillin. However, *C. glutamicum* protoplast cells could not be generated using the same method as *E. coli* due to the presence of a thick mycolic acid layer. Therefore, instead of protoplast formation, *C. glutamicum* L-form cells were obtained. Briefly, 10^9 *C. glutamicum* cells were spread onto medium containing BHI/MSM buffer (20 mM $MgCl_2$, 0.5 M Sucrose, 20 mM maleic acid, pH 7), 400 μg/ml D-cycloserine, and 2% agar and incubated for 5 days at 30°C. Heterologous cell fusion was then performed as follows. *E. coli* protoplast cells and *C. glutamicum* L-form cells were suspended into 40% PEG6000 buffer (40% PEG6000, 20 mM $MgCl_2$, 0.5 M Sucrose, 20 mM maleic acid, 10 mM $CaCl_2$, 5% DMSO, pH7) and incubated at 25°C for 5 min. The fused cells were further

subjected to protoplast regeneration by suspending in GP medium containing 1% BSA and incubating at 30°C for 2 days.

Site-directed recombination of a large heterologous genome segment using protoplast fusion and the CRISPR/Cas9 system

To insert the homologous arms of the recombination sites into the genome of an *E. coli* strain, approximately 1 kbp of NCgl1683 and NCgl1768 flanking regions derived from the *C. glutamicum* ATCC13032 strain was inserted into the DH5α strain. The newly constructed strain was named ECH2. Two gRNA genes targeting the NCgl1683 and NCgl1783 genes were inserted into the ECH2 genome and the resulting newly constructed strain was named ECHG2. A plasmid pRedCas9DG was constructed to express Cas9 and λRED and the ECH2 strain was transformed using this plasmid. Next, the *cglIR gene* encoding a type II restriction endonuclease was disrupted by inserting the chloramphenicol resistance gene, *cat*, into the ATCC13032 strain genome. The newly constructed strain was named as the *cglIR*::*cat* strain. Heterologous genomic recombination between *E. coli* and *C. glutamicum* was then attempted by the expression of gRNAs, Cas9, and λRed recombinase during protoplast fusion.

Results

Protoplast cells of ECHG2/pRedCas9DG and L-form cells of *cglIR*::*cat* were fused using the protoplast fusion technique. However, recombination of the ~100 kbp genome segment derived from the *C. glutamicum* genome into the *E. coli* genome was not observed. On the contrary, markerless gene deletions and heterologous gene insertion using this pRedCasDG plasmid were successful. This suggests a short duration of the co-existence of two heterologous genomes during heterologous protoplast fusion. To replicate the *C. glutamicum* genome as a large single-copy plasimid in *E. coli* cells, an *E. coli* single-copy plasmid derived from miniF was inserted into the *cglIR*::*cat* genome. To observe the co-existence of two heterologous genomes, the mCherry and EGFP genes were inserted into the *E. coli* and *C. glutamicum* genomes respectively. The newly constructed cells were then subjected to a heterologous protoplast fusion experiment. The obtained single colonies were isolated and examined by fluorescent microscopy and PCR to determine whether the fusant clones carried two heterologous genomes (Fig. 1). As shown in Fig. 1, the fusant cells expressed both mCherry and EGFP and carried both *E. coli* and *C. glutamicum* genes (randomly selected genes *mreB* and *leuA*, respectively). However, after subculturing three times, the fusant cells were confirmed to have lost the *C. glutamicum* genome. These results suggest that the miniF-derived replication system can maintain the heterologous genome for several generations, even though maintenance of the heterologous genome is unstable.

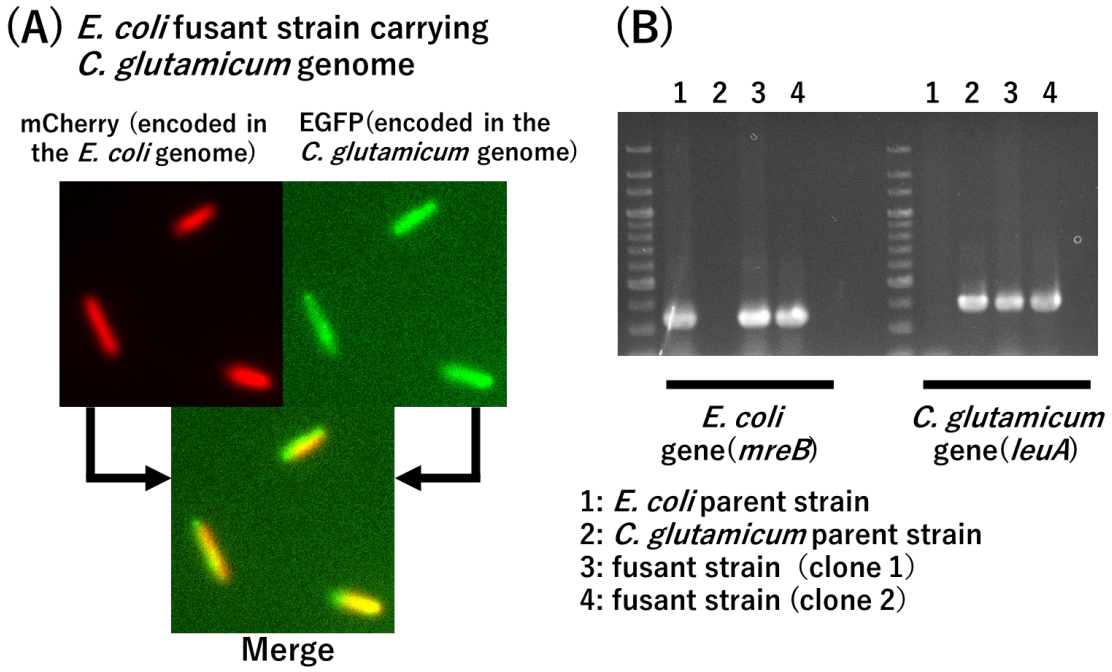

Fig. 1. Heterologous protoplast fusion of *E. coli* and *C. glutamicum*. (A) Fluorescence microscopy of the *E. coli* and *C. glutamicum* fusant cells. (B) PCR product derived from the fusant cells.

Conclusion

In this study, I showed that the *C. glutamicum* genome can be introduced into *E. coli* cells using the heterologous protoplast fusion technique. The results suggested that the miniF-derived replication system can maintain the heterologous genome for several generations in *E. coli*, even though the maintenance of the heterologous genome is unstable. Therefore, developments in the methodology to maintain the heterologous genome stably and for site-directed recombination between two heterologous genomes are needed in future studies.

References

1) Hopwood, D.A. (1981) Genetic studies with bacterial protoplasts. *Annu Rev Microbiol.* **35:** 237–272.