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Shuttle Vectors Derived from pN315 for Study of Essential Genes in *Staphylococcus aureus*

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Using the *par* to *rep* region of the 24653 bp plasmid pN315, which is present in *Staphylococcus aureus* strain N315, we constructed three vectors that can be shuttled between *Escherichia coli* and *S. aureus* and maintained stably in *S. aureus*. Due to plasmid incompatibility, the resident plasmid in *S. aureus* cells can be replaced via transformation with an entering plasmid, which carries a different drug resistance gene. To evaluate the applicability of this plasmid-based approach for identifying genes essential for *S. aureus* cell growth, the chromosomal *mraY* gene, which is involved in peptidoglycan biosynthesis, was deleted in cells harboring a resident plasmid with an intact *mraY* gene. The resultant disruptant was then transformed with an empty vector. Cells with a chromosomal *mraY* deletion but lacking the plasmid supplying *mraY* could not be recovered, suggesting that *mraY* is indispensable for staphylococcal cell growth or viability. In contrast, other two genes were shown to be dispensable by this system. Thus, the pN315-based plasmids appear to be useful for studying genes essential for *S. aureus* cell growth.

Key words *Staphylococcus aureus*; plasmid shuffling; incompatibility; *mraY*; pN315

Plasmids that replicate in bacterial cells can be classified into incompatibility groups, and those within a group will not coexist in a single cell. In general, genes and loci required for plasmid replication, copy number control, and partition are also involved in incompatibility.^{2–4)} Thus, replacement of a resident plasmid with an entering can be accomplished when the two plasmids belong to the same incompatibility group but have a different antibiotics resistance marker gene. This method is called “plasmid shuffling” and has been used to carry out site-directed mutagenesis and screens for genes essential for cell growth in both *Escherichia coli* and yeast.^{5–7)} This system can be used to determine the essential nature of a target gene via the following three steps. First, the target gene on the chromosome is disrupted in cells harboring a plasmid that contains the intact target gene. Second, the cell is transformed with an empty vector that is incompatible with the resident plasmid and contains a different drug resistance marker gene from the one of the target gene-containing plasmid. Finally, if cells carrying the entering vector cannot be recovered (*i.e.* cells only grow in the presence of either the chromosomal or a plasmid-based copy of the target gene), then one can conclude that the target gene is required for cell growth. One of strong merits of this strategy is that genes essentiality can be examined under various culturing conditions such as temperature, osmosis, pH, salts, drugs, and host or environmental factors. More, conditional lethal mutations for above factors could be isolated if error-prone polymerase chain reaction (PCR)-amplified genes are cloned and used for shuffling.⁵⁾ Although the method has been successfully used in other species, to the best of our knowledge there is no previous report that the method can be used in *Staphylococcus aureus*. *S. aureus* is a pathogenic bacterium that causes opportunistic infections and has received increasing attention as it has rapidly gained resistance to various antibiotics, which makes it difficult to treat infections.⁸⁾ Gaining a better understanding of genes and proteins in *S. aureus* is important to develop treatments

for *S. aureus*.

Due to the complete genome sequencing of clinically important Staphylococcal strains, number of larger plasmids has been revealed. Among them, pN315 is a 24653 bp plasmid harbored in multi-drug resistant *S. aureus* N315 strain⁹⁾ and belongs to pSK1-type replicon being presumed to replicate via the theta mode.^{10,11)} In this study, we constructed a set of pN315-based vectors and used the plasmid shuffling assay to study the representative example of the *mraY* gene and as expected, found that *mraY* is required for cell growth or viability in *S. aureus*. Our initial findings suggest that the pN315-based plasmids are suitable for identification of genes essential for staphylococcal cell growth.

MATERIALS AND METHODS

Genetic Manipulation of Bacteria and Reagents *S. aureus* and *E. coli* cells were cultured at 37 °C in LB medium [1% (w/v) bactotryptone (Difco), 0.5% (w/v) yeast extract (Difco), and 1% (w/v) NaCl]. Transformation of plasmids into *S. aureus* cells was performed by electroporation as described previously.¹²⁾ *S. aureus* cells were cultured in the presence of chloramphenicol (12.5 µg/ml), kanamycin (25 µg/ml), erythromycin (10 µg/ml), or phleomycin (20 µg/ml, Sigma); *E. coli* cells were in the presence of chloramphenicol (25 µg/ml), kanamycin (25 µg/ml), or ampicillin (50 µg/ml), as appropriate. A ligation kit (ver. 2) was purchased from Takara; and the KOD DNA polymerase from Toyobo.

