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Improved single-copy integration vectors for *Staphylococcus aureus*

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Abstract

We have previously reported the construction of *Staphylococcus aureus* single-copy integration vectors based on the lysogenic bacteriophage L54a site-specific recombination system. These vectors can replicate autonomously in *Escherichia coli*, which allows DNA manipulations. In *S. aureus*, the vectors, which do not possess staphylococcal replication function, can only be maintained by integrating into the chromosome. However, the original vectors have limited cloning sites and do not have protection from potential transcription of external promoters. Here we report the improved version of these vectors that circumvent these shortcomings. In addition, a second integration site based on the bacteriophage ϕ 11 site-specific recombination system has been added such that the vectors can integrate either at the L54a attachment site or at the ϕ 11 attachment site.

Staphylococcus aureus is an important human pathogen that can cause a wide variety of diseases. The advent of molecular genetics has greatly facilitated our understanding of the pathogenesis of this organism. Plasmid vectors have been used extensively in carrying out such studies. However, self-replicating plasmid vectors are multiple-copy in nature, which are likely to result in artifacts due to high gene dosage (Hoang et al, 2000). Previously, we have developed a set of integration plasmid vectors that allow cloning of genes in *S. aureus* in chromosome-equivalent copy number (Lee et al., 1991). The single-copy integration vectors, pCL83 and pCL84 have been widely used in various genetic studies of *S. aureus*. These vectors are based on the lysogenic bacteriophage L54a integration recombination system that efficiently promotes integration of the vectors (and the gene cloned therein) into the *S. aureus* chromosome at the L54a *attB* site located within the *geh* locus. As with most of lysogenic bacteriophage, the integration of L54a requires the phage-encoded *int* gene and the corresponding phage attachment site *attP*. Two kinds of vectors were constructed in the initial report (Lee, 1991); the plasmids pCL83 and pCL84 contain the *attP* site but not the *int* gene, whereas the plasmid pCL55 contains both the *attP* site and the *int* gene. Integration of pCL83 or pCL84 therefore requires the *int* gene provided in trans. RN4220 with pYL119 Δ 19 containing the L54a *int* gene (i.e., strain CYL316) was constructed for this purpose. It was found that pCL83/pCL84 had higher efficiency of transformation than pCL55, which is likely due to *int* gene dosage effect since pYL119 Δ 19 is a multiple copy plasmid. The integration disrupts the *geh* gene encoding for the lipase enzyme (Lee and Iandolo, 1986a;1986b) providing a convenient method of validating correct integration. Once integrated, the plasmids are stably maintained since excision gene, *xis*, is intentionally excluded in the system. The integrated plasmid could easily be transferred by phage transduction to desired strains for further analysis. However, since the

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integration site is located in the *geh* gene and the cloning sites are close to the *attP* site, a cloned gene in these integrated vectors could potentially be affected by transcription from the *geh* promoter in the chromosome. The disruption of the *geh* gene could also affect the virulence of the host organism thus interfering with pathogenic studies in which the lipase gene may play a role. Furthermore, the original plasmid vectors are limited in available cloning sites. In this communication, we report the development of new improved single copy integration vectors that circumvent these drawbacks.

To improve the single-copy integration vectors pCL83 and pCL84, we first constructed plasmid pCL25 (Fig. 1). Two features of pCL25 are distinct from pCL83 or pCL84. First, the L54a *attP* site in pCL25 is located away from the multiple cloning sites. By positioning the *attP* site away from the cloning sites, it reduces the possibility of transcription of the cloned gene(s) from the *geh* promoter after integration of the plasmid in the chromosome. Indeed, any promoter transcribing in the direction of *geh* gene in the chromosome would have to transcribe additional 6 kb to reach the multiple cloning sites (the arrow of the L54a *attP* site in Fig. 1 corresponds to the direction of the *geh* transcription). This feature makes it unlikely that a cloned gene at the multiple cloning sites could be affected transcriptionally by the *geh* promoter in the chromosome. Second, pCL25 contains the pUC18 multiple cloning site, which provides more unique cloning sites than the original vectors.

