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Genomic engineering of *Lactococcus lactis* IL1403

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Research aims

A set of single-gene deletion mutants of all nonessential genes in *Escherichia coli* K-12 and *Bacillus subtilis* 168 have been constructed. In addition, we previously constructed a series of consecutive chromosomal deletion mutants of *E. coli*. From these results, around 300 essential genes for growth in each organism were estimated[1–3]. In the case of *E. coli*, which has around 4,400 genes on the chromosome, tens of thousands mutants would be needed to disrupt all of the nonessential genes by using random mutagenesis methods. Therefore simple assay have to be used for screening of the mutant library. On the other hand, a set of 4,100 single gene knockout mutants, which includes mutants defective in each of the nonessential genes, can cover all of the nonessential genes of *E. coli*. Furthermore, 551 chromosomal deletion mutants cover the all of *E. coli* genome. Since these mutant libraries can cover the nonessential genes by less number of mutants, it is possible to screen them by complicated assay methods. Indeed, these mutant libraries were applied to various screening for genes, for instance, genes involved in biofilm formation, swarming, or ATP generation.

Lactic acid bacteria (LAB) are a heterogeneous group of microorganisms able to convert carbohydrates into lactic acid. Useful LAB are industrially important that are used all over the world in large variety of industrial food fermentations. In addition, LAB are also used to produce a material for poly lactic acid which is a kind of bioplastic, and are featured as probiotic bacteria. From such a point of view, many complete genomic sequences and genome-scale metabolic engineering of LAB have been reported. However, although random mutagenesis libraries of LAB were constructed by using transposon or insertion sequence, to our knowledge, there is no report about a set of single-gene knockout mutants or consecutive chromosomal deletion mutants. *Lactococcus lactis* is a non-pathogen bacterium and used by dairy industry as starters in cheese fermentation. *L. lactis* strain IL1403 was the first LAB whose complete genome sequence has been determined[4]. We constructed an efficient chromosomal deletion system for this bacterium and are trying to construct a set of consecutive chromosomal deletion mutants.

Methods and Results

The chromosomal deletion mutants were constructed with a *L. lactis* homologous recombination system using temperature sensitive replication plasmid (Fig. 1A). A vector plasmid pGhT000 was derived from pGh9[5]. To construct pGhT000, a restriction enzyme *Xcm*I site in an erythromycin resistance gene of pGh9 was point mutated to remove the site without amino acid substitution. Next, *upp* gene from *L. lactis* IL1403 was cloned into the pGh9ΔXcmI to construct pGhT00. The *upp* gene encodes an uracil phosphoribosyltransferase enzyme catalyzing the conversion of uracil to UMP. Whereas wild type *L. lactis* is sensitive to growth inhibition by the toxic base analog 5-fluorouracil (5FU), a deletion mutant of *upp* is resistant[6]. Therefore the *upp* gene can be used as a negative selection marker. Then, a kanamycin resistant gene flanked by *Xcm*I sites was cloned into the multicloning site of pGhT00 to construct pGhT000. The plasmid was designed to produce single thymidine overhangs at both ends of the linearized fragment when digested with *Xcm*I, and thus enabled TA-cloning of PCR products which have adenosine overhangs

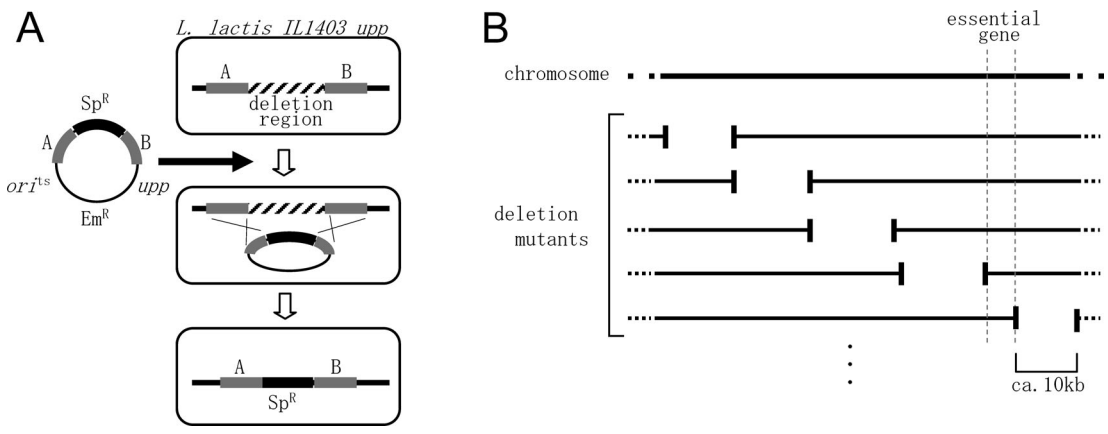

Fig. 1. (A) The chromosomal deletion system. (B) Schematic drawing of a series of consecutive chromosomal deletion mutants.

at both ends. To make a deletion, a DNA fragment in which chromosomal regions flanking the region to be deleted were joined to the ends of a spectinomycin resistance gene was constructed by two round PCR, and it was cloned into pGhT000. The resulting plasmid was then introduced into *L. lactis* IL1403 *upp*. A spectinomycin resistant (Sp^R) transformant was cultured at 28°C for 150 min, followed by cultured at 35°C for 150 min. An aliquot of the culture was plated and incubated at 35°C to obtain SpR recombinant. Then, an aliquot of overnight culture of the Sp^R recombinant at 28°C were plated and incubated at 35°C to obtain Sp^R and 5FU resistant clones, which were the expected chromosomal deletion mutants. This system allowed for efficient construction of deletion mutants of *L. lactis* IL1403. The obtained recombinants were confirmed for the presence of the expected deletion by PCR.

To construct a series of consecutive chromosomal deletion mutants efficiently, it is important that the region to be deleted should not include essential genes. However, only few essential genes of *L. lactis* are known. As described above, 271 genes of *B. subtilis* 168 was estimated as essential genes. Then, we compared the all genes of *B. subtilis* 168 and *L. lactis* IL1403, and identified around 300 genes of *L. lactis* as candidates for essential genes, which include RNA coding genes. Considering the estimated number of essential genes and chromosomal length (2.4 Mb) of *L. lactis* IL1403, the length of deletion to be made in each mutant was set to around 10 kb (Fig. 1B). We are progressing to construct the series of consecutive deletion mutant of *L. lactis* IL1403.

Conclusion

A plasmid vector pGhT000 for deletion of *L. lactis* IL1403 chromosome was constructed. The vector plasmid contains a temperature sensitive replication origin, *upp* gene from *L. lactis* IL1403 as a negative selection marker, and restriction enzyme *XmaI* sites for TA-cloning of PCR product. By using the plasmid with the ts replication origin and negative selection marker, we succeeded in efficiently constructing chromosomal deletion mutants of *L. lactis* IL1403. Essential genes of *L. lactis* were estimated from those of *B. subtilis*. Construction of a series of consecutive chromosomal deletion mutants of *L. lactis* IL1403 is progressing.

References

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