Bacterial Strains and Plasmids *S. aureus* strains and plasmids used in this study are listed in Table 1. *E. coli* strain JM109 (Takara) was used for cloning of the shuttle vectors and suicide targeting plasmids.

To construct pM101 (Fig. 1A), the *SAP031(par)-SAP001(rep)-SAP002* region (2831 bp) of pN315 was amplified using primers mini-P1 (5'-ACGGCATTATGGCTTTT-3') and mini-P2 (5'-TTCCTGCAGGGCTTTTCTTA-3'), and was inserted into the *Sma*I site of pSF151.¹³⁾

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The resulting plasmid, which contains the cloned *rep* gene and a kanamycin resistance gene in the same orientation, was named pM101. Two derivatives of pN315 were also constructed, pM102 and pM103. To construct pM102 (Fig. 1B), the *SAP031* to *SAP002* region described above was inserted into the *HincII* site of pCM, which also contains the *E. coli* pUC origin and a chloramphenicol resistance (*cat*) gene as described below. The resultant plasmid pM102 contains the cloned *rep* gene and the *cat* gene in the reverse orientations. The plasmid pM103 (Fig. 1C) was constructed by inserting the *cat* gene region from pND50,¹⁴ which was amplified by PCR using primers Cm-P1 (5'-GGGGGCGTCGTCTGCATCATCGCATCTGT-3') and Cm-P2 (5'-GGGGGCGTCTAACGGGCGAGGTAGTGAC-3') and then digested with *HinII*, into the *HinII* site of pM101, disrupting the kanamycin resistance gene in the parent vector.

The pCM plasmid was constructed from pND50 by deleting the two *S. aureus* pUB110-derived origin regions in two

steps. First, one was removed from pND50 by digestion with *AlfII* and *AvaII* and self-ligation. The other was removed using a PCR approach with the primers pCK20-P1 (5'-GATCTGGAGCTGTAATATAAAACC-3') and pCK20-P2 (5'-TGAGCAAAAGGCCAGCAAAAGG-3') followed by self-ligation to circularize the PCR product. To construct the pM103-*mraY* plasmid, we first amplified *mraY* by PCR using the primers *mraY*-P5 (5'-GGGGTTCGACGAATGGCTACATTAATAAGGGTGT-3') and *mraY*-P6 (5'-GGGGGATCCTTGCCAAACCGACGACTAAT-3') with RN4220 genomic DNA as a template, then digested the PCR product with *Sall* and *BamHI*, and finally inserted the *mraY* fragment into *Sall*, *BamHI*-digested pM103. To construct the pM103-*SA0172* plasmid, amplified *SA0172* regions using primers *SA0172*-P5 (5'-GGGGGTACCATGCTGGGCTAGCGATTAAAC-3') and *SA0172*-P6 (5'-GGGGGTACCGACCAATTAATAGGTTTTCAT-3') was digested with *KpnI* and inserted into the *KpnI* site of pM103. To construct the pM103-*SA1839* plasmid, we used primers of *SA1839*-P5 (5'-GGGGGTACCAAGCCCATAATTATTGCTCCTTC-3') and *SA1839*-P6 (5'-GGGGGTACCAACACGGATTATCGTCGTGTGATTTCG-3'); those also contained the *KpnI* site in order to insert the resulting PCR fragment into the *KpnI* site of pM103.

The pKE516 plasmid was constructed by replacement of a *cat* gene of pKE515¹⁵ with an erythromycin resistance gene in two steps. First, the *cat* gene open reading frame of pKE515 was deleted by PCR using primers of pKE-Cm-L (5'-ATCATATAAATTCCTCCTAAATTTTAAATC-3') and pKE-Cm-R (5'-ATCTTTTATGATTGTTGGGAGTGAATTAAAT-3') and following self-ligation, which produced the *EcoRV* site. Second, the erythromycin resistance gene open reading frame from pMUTIN-T3¹⁶ was amplified by PCR with primers of *Erm*-P1 (5'-GAAGGAGTGATTACATGAACAAAA-3') and *Erm*-P2 (5'-CCAAATTTACAAAAGCGACTCA-3') and inserted into the *EcoRV* site. In the resulting plasmid, pKE516, the erythromycin resistance open reading frame is in the same orientation as the preexisting *cat* open reading frame.