As with the original single-copy vectors pCL83 and pCL84, pCL25 integrates at the L54a *attB* site located within the *geh* gene. However, for some experiments, it may be beneficial to integrate the plasmid at a different chromosomal site. For example, if a strain of interest has already carried a prophage integrated at the L54a *attB* site, transfer of the integrated plasmid by transduction from the primary host CYL316 to this strain may be difficult. In addition, as indicated above, disruption of *geh* may affect virulence of *S. aureus*. Pathogenesis studies in which the *geh* gene may be involved will be inadvertently affected if these vectors are used. Thus, it would be desirable to have a single-copy vector capable of integrating at a different chromosome site. To this end, we constructed plasmid pLL29 (Fig. 1) by placing the *attP* site derived from the lysogenic bacteriophage ϕ 11 next to the L54a *attP* site in pCL25. The ϕ 11 *attB* site is located within the intergenic region between ORF2016 and 2017 of the NCTC8325 annotated genome (Iandolo et al., 2002; Gillaspay et al., 2006). The integration of ϕ 11 does not result in apparent phenotype change on the host (Gillaspay et al. 2006). As in pCL25, potential transcription from a promoter in the chromosome is also minimized in pLL29 since the *attP* sites of pLL29 is distantly located from the multiple cloning sites. Nonetheless, to further eliminate the possibility of read through of the cloned genes from an external promoter, we added a 965-bp fragment containing five tandem repeats of the *T1* transcriptional terminator (*T1*₅) from the *E. coli* *rrnB* operon (Simons et al., 1987) at the *EcoRI* site on pLL29. This plasmid, pLL39, should effectively prevent any read through into the cloning sites.

Since pLL29 and pLL39 does not have an integrase gene but contain the L54a *attP* and ϕ 11 *attP* sites, these plasmids are unable to integrate to the L54a *attB* site or the ϕ 11 *attB* site on the chromosome unless the L54a *int* gene or the ϕ 11 *int* gene is provided in the host. Strain CYL316 that we previously constructed, which contains the L54a *int* gene cloned in a plasmid vector (pYL112 Δ 19), would be a suitable host for integration of pLL29 at the L54a *attB* site (Lee et al., 1991). To construct a host for integrating pLL29 at the ϕ 11 *attB* site on the chromosome, we cloned a PCR fragment containing the ϕ 11 *int* gene in an *S. aureus*-*E. coli* shuttle vector pCL15 (Luong and Lee, 2006) using two primers (CTGCAGCCAATTTTGAGGAGGGAGAA; GGATCCAATTTGATACCGTTTAATCAA) such that the *int* gene is under the control of the IPTG-inducible *Pspac* promoter (Yansura and Henner, 1984). The cloned ϕ 11 *int* gene was verified by sequencing. The resulting plasmid pLL2787 was electroporated into strain RN4220 to form strain CYL557.

To demonstrate that pLL29 is a useful single-copy integration vector that could integrate into either the L54a *attB* site or the ϕ 11 *attB* site, the plasmid was transformed by electroporation into competent CYL316 or CYL557 prepared from logarithmically growing cultures in the presence of chloramphenicol at 10 μ g/ml according to the procedure of Kraemer and Iandolo (1990). The transformants were selected in Trypticase soy agar plates containing tetracycline at 3 μ g/ml. To confirm that pLL29 integrated at the L54a *attB* site in CYL316, we performed two PCR reactions using primer scv2.1 (TGTGCCATGATAACAGCAGC) paired with scv4 (ACCCAGTTTGTAAATTCAGGAG) and primer scv8 (GCACATAATTGCTCACAGCCA) paired with scv1 (GCAACACCACATAATGGTTCAC). These primers were designed such that defined DNA fragments could be amplified only when the vector is integrated at the correct L54a *attP* site (Fig. 2A). Previously, we found that pCL83 and pCL84 had high degree of correct integration at the chromosomal L54a *attB* site. Based on these results, we randomly selected three colonies from the transformants for verification by PCR. All three colonies gave the same gel pattern and the results from one of the colonies are shown in Fig. 2B. As shown, both pairs of primer gave rise to correct DNA fragments when CYL316(pLL29) DNA was used as the template but not when the control CYL316 DNA was used as the template. Similarly, we confirmed the integration of pLL29 in CYL557 by PCR amplification from three randomly selected transformants using primer scv4 paired with scv10 (TATACCTCGATGATGTGCATAC) and primer scv8 paired with scv9 (GCTGATCTAACAATCCAATCCA). The PCR results from one of the CYL557(pLL29) colonies are also shown in Fig. 2B.

