Published in final edited form as:
Plasmid. 2013 May ; 69(3): 211–222. doi:10.1016/j.plasmid.2013.01.002.

Construction of new cloning, *lacZ* reporter and scarless-markerless suicide vectors for genetic studies in *Aggregatibacter actinomycetemcomitans*

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Abstract

To elucidate the putative function of a gene, effective tools are required for genetic characterization that facilitate its inactivation, deletion or modification on the bacterial chromosome. In the present study, the nucleotide sequence of the *Escherichia coli/Aggregatibacter actinomycetemcomitans* shuttle vector pYGK was determined, allowing us to redesign and construct a new shuttle cloning vector, pJT4, and promoterless *lacZ* transcriptional/translational fusion plasmids, pJT3 and pJT5. Plasmids pJT4 and pJT5 contain the origin of replication necessary to maintain shuttle vector replication. In addition, a new suicide vector, pJT1, was constructed for the generation of scarless and markerless deletion mutations of genes in the oral pathogen *A. actinomycetemcomitans*. Plasmid pJT1 is a pUC-based suicide vector that is counter-selectable for sucrose sensitivity. This vector does not leave antibiotic markers or scars on the chromosome after gene deletion and thus provides the option to combine several mutations in the same genetic background. The effectiveness of pJT1 was demonstrated by the construction of *A. actinomycetemcomitans* isogenic *qseB* single deletion (Δ*qseB*) mutant and *lsrRK* double deletion mutants (Δ*lsrRK*). These new vectors may offer alternatives for genetic studies in *A. actinomycetemcomitans* and other members of the HACEK (*Haemophilus spp.*, *A. actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*) group of Gram-negative bacteria.

1. Introduction

Aggregatibacter actinomycetemcomitans is a Gram-negative, facultative bacterium, and non-motile coccobacillus of the Pasteurellaceae familly that is found in the oral cavity, and it is implicated in the etiology of aggressive and chronic periodontitis, but also is associated with extra-oral infections such as infective endocarditis, soft tissue abscesses, meningitis, pneumonia, septicemia, urinary tract infections, and osteomyelitis (Haffajee and Socransky, 1994; Henderson et al., 2003; Paturel et al., 2004; Nørskov-Lauritsen and Kilian, 2006; Rahamat-Langendoen et al., 2011 Wang et al., 2010; Hyvärinen et al., 2012). *A. actinomycetemcomitans* expresses a variety of virulence factors to survive in the oral cavity and causes tissue inflammation, tissue destruction, immune suppression and bone resorption

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(Han et al., 2006; Rogers et al.,2007). The molecular mechanisms by which *A. actinomycetemcomitants* successfully colonizes and persists in the oral cavity, and its ability to disseminate to other organs of the body have not been well defined. In part, this is due to the limited repertoire of molecular genetic tools that are available to manipulate *A. actinomycetemcomitans*.

Several molecular biological approaches have been reported for studies of *A. actinomycetemcomitans* gene function including conjugation-based transposon-insertion mutagenesis (Thomson et al., 1999) which was applied for the mutagenesis of the *katA* gene and the characterization of genes involved in the tight adherence (*tad*) locus Kachlany et al., 2000; Kachlany et al., 2001a; Kachlany et al., 2001b; Planet et al., 2003). Gene inactivation by a single recombinant event to insert an antibiotic marker carried on a plasmid vector has been performed using mobilizable vectors such as pVT1461 (R6K *ori*) to inactivate *recA* (Mintz et al., 2002), and vectors such as pUC19 that do not replicate in *A. actinomycetemcomitans* have been used to inactivate the *lsrB* gene (Shao et al., 2007a). Gene replacement by an antibiotic marker, generated by double recombination events, using plasmids based on the pUC replicon and containing the counter-selectable marker *sacB* for sucrose-sensitivity (Reyrat et al., 1998) have also been reported to produce genetic deletions of *orfA* (Schaeffer et al., 2008) and *ihfB* (Kolodrubetz et al., 2010). Finally, the Cre/*loxP* recombinase system, which represents a step forward in the generation of markerless deletion mutations in *A. actinomyctemcomitans*, has been applied to generate a deletion mutation of the SPA-a gene cluster, and the mutant strain was complemented by the reinsertion of the SPA-a gene cluster. In addition, a deletion mutation of the *pagBC* genes was performed using this methodology (Fujise et al., 2004). However, these approaches may have limitations for the construction of multiple deletion mutations arising the antibiotic markers or the scars (i.e, the *loxP* sequence left in the genome after the recombination event between two *loxP* sequences) generated by the Cre recombinase system. For example, the *loxP* sequences remaining in the chromosome may be substrates for future recombination events during the construction of new deletion mutations in the same genetic background unless *loxP* with different spacer sequences are used (Lee and Saito, 1998)