Deletion of the Chromosomal *mraY*, *SA0172*, or *SA1839* Gene The chromosomal *mraY* gene was replaced with a phleomycin resistance gene as described previously,¹⁷

Table 1. Bacterial Strains and Plasmids in This Study

Strain or plasmid	Description ^{a)}	Source or reference
Strains		
RN4220	<i>S. aureus</i> strain derivative from NCTC8325, restriction negative	Novick R. P. 1991
WM112	<i>mraY::phleo</i> as RN4220	This study
Plasmids		
pN315	A 24653 bp plasmid found in <i>S. aureus</i> N315 strain	Kuroda M., et al. 2001
pM101	<i>S. aureus-E. coli</i> shuttle vector, Km ^r	This study (Fig. 1A)
pM102	<i>S. aureus-E. coli</i> shuttle vector, Cm ^r	This study (Fig. 1B)
pM103	<i>S. aureus-E. coli</i> shuttle vector, Cm ^r	This study (Fig. 1C)
pM103- <i>mraY</i>	pM103 carrying the intact <i>mraY</i> gene	This study
pM103- <i>SA0172</i>	pM103 carrying the intact <i>SA0172</i> gene	This study
pM103- <i>SA1839</i>	pM103 carrying the intact <i>SA1839</i> gene	This study
pND50	<i>S. aureus-E. coli</i> shuttle vector, Cm ^r	Yamagishi J., et al. 1996
pCM	<i>E. coli</i> pUC plasmid, Cm ^r	This study
pUC19- <i>upp-phleo</i>	pUC19 carrying the <i>upp-phleo</i> cassette	Fabret C., et al. 2002
pKE515	<i>S. aureus-E. coli</i> shuttle vector, Cm ^r , Amp ^r	Li Y., et al. 2004
pKE516	<i>S. aureus-E. coli</i> shuttle vector, Erm ^r , Amp ^r	This study
pSF151	<i>E. coli</i> pUC plasmid, Km ^r	Tao L., et al. 1992
pMUTIN-T3	<i>E. coli</i> pUC plasmid, Erm ^r , Amp ^r	Vagner V., et al. 1998

a) Abbreviations used are: Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Erm^r, erythromycin resistance.

Fig. 1. The Plasmid Vectors pM101, pM102, and pM103

(A) To construct the pM101 plasmid, the *SAP031*(*par*)-*SAP001*(*rep*)-*SAP002* region of pN315 (2831 bp) was amplified by PCR and inserted into the *SmaI* site of pSF151. (B) To construct pM102, the *par*-*SAP002* region was inserted in the *HincII* site of pCM. (C) The pM103 plasmid was generated from pM101 by inserting the chloramphenicol resistance gene (Cm) into the *HinII* site in the kanamycin resistance gene (Km). Arrows indicate the direction of transcription of each gene.

with some modifications. First, an approximately 1.5 kbp DNA fragment of upstream or downstream of the *mraY* gene was amplified by PCR using the primers *mraY*-P1 (5'-TCAAGAAGGTGCTTTTGATCC-3') and *mraY*-P2 (5'-TATTGGATCCTCTCCTCTTCTTAATCCAGACTTTT-3') or *mraY*-P3 (5'-GTTCAGCAATCGCGTTTATGGATTGAGTGC-3') and *mraY*-P4 (5'-GCCTTTGACCTTTTTC-TTTC-3'), respectively. Second, the phleomycin resistance gene region was amplified using primers that had sequence complementary to *mraY*-P2 or *mraY*-P3 and to the pUC19-*upp-phleo* template sequence. Next, the three amplified fragments were connected by joining PCR¹⁷⁾ and the resultant fragment was inserted at the *Sma*I site of pSF151.

The targeting vector obtained was introduced into RN4220 cells harboring pM103-*mraY* and kanamycin resistant transformants at 50 µg/ml, generated *via* first site homologous recombination, were selected. From these cells, kanamycin-sensitive strains with both phleomycin resistance and chloramphenicol resistance, formed *via* a second site homologous recombination event, were selected and named WM112/pM103-*mraY* (see Fig. 3A). Deletion of the chromosomal *mraY* gene in WM112/pM103-*mraY* was confirmed by PCR using the primers *mraY*-P1 and *mraY*-P4 (data not shown).