Using the primer pairs shown in Fig. 2, we also demonstrated that pCL25 could integrate at the L54a *attB* site whereas pLL39 could integrate at either the L54a *attB* or the ϕ 11 *attB* site in the chromosome (data not shown). It should be noted here that although the ϕ 11 *int* gene in pCL15 is under the control of the IPTG-inducible *Pspac* promoter, we found induction by IPTG was not necessary for electroporation of pLL29 in strain CYL557 suggesting that the ϕ 11 Int protein is expressed at a substantial basal level from the *Pspac* promoter.

As in other bacteria, transfer of DNA into *S. aureus* is hampered by restriction barrier. To circumvent this problem, a restrictionless strain, RN4220 (Kreiswirth et al.), was used for the initial uptake of the vectors and the gene cloned therein. The integrated plasmid was then transferred by bacteriophage transduction to other strains for further study. The ϕ 11 *attB* site is conserved in all eight sequenced genomes indicating that integration of pLL29 (or pLL39) at the ϕ 11 *attB* site could be transferred readily to most other strains by bacteriophage transduction. Indeed, in a separate study to investigate capsule gene regulation, we have cloned a 6-kb fragment in pLL29 and have successfully moved the plasmid integrated at the ϕ 11 *attB* site into strains Newman and Becker (data not shown).

In conclusion, in this communication, we reported the modification of the vectors useful for cloning DNA fragments into *S. aureus* in chromosome-equivalent copy number. These vectors have several advantages over the previously published vectors. First, they have more unique cloning sites, which facilitate cloning of genes into these vectors. Second, the location of the multiple cloning sites and the *attP* site are distantly separated such that read through from the chromosome is less likely than the previous vectors. Third, pLL29 and pLL39 have an additional *attP* site that can integrate into a different chromosomal location. Most recently, a pair of integration vectors based on the pathogenicity island SaPII integration system have been published (Charpentier et al., 2004). The availability of additional specific chromosomal sites for integration vectors will facilitate genetics studies in *S. aureus*.

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Fig 1.

Plasmid maps of improved integration vectors. The nucleotide sequences of the vectors have been deposited to GenBank under the accession numbers EF540894 (pCL25), EF540895 (pLL29) and EF540896 (pLL39).

Fig 2.

Panel A. Schematic illustration of integration of pLL29 at the *L54a attB* site or at the $\phi 11$ *attB* site. The locations of the primers used for verifying the integration are shown. The distance between the primers is also shown. **Panel B.** PCR-amplified products verifying the integration of pLL29 at the *L54a attB* site in CYL316 (left) or at the $\phi 11$ *attB* site in CYL557 (right). Lane 1, CYL316(pLL29) DNA amplified with *scv1* and *scv8* primers; Lane 2, CYL316 DNA amplified with *scv1* and *scv8* primers. Lane 3, CYL316(pLL29) DNA amplified with *scv2.1* and *scv4* primers; Lane 4, CYL316 DNA amplified with *scv2.1* and *scv4* primers. Lane 5, CYL557(pLL29) DNA amplified with *scv4* and *scv10* primers; Lane 6, CYL557 DNA amplified with *scv4* and *scv10* primers. Lane 7, CY557(pLL29) DNA amplified with *scv8* and *scv9* primers; Lane 8, CYL557 DNA amplified with *scv8* and *scv9* primers. DNA size markers (M) are 100bp ladder from Invitrogen (USA)