A. actinomycetemcomitans is genetically hetereogeneous and comprises strains of six clonal populations expressing distinct serotypes that exhibit variation in natural competence for DNA uptake Kittichotirat et al., 2011). Serotypes a, d and e are naturally competent (Fujise et al., 2004), but serotypes b, c and f contain an insertional inactivation of the *comM* gene that results in the inability of these strains to be readily transformed (Mena and Chen, 2007; Kittichotirat et al., 2011). This is important since serotype b and c strains are commonly associated with human oral infections. The Cre/*loxP* recombinase system has been successfully used in naturally competent *A. actinomycetemcomitans* strains, but its efficacy in non-natural competent strains has not yet been reported. In an attempt to overcome the limitation presented by non-naturally competent strains, Bhattacharjee et al. (2007) reported that inducible expression of the *tfox* gene from a plasmid restored the natural competent phenotype in *A. actinomycetemcomitans* strains except serotype f. While this system has utility, the approach also has several limitations in that the DNA used for the transformation must contain a 9-bp uptake sequence (USS), it relies on selection by antibiotic resistance, and the replicating plasmid containing *tfoX* must be eliminated before the performing genetic studies. In general, there remains a strong need for additional genetic tools to manipulate *A. actinomycetemcomitans* that are effective in naturally competent as well as non-naturally competent strains.

Plasmid pYGK (Brogan et al., 1996) is an *A. actinomycetemcomitans-E. coli* shuttle vector that was derived from plasmid pYG10 extracted from *A. pleuropneumoniae* 80-8141 Lalonde et al., 1989). It has been used as a transcriptional reporter plasmid and cloning

vector for complementation studies in *A. actinomyctemcomitans* James et al., 2006; Novak et al., 2010; Shao et al., 2007a; Shao et al., 2007b). However, the nucleotide sequence of pYGK has not been determined, nor has its origin of replication been identified which has limited its further development. In the present study, we report the nucleotide sequence of pYGK and define the minimal origin of replication required to maintain the plasmid in *A. actinomycetemcomitans* and *E. coli*. This allowed us to redesign and construct three new pYGK-derived plasmids including the cloning vector pJT4 and the transcriptional/translational reporter plasmids pJT3 and pJT5. In addition, we describe the construction of pJT1, a suicide plasmid that facilitates the generation of scarless and markerless chromosomal deletion mutations in *A. actinomycetemcomitans*. The utility of each plasmid was demonstrated in the serotype c strain *A. actinomycetemcomitans* 652.

2. Materials and methods

2.1 Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. LB (Luria-Bertani) broth, LB agar (LB broth plus 1.5% agar), brain heart infusion (BHI) broth, BHI agar, TYE (1% tryptone, and 0.5% yeast extract) broth, and TYE agar (all from DIFCO) were routinely used for propagation and plating of bacteria. SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) (Hanahan, 1983) was used for the recovery post-transformation bacterial cells. *A. actinomycetemcomitans* strain 652 (serotype c) that was grown at 37°C under microaerophilic conditions. When required the medium was supplemented with 10 % sucrose, 1 mM IPTG, 25 μg ml^{-1} kanamycin (Km), 12.5 μg ml^{-1} tetracycline, 100 μg ml^{-1} ampicillin (Ap), or 50 μg ml^{-1} spectinomycin (Sp).