Deletion of each of the chromosomal *SA0172* and *SA1839* genes was done in the same manner as the *mraY* gene. Primers used to construct a targeting vector for *SA0172* were *SA0172*-P1 (5'-CGTGGGTAAAGGACCCAATA-3'), *SA0172*-P2 (5'-TATTGGATCGCTAGCCAGCATCCTTATTC-3'), *SA0172*-P3 (5'-GTTCAGCAATCGTATTCGGGGACAAAAACAA-3'), and *SA0172*-P4 (5'-TGCGCATCATCTATCTCAGG-3'). For *SA1839*, primers used were *SA1839*-P1 (5'-CTGTTGCAGTTGTCTGACCG-3'), *SA1839*-P2 (5'-TATTGGATCCAGCGACGATAATCCGTGTGT-3'), *SA1839*-P3 (5'-GTTCAGCAATCGTCACGAGACACCTCCCTTTT-3'), and *SA1839*-P4 (5'-AGCACCATTACGAGCAGGAT-3'). Each of the targeting vectors obtained was introduced into RN4220 cells harboring pM103-*SA0172* or pM103-*SA1839*, respectively, and double crossover homologous recombinants were selected. Replacement of each the chromosomal gene with the phleomycin resistance gene was confirmed by PCR (data not shown).

Measurement of the Percentage of Plasmid Maintenance The cells harboring a target plasmid cultured in liquid LB medium containing appropriate antibiotics at 37 °C overnight were defined as the starting test culture sample (*i.e.* the 1st generation). The starting cultures were diluted 100 to 1000-fold with LB medium without antibiotics. Dilution and cultivation were repeated to maintain cells in the logarithmic cell growth phase. Samples were isolated, successfully diluted, plated on LB plates in the presence or absence antibiotics, and cultured at 37 °C overnight. The number of colonies was counted and then what proportion of cells maintained the plasmid was determined.

RESULTS

Development of a Pair of Plasmids for Quick Shuffling We first asked if replacement *via* incompatibility of pKE515-based plasmids in *S. aureus* cells can take place in transformants after overnight cultivation. Based on homology search, its initiator protein may act by introducing a site-specific nick

in the leading strand replication origin, and it may replicate *via* rolling circle mode and be maintained without active partitioning system.²⁾ For this purpose, pKE516, in which the *cat* gene of pKE515¹⁵⁾ was replaced with the erythromycin resistance open reading frame, was electroporated into RN4220 cells harboring pKE515, and transformants were selected by erythromycin. We found that more than 90% of the resultant erythromycin-resistant transformants also had chloramphenicol resistance, suggesting that the resident pKE515 plasmid co-exists in RN4220 cells with the newly introduced pKE516 plasmid under the conditions we tested. Loss of the resident pKE515 *via* incompatibility did occur, but it required continuous cultivation of more than 40 generations in liquid medium containing erythromycin but not chloramphenicol (data not shown). Based on co-existence of the plasmids, we concluded that the rolling circle replication-type pKE515-based plasmids are inappropriate for the plasmid shuffling assay.

Next, we turned our attention to another incompatibility group plasmid, the pN315 plasmid, which was identified in the clinically isolated methicillin-resistant *S. aureus* (MRSA) strain N315. The nucleotide sequence of the 24653 bp plasmid had already been determined and putative function of proteins encoded in pN315 had been assigned by a comparative analysis.⁹⁾ From the previous studies, SAP001 protein is estimated to be Rep protein for plasmid replication initiation enabling the replicon to replicate *via* theta mode and SAP031 protein is Par protein for stable maintenance of the plasmid.^{10,11,18,19)} However, it is unknown if plasmid shuffling occurs quickly or requires a longer time-period when a DNA region for replication and partition of the plasmid is subcloned in order to construct shuttle vectors.

To construct a shuttle vector capable of being replicated and maintained in both *E. coli* and *S. aureus* using pN315 *rep* and *par* region, we amplified the *SAP031-SAP001-SAP002* DNA region by PCR and cloned the region into an *E. coli* pUC-derived plasmid. In this amplified region, the *SAP031* (*par*) gene is upstream of the *SAP001* (*rep*) gene and the genes are in opposite orientations and the 106 amino acid SAP002 protein, which is downstream of *SAP001*, overlaps with what is the last 20 bp of the *SAP001* open reading frame (Fig. 1). The SAP002 protein has amino acid similarity to Rep proteins, while it is a truncated form. The resultant plasmid, pM101 (Fig. 1A), can be propagated in *E. coli*. To test the ability of pM101 to replicate and partition in *S. aureus*, pM101 was electroporated into *S. aureus* RN4220 cells and presence of the plasmid was confirmed based on recovery of kanamycin-resistant transformants. Extraction and agarose gel electrophoresis of plasmids revealed that the transformants contained a covalently closed circular form of the plasmid. Recovered amount of the pM101 DNA from RN4220 cells was much similar to that of pUB110-derived plasmid pND50, implying that the copy number of pM101 might be 5.²⁾ Also, the plasmid was stably maintained, as more than 80% of cells carried the plasmid even after cultivation for 60 generations in the absence of kanamycin. Thus, these results suggest that the *SAP031-SAP002* region is sufficient for autonomous replication and stable maintenance of pM101 in *S. aureus*.