2.2 DNA procedures

DNA manipulations were carried out as described (Sambrook and Russell, 2001). Transformation of *E. coli* and *A. actinomycetemcomitans* was done by electroporation (Bio-Rad) [2 mm cuvette, voltage (V) 1800, capacitance (μF) 25, resistance (ohm) 200]. For replicative plasmids the ~2μg was used for electroporation whereas for non-replicative plasmids, ~20 μg was used. For this study, smooth colony strains of *A. actinomycetemcomitans* were used. However, we previously inactivated *luxS* in both a smooth strain and a clinical isolate via electroporation (Demuth et al., 2011) and observed little difference in transformation efficiency. Transformant cells containing plasmids were selected on LB agar plates supplemented with appropriated antibiotics. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Restriction enzymes were used as recommended by the manufacturer (New England Biolabs). All PCR products were amplified with the Platinum PCR Super Mix High Fidelity used as recommended by the manufacturer (Invitrogen). All primers used in this study (Integrated DNA Technology) were flanked with restriction enzyme recognition sites (underlined in the primer sequences) and are shown in Table 2. Primer sequences were designed based on the genome sequence of *A. actinomycetemcomitans* D11S-1 strain available from the Pathosystems Resource Integration Center (http://patricbrc.vbi.vt.edu). All nucleotide substitutions in the recombinant plasmids were performed using a Quick-Change site-directed mutagenesis kit (Stratagene). All constructs, and the presence of a desired mutation were verified by DNA sequencing (University of Louisville Facilities).

2.3 Nucleotide sequence and analysis of the pYGK-lacZ shuttle vector

The initial plasmid sequence was obtained by using primers designed based on the *lacZ* and *aph*(*3′*)*Ia* genes contained in pYGK-*lacZ*. Subsequently, the sequencing of the whole plasmid was performed using new primers designed on the new sequence revealed.

Sequencing was carried out at the University of Louisville Core Sequencing Facility. The sequence was submitted to GenBank with the accession numbers, **JX826404** for pYGK and **JX826405** for pYGK-l*acZ*. ORFs were predicted with the ORF finder tools (National Center for Biotechnology Information). The DNA and putative protein sequences were compared to the public sequence databases with the Blast program.

2.4 Construction of the physical map of the pYGKlacZ shuttle vector

The pYGK-*lacZ* is a pYG53-derived plasmid that contains the *lacZ* and *aph*(*3′*)*Ia* genes from *E. coli* C600 strain (Brogan et al., 1996) and pUC4K (Vieira and Messing, 1982), respectively. The pYG53 is in turn a pYG10-derived plasmid (Lalonde et al., 1989). To construct the physical map of pYGK-*lacZ*, purified plasmid DNA was digested with *Xba*I, *Pst*I, *EcoR*I, *Hin*dIII *Kpn*I *Bam*HI, or various combinations of these enzymes. Digested DNAs were analyzed by conventional agarose gel electrophoresis in 1 % agarose gels (Bio-rad).

2.5 Construction of pJT4 and pJT6 shuttle plasmids

The construction of pJT4 was performed in several steps that involved the construction of precursor plasmids pJT4-A and pJT4-B, described below. The fragment containing the putative origin of replication, two ORFs as well as the cluster of genes involved in the mobility of pYGK-*lacZ*, was PCR amplified using primer set ATE-107F and ATE-108R. The 2484-bp PCR product was digested with *Xba*I-*Bam*HI and ligated with the 1214 bp *Xba*I-*Bam*HI-digested fragment containing the *aph*(*3′*)*Ia* from pYGK-*lacZ*. This latter fragment was amplified with the primer set ATE-105F and ATE-106R to create pJT4-A. Next, the unique *Bam*HI site in pJT4-A plasmid was replaced with a *Sal*I site by site-directed mutagenesis using primer set ATE-115F and ATE-116R, to create pJT4-B. Three additional unique restriction sites, *Kpn*I, *Apa*I and *Bam*HI sites, were then introduced downstream of the *mobA* gene in the pJT4-B by site-directed mutagenesis using primer set ATE-117F and ATE-118R, the resulting plasmid was designated pJT4. The fragment containing the putative origin of replication and the two ORFs of pYGK-*lacZ*, was PCR amplified using primer set ATE-177F and ATE-178R. The 820-bp PCR product was digested with *Xba*I-*Sal*I and ligated with the 1214 bp *Xba*I-*Sal*I-digested fragment containing the *aph*(*3′*)*Ia* from pYGK-*lacZ*. This latter fragment was amplified with the primer set ATE-179F and ATE-106R to create pJT6.

2.6 Construction of pJT3, pJT5, pATE12 and pATE65, transcriptional/translational lacZ gene reporter plasmids

Plasmid pJT3 was constructed from pYGK-*lacZ* by excising a 3167-bp *Bam*HI-*Xba*I fragment and replacing it with a 3537-bp *Bam*HI-*Xba*I fragment containing the promoterless *lacZ* gene and the transcriptional terminator *rrnB*T1 and *rrnB*T2, amplified from pBAD-*lacZ* (Invitrogene) using the primer set ATE-10F and ATE-12R. This terminator product was also cloned into *Bam*HI-*Xba*I-digested pJT4 to create pJT5. The promoter region of *A. actinomycetemcomitans lsrR* was then PCR amplified with the primer set lsrR255-f3 and lsrR255-r6. The 255-bp PCR product was digested with *Kpn*I-*Bam*HI and cloned into *Kpn*I-*Bam*HI-digested pYGK-*lacZ* and pJT5 to create pATE12 and pATE65 respectively.

2.7 Construction of pATE52, pATE60 plasmids

The promoter region and structural *lsrRK* genes was amplified from *A. actinomycetemcomitans* genomic DNA using primer sets lsrR-f109 and lsrK-r110, and the resulting 3090 bp fragment was digested with *Bam*HI-*Xba*I and cloned into *Bam*HI-*Xba*I-digested pJT4 (see Table 2) to create pATE52. Similarly, the promoter region and structural *crp* gene was amplified from *A. actinomycetemcomitans* genomic DNA using primer sets

crp-F151 and crp-r152 and the resulting 1120-bp product was digested with *Kpn*I-*Xba*I and cloned into *Kpn*I-*Xba*I-digested pJT4 (see Table 2) to create pATE60.

2.8 Growth kinetics

A single colony of *A. actinomycetemcomitans* harboring each recombinant plasmid was independently inoculated into 10 ml of BHI media supplemented with 25 μg ml^{-1} Km and was grown standing for 24 hour at 37°C. The next day, the overnight culture (optical density at 600 nm [OD_{600nm}] of 0.6) was diluted at a 1:30 ratio to inoculate 12 ml of BHI (12 ml in 15-ml conical centrifuge tubes) with 25 μg ml^{-1} Km and grown standing at 37°C. For the first hours of growth, an aliquot of 1.1 ml was taken from each culture until 9 h. Additional aliquots were taken from each culture at the 24 h (1 ml to read the OD_{600nm} and 0.1 ml for the β-galactosidase activity assay. β-galactosidase activity was also determined for each aliquot as described below.

2.9 β-galactosidase assays

β-galactosidase (β-gal) activity was qualitatively assessed on BHI agar plates that were supplemented with 50 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Quantitative evaluation of β-gal activity was carried out using permeabilized cells incubated with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate (Sigma) as previously described (Miller, 1972). Average values (± the standard deviations) for activity units were routinely calculated from three independent assays using GraphPad Prism v5 software.

2.10 Determination of plasmid stability

The plasmid stability of strain *A. actinomycetemcomitans* harboring pATE3 or pJT4 or pJT5 or pJT6 was performed essentially as described previously by Torres-Escobar et al. (2010) except that *A. actinomycetemcomitans* harboring each recombinant plasmid was determined with BHI media supplemented with 25 μg ml^{-1} Km.