Next, we asked if pM101 can be used in a 'plasmid shuffling' approach to replacement of one plasmid with another.

Fig. 2. Replacement of the Resident pM101 Plasmid by pM102

pM102 (Cm^r) was electroporated into RN4220 cells harboring pM101 (Km^r) and transformants were selected on LB agar plates containing 12.5 µg/ml chloramphenicol at 37 °C for overnight. The resultant colonies were picked randomly, suspended in buffer, and grown on LB agar plates containing 12.5 µg/ml chloramphenicol (upper left), 25 µg/ml kanamycin (upper right), or without antibiotics (lower left) at 37 °C for overnight. One of Cm-resistant transformants and recipient RN4220/pM101 cells are shown.

To do this, we constructed another plasmid, pM102 (Fig. 1B), which contains the same *SAP031-SAP002* region but a different antibiotic resistance gene (namely, Cm^r). The pM102 plasmid was electroporated into *S. aureus* RN4220 cells harboring pM101 (Km^r), and transformants were selected on chloramphenicol-containing media. When the resultant sixty chloramphenicol-resistant transformants were subsequently tested for kanamycin resistance, we found that all of them tested were sensitive to kanamycin (Fig. 2). Thus, the results suggest that the resident pM101 plasmid was quickly replaced by the entering pM102 plasmid during selection of transformants, which fulfilled our requirements for use in other studies.

Study of the *mraY* and Other Two Genes Using Plasmid Shuffling Next, we asked if pN315-based plasmid shuffling can be used to examine whether specific *S. aureus* genes are essential for cell growth. We chose to analyze the *mraY* gene as a representative example, as the *mraY* gene product is involved in peptidoglycan biosynthesis^{20,21)} and thus, is an important target for the development of antibiotics.²²⁾ Additionally, we were interested to explore the requirement of *mraY* for staphylococcal cell growth, a topic that had not previously been examined.

Before disrupting the chromosomal *mraY* gene, we cloned the intact *mraY* gene and its promoter region into pM103 (Cm^r), which like pM101 and pM102 is a pN315-derived plasmid, and electroporated the resultant pM103-*mraY* plasmid into *S. aureus* RN4220 cells. Next, the chromosomal *mraY* gene was replaced with a phleomycin resistance gene via double crossover homologous recombination, and the resultant strain was named WM112/pM103-*mraY* (Fig. 3A). Then, the empty vector pM101 (Km^r) was electroporated into WM112/pM103-*mraY* cells and we tested growth of transformants on LB agar plates containing kanamycin (Fig. 3A). Kanamycin-resistant transformants were not obtained (Fig. 3B). As a control, pM101 was electroporated into RN4220 cells harboring pM103-*mraY* and as expected, more than a

Fig. 3. Plasmid Shuffling Reveals That *mraY* Is Essential for *S. aureus* Cell Growth

A schematic representation of the protocol is shown in (A). In WM112/pM103-*mraY* cells, the chromosomal *mraY* gene was replaced by a phleomycin resistance gene via double crossover homologous recombination in the presence of pM103-*mraY* (Cm^r), which contains an intact *mraY* gene. This strain was then transformed with the empty vector pM101 (Km^r) and the presence of kanamycin resistance transformants after an overnight of growth at 37 °C was assayed. The left panel of (B) shows that transformants in which WM112 cells harbor the pM101 empty vector could not be obtained. In contrast, as shown in the right panel of (B), more than one thousand transformants per plate appeared after transformation of pM101 into the parental RN4220 cells harboring pM103-*mraY*.

thousand of kanamycin-resistant transformants were obtained (Fig. 3B). These results suggest that *mraY* cannot be depleted from *S. aureus* cells; in other words, that the *mraY* gene is indispensable for *S. aureus* cell growth or viability.