2.11 Construction of the suicide vector pJT1, for the generation of scarless and markerless deletion mutations

The construction of the pJT1 suicide vector was performed in several steps that involved the construction of precursor plasmids pJT-A, pJT-B, and pJT-C, described below. A 240 bp DNA fragment containing a multiple cloning site (MCS) flanked by *Eco*RI and *Hin*dIII overhanging sequences was obtained by annealing three sets of complementary single-stranded 80-b oligonucleotides, (MDJR-16F and MDJR-17R), (MDJR-18F and MDJR-19R) and (MDJR-20F and MDJR-21R). The 240 bp synthetic DNA was cloned into *Eco*RI-*Hin*dIII-digested pUC18 (Yanisch et al., 1985) to create pJT-A. A 87-bp DNA fragment containing the synthetic *trc* promoter (P_{trc}) (Amann et al., 1983) with by *Sac*I and *Bgl*II sites was obtained by annealing two complementary single-stranded 80 base oligonucleotides, (MDJR-22F and MDJR-23R). The resulting 87 bp synthetic DNA was cloned into *Sac*I-*Bgl*II-digested pJT-A to create pJT-B. Finally, the *sacB* gene was PCR amplified from pRE112 (Edwards et al., 1998) using primer set MDJR-24F and MDJR-27R. The 1552 bp PCR product was digested with *Bgl*II-*Mlu*I and cloned into *Bgl*II-*Mlu*I-digested pJT-B to create pJT-C. pJT-C was then digested with *Nhe*I-*Mlu*I to produce a 1777 bp fragment containing the MCS-P_{trc} -*sacB* region. To produce pJT-1, this fragment was simultaneously ligated with two other fragments; an 857 bp fragment containing the pUC replication origin that was PCR amplified from pUC18 using primer set MDJR-34F and MDJR-35R, and a 1191 bp fragment containing the spectinomycin resistance marker that was PCR amplified from pVT1461 (Mintz et al., 2002) using primer set MDJR-28F and MDJR-29R.

2.12 Generation of A. actinomycetemcomitans markerless deletion mutants

The construction of *A. actinomycetemcomitans* harboring a markerless deletion mutation of *qseB* gene was carried out by amplifying the upstream and downstream flanking regions of *qseB* with primer sets (MDJR-49F and MDJR-82R) and (MDJR-83F and MDJR-84R; Table 2). The respective 1630-bp and 1890-bp PCR products were digested with *Not*I-*Xho*I, and *Xho*I-*Pst*I and cloned adjacently (joined by the *Xho*I restriction site) into *Not*I-*Pst*I-digested pJT1 suicide vector to create pDJR37 (Table 1). A similar approach was used to generate the *A. actinomycetemcomitans* markerless double-deletion mutant in *lsrRK* by constructing and using the suicide plasmid pATE47 (Table 1). Primer sets used to amplify the flanking regions of *lsrRK* were (ATE-86F and ATE-87R) and (ATE-88F and ATE-89R) (Table 2). Each recombinant suicide plasmid (~20 μg) was introduced individually into *A. actinomycetemcomitans* by electroporation. Electroporated cells were incubated in 0.5 ml of SOC broth standing for 5 h at 37°C in microaerophilic conditions (anaerobic jar). Bacterial cells with single recombinant event were selected onto BHI agar containing 50 μg ml^{-1} spectinomycin (Sp) at 37°C under microaerophilic conditions. Ten spectinomycin-resistant (Sp^r) colonies were randomly selected and subcultured daily for 24 h at 37°C for three consecutive days, except that the final cultures were grown in presence of 1mM IPTG (IPTG-induced P_{trc}-*sacB*). To select for bacteria that had undergone a second recombination event, the culture was diluted 10-fold and spread onto TYE agar supplemented with 1 mM IPTG and 10% sucrose, and grown at 37°C in microaerophilic conditions. One thousand sucrose-resistant (Suc^r) colonies were replica plated onto TYE agar supplemented with sucrose and onto BHI agar supplemented with spectinomycin. Spectinomycin-sensitive ($Sp^{S)}$ colonies were selected to perform PCR for the deletion mutation of the target genes, using the primer sets for *qseB* deletion (MDJR-63F and MDJR61R) or for *lsrRK* deletion (ATE-62F and ATE-102R). Sp^S colonies that were PCR positive were selected for further analysis.

3. Results

3.1 Nucleotide sequence of the shuttle vector pYGKlacZ

The complete nucleotide sequence of pYGK was determined as described in Materials and Methods and a map of the plasmid containing the *lacZ* gene of *E. coli* C600 is shown in Fig. 1A. Through comparative analysis with the nucleotide/protein sequences in the GenBank database, four regions were identified on plasmid. As shown in Fig. 1B, pYGK contains several open reading frames (orfs) that are conserved in other organisms of the *Pasteurellaceae* and *Enterobacteriaceae* (Kehrenberg and Schwarz, 2002; Matter et al., 2008; Kang et al., 2009). The first region harbors genes encoding antibiotic resistance markers such as *catII*, which encodes chloramphenicol acetyltransferase. This gene is truncated in pYGK as a result of the previous cloning by Brogan et al. (1996) of a 1252-bp DNA fragment harboring the *aph*(*3′*)-*Ia* gene (encoding aminoglycoside phosphotransferase [Km]) from pUC4K. This region of pYGK also contains *strA*, encoding aminoglycoside 3′-phosphotranferase [APH0(3″)-Ib], and a truncated *strB* gene that encodes a portion of aminoglycoside 6′-phosphotranferase (APH0(6)-Id). The second region comprises three ORFs encoding proteins involved in plasmid mobilization (MobA, MobB and MobC) that share high sequence similarity to related genes identified in other organisms in the family *Pasteurellaceae* (Matter et al., 2008). The *mobC* and *mobA* genes overlap by four bases. In addition, *mobB* resides completely within *mobA,* but is translated in a different reading frame. The third region revealed two ORFs (*orf1* and *orf2*) encoding proteins with no significant homologies to amino acid sequences available in the Gene-Bank database. The fourth region contained a potential origin of replication that shares 100% identity to the 1160 bp region containing the origin of replication of plasmid pD70 from *Mannheimia hemolytica* serotype 1 (Briggs and Tatum, 2005). In addition, two putative *ori*T sequences,

four inverted repeat (IR1-4) sequences, and 2 direct repeat (DR) sequences that reside within the IR_1 (Briggs and Tatum, 2005) were identified (see Table 3). The entire nucleotide sequence of pYGK is 5469 bp with position 1 defined as the guanine residue that corresponds to the first nucleotide of the unique restriction site *Eco*RI. The length of pYGK containing *lacZ* as shown in Figure 1A is 8528 bp.

3.2 Mapping the replication origin of pYGK

A series of four fragments of 2484 bp, 1493 bp, 820 and 809 bp (see Fig 2) flanked with appropriate restriction enzymes sites were amplified (as described in Material and Methods) from pYGK and each was individually ligated with a 1252 bp fragment (flanked with restriction sites) containing a kanamycin resistance marker. Each ligation was introduced into *E. coli* XLI-blue MRF′ strain by electroporation and the cells were spread onto LB agar supplemented with 20 $\mu g\ ml^{-1}$ kanamycin and incubated at 37 °C. Km^r colonies were detected in transformants harboring the 2484 bp or 820 bp fragments. This recombinant plasmid, designated pJT4-A and pJT6, respectively, also replicated in *A. actinomycetemcomitans* 652. No recombinants were recovered from ligations using the 1493 bp or 809 bp fragments, indicating that the 820-bp (containing the two ORF plus the four IR sequences) that resides into the 2484-bp fragment is enough for supporting the replication of this plasmid in both *E. coli* and *A. actinomycetemcomitans* cells and that the *mob* genes region is unnecessary for pYGK replication.

3.3 Improvement of the shuttle plasmid pYGK-lacZ

pYGK-*lacZ* is a transcriptional reporter plasmid that has been successfully used to characterize the leukotoxin promoter of *A. actinomycetemcomitans* (Brogan et al., 1996). To improve this vector and to minimize potential toxic effects arising from run-on transcription from strong promoters fused to *lacZ,* the *lacZ* gene from *E. coli* C600 was replaced with the promoterless *lacZ* gene from pBAD-*lacZ* that includes the strong transcriptional terminator sequences T1 and T2 derived from the *rrnB* gene (Orosz et al., 1991). The resulting plasmid was named pJT3 (Fig. 3A). The utility of the pJT3 transcriptional/translational reporter plasmid was recently validated through the determination of the minimal regulatory region of the *A. actinomycetemcomitans lsrCADBFG* and *lsrRK* operons (Torres-Escobar et al., 2013) as well as *ygiW-qseBC* operon (Juarez-Rodriguez et al., unpublished results). Since pJT4-A contains a replication origin that is required for maintenance in both *E. coli* and *A. actinomycetemcomitans*, further modifications of this plasmid were performed through deletion/insertion of new restriction sites (see Material and Methods) with the aim to conserve the same unique restriction sites *Kpn*I and *Bam*HI previously mapped on pYGK-*lacZ*. The resulting new cloning vector was designated pJT4 (Fig. 3B) and this plasmid lacks all sequences of pYGK that are not required for replication or selection. To validate its utility as shuttle cloning vector, the *A. actinomycetemcomitans lsrRK* operon and *crp* gene were cloned individually into pJT4 (see Material and Methods) and the resulting recombinant plasmids pATE52 and pATE60, respectively, were selected for in *E. coli* and analyzed by restriction digestion. Subsequently, these plasmids were introduced by electroporation into *A. actinomycetemcomitans* 652, re-isolated from recombinant cells and shown to be identical to the input plasmid by restriction analysis (data not shown). These results confirm that the pJT4-derivative plasmids replicate and are maintained in both *E. coli* and *A. actinomycetemcomitans* cells. Finally, the *lacZ* gene from pBAD-*lacZ* was introduced into pJT4 resulting in the transcriptional/translational reporter plasmid pJT5 (Fig. 3C). To demonstrate the utility of pJT5, the 255 bp *A. actinomycetemcomitans lsrR* promoter was cloned into the plasmid to generate pATE65. pATE65 was introduced by electroporation into *A. actinomycetemcomitans* 652 and β-galactosidase activity produced by this strain was compared to a similar strain harboring pATE12 (pYGK-*lacZ* fused to the *lsrR* promoter) and pATE23 (pJT3, *lsrR* promoter-*lacZ*) (Torres-Escobar et al., 2013). The

lsrR promoter expressed similar levels of activity (see Table 4), indicating that pJT5 functions as a transcriptional/translational reporter plasmid for studies of gene expression in *A. actinomycetemcomitans*. Plasmid pJT3, pJT4, pJT5 and pJT6 were stably maintained for 50 or more generations in *A. actinomycetemcomitans* under selective conditions and *A. actinomycetemcomitans* transformant cells [OD_{600nm} of 0.6 (1.15×10^9 colony forming, CFU, units ml^{-1})] produced the same amount of each recombinant plasmid (~0.75 $\mu g\ ml^{-1}$) as pYGK (not shown). pJT4 and pJT5 are both almost 2 kbp smaller than the pYGK plasmids from which they were derived.

3.4 pJT1, a scarless-markerless suicide plasmid

The suicide plasmid pJT1 (Fig. 3D) carries the pUC origin of replication and does not replicate in *A. actinomycetemcomitans*. In addition, pJT1 is a sucrose-sensitive suicide vector containing the *Bacillus subtilis sacB* gene encoding levane saccharase. This gene is lethal in most Gram-negative bacteria in the presence of sucrose (Gay et al., 1983) and thus provides strong selection for isolation of organisms that have undergone both recombination events required for gene deletion mutagenesis. The expression of *sacB* is under the control of the *trc* promoter (P_{trc}), and thus is inducible in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). In its present form, pJT1 also contains a spectinomycin resistance gene and a multiple cloning site (MCS) with thirteen unique restriction endonuclease sites for cloning the DNA fragments flanking the target gene to be deleted or inactivated. However, as described in the Materials and Methods, pJT1 was constructed from individual fragments containing a multiple cloning site (MCS), *trc* promoter and *sacB*; the pUC origin of replication; and an antibiotic resistance marker. The use of these separate functional cassettes readily facilitates additional modifications to develop new vectors with different selectable markers, origins or reporter genes.

3.5 Construction of the A. actinomycetemcomitans deletion mutants

To demonstrate the utility of pJT1, we generated markerless deletion mutants of the *lsrRK* operon and the *qseB* gene of *A. actinomycetemcomitans*. DNA fragments flanking *qseB* or *lsrRK* were cloned individually into pJT1 (as stated in Materials and Methods) to create the suicide plasmids pDJR37 and pATE47, respectively. Twenty micrograms of each plasmid were introduced into *A. actinomycetemcomitans* 652 cells by electroporation. Transformed bacterial cells with the suicide plasmid integrated in the chromosome by a single homologous recombination event were selected on medium containing 50 $\mu g\ ml^{-1}$ spectinomycin. The transformation efficiencies were about 10 CFU μg^{-1} DNA using 1×10^{10} electroporated cells. Ten Sp^r colonies were randomly chosen and sub-cultured daily for three consecutive days. To identify gene deletion mutants that had undergone a second event of recombination, cells were diluted and spread on TYE agar with 1mM IPTG and 10% sucrose. Approximately 1,000 Suc^r colonies were replica plated onto agar medium containing sucrose in the presence and absence of spectimomycin. Twenty one and twenty Suc^R-Sp^S colonies were obtained with pDJR37 and pATE47, respectively. About 85 % of Suc^R-Sp^S colonies contained the expected modified allele, as shown in Figure 4B. Deletion of *qseB* was confirmed in ten independent Suc^R-Sp^S colonies by the generation of a 929 bp amplicon. A similar PCR approach was used to confirm deletion of lsrRK (data not shown). Subsequently, markerless deletion mutagenesis using pJT1 was used successfully to generate deletion mutants lacking *qseC*, *qseBC*, and *ygiW* (Juarez-Rodrigues et al., unpublished results) and *lsrR*, *lsrK*, and *crp* (Torres-Escobar et al., 2013). The transformation efficiency of each of these suicide plasmids was similar to that reported above. In addition, the yield of clones that contained the expected modified allele was similar to that obtained in the experiment described above.

4. Discussion

To continue to make progress in understanding the genetic and molecular basis of *A. actinomycetemcomitans* pathogenicity and virulence, new molecular tools and genetic techniques, and/or improvements on the currently available vectors are needed to manipulate the *A. actinomycetemcomitans* genome. In this study, we focused on the construction of vectors that will facilitate gene cloning, gene deletion and the analysis of gene expression in *A. actinomycetemcomitans*. To accomplish this, the complete sequence of the *A. actinomycetemcomitans-E. coli* shuttle vector pYGK was determined in order to further characterize the plasmid and improve its stability by eliminating sequences that are non-essential for its replication. Analysis of the nucleotide sequence of pYGK revealed that the original parent plasmid pYG10, isolated from *A. pleuropneumoniae* s 80-8141 obtained from a clinical case of swine pleuropneumoniae (Lalonde et al., 1989), is a mobile plasmid that mediates antibiotic resistance. The nucleotide sequence also allowed us to identify elements that may represent the origin of replication required for maintenance of the plasmid in both *A. actinomycetemcomitans* and *E. coli.* Indeed, the overall structure of the pYGK repeat region was similar to the 720 bp minimal replication unit identified by Briggs and Tatum, (2005) in plasmid pD70 from *Mannheimia haemolytica*. Similar 720 bp origins also exist in plasmid pIG1 from *Pasteurella multocida* (Gene Bank accession No. U57657.1). Briggs and Tatum, (2005) determined that pD70-3′Δ400*ori*Km^r can be replicated in the *Pasteurella multocida* NADC TT94 strain, but they did not determine if the 720 bp origin of pD70 was functional in *E. coli*. Our results indicate that a 809 bp fragment containing the repeat region of pYGK (but excluding IR_1) was insufficient to support replication in either *A. actinomycetemcomitans* or *E. coli*. Similarly, a 1493 bp fragment harboring only the mobilization genes was unable to support replication, whereas a 820 bp fragment containing the two ORF's plus the four IR sequences as well as a 2484 bp fragment containing both the cluster of mobilization genes and inverted repeat sequences allowed replication. Therefore, this fragment was subsequently used in the construction of cloning and *lacZ* reporter vectors. These