Further, we asked if other two genes are required for staphylococcal cell growth. They were the *SA0172* and *SA1839* genes and encode unknown function putative membrane proteins. Using the above-mentioned approach, we replaced each of the chromosomal genes with the phleomycin resistance gene in cells harboring the respective intact gene on pM103 plasmid, and the constructed cells were electroporated with the empty pM101 vector. In contrast to the *mraY* gene, transformants with empty vector were obtained for each of two deletion mutants at any temperature tested including 30 °C, 37 °C, and 43 °C without apparent retardation of colony formation, suggesting that the *SA0172* and *SA1839* genes are dispensable for *S. aureus* cell growth or viability. Taken together, these results suggest that the pN315-based plasmid shuffling method is useful to identify and analyze *S. aureus* genes that are essential for cell growth. In addition, successful construction of WM112/pM103-*mraY* suggests that the pN315-based plasmid will also be useful for other

molecular genetic analyses, including exogenous gene expression useful for complementation analysis.

DISCUSSION

In this study, we used the *rep* to *par* region of pN315 to construct three vectors (pM101, pM102, and pM103). These vectors replicate and are maintained in both *E. coli* and *S. aureus*. Furthermore, we took advantage of incompatibility among the plasmids to test if an *S. aureus* gene is essential for cell growth. Specifically, use of the plasmid shuffling method revealed that the *mraY* gene couldn't be lost from *S. aureus* cells. In contrast, the *SA0172* and *SA1839* genes could be deleted from *S. aureus* cells. Because *mraY* is known to be involved in biosynthesis of peptidoglycan, a bacteria-specific architectural component, we predicted that it is essential and accordingly, the results of this study revealed that *mraY* is essential for *S. aureus* cell growth or viability. Quick shuffling among the pN315-based plasmids may be attributed to its elements controlling the replication initiation and partition, and the failure of it among the pKE515-based plasmids is presumably due to differences in their replication and partition modes. The pN315-based plasmids described in this work may be useful to examine specific requirement for cell growth or viability of genes. In addition, the plasmids may be useful for further analysis of the growth requirements for essential genes, as has been described for other species.^{5,6} Given to the worldwide spread of MRSA, the development of novel anti-staphylococcal agents has become a very important issue and thus, the method described here provides a powerful tool that will help inform drug development aimed at targeting essential staphylococcal gene products.

The system described here works fine in RN4220 strain that is restriction-negative and does not have other plasmids, but needs to make a suitable response in case of using in other *S. aureus* strains of interest. Specifically, modification of all the vector plasmids by passing RN4220 strain prior to transformation is required. This also applies to the introduction of the deletion mutation in the chromosome allele of the gene, so temperature-sensitive plasmids such as pKOR1²³ than our non-replicative vector is desired for targeting. Alternatively, phage transduction-mediated transfer of chromosomal mutations or plasmids from RN4220 strain to the other strains of interest is doable. There are other methods to validate growth requirement of bacterial genes but each of these alternative methods has one or more significant weak points. For example, insufficient repression of gene expression *via* anti-sense^{24,25} or *via* the LacI repressor method²⁶ can mean that some essential genes are not identified, as sufficient gene product 'escapes' to maintain cell growth. The plasmid shuffling method described here, by contrast, excludes the possibility of insufficient repression of the target gene, as the introduction of a non-compatible second plasmid drives the target gene (on the first plasmid) out of the cells. In other case, very rare frequency to generate deletion mutants *via* recombination might make one inappropriately deduce that the target gene is essential when the process is omitted for complementation. Also, the effect on neighboring gene expression caused by deletion or modification of the target gene locus can also explain an inability to obtain a strain in which the

target gene is deleted. Additionally, if a deletion of the target gene is obtained, there is a possibility that a suppressor mutation also occurred during the process of isolating the recombinant mutation, which usually takes several days of cultivation. These problems can be solved by using instead the plasmid shuffling method, as with this method, cell growth is validated under conditions that form more than a thousand transformants and the process itself requires only an overnight incubation (Fig. 3). Moreover, plasmid shuffling includes a process complementation step that helps to confirm that a target gene-dependent phenotype is observed. One may assume that genes requirement for cell growth can be examined by using temperature-sensitive plasmids instead of our plasmids established here. Truly, temperature-sensitive plasmids make us judge it under high temperature conditions, however, they cannot do it at low temperatures at which they are maintained in cells. As an illustration, we recently have presented evidence, by using this plasmid shuffling system, that the *ltaS* gene for lipoteichoic acid synthesis is essential for *S. aureus* cell growth at 37 °C or higher temperature but not at low temperature (30 °C).²⁷ Finally, the applicability of the method for isolation of conditional lethal mutants of the essential gene is an additional advantage of the plasmid shuffling method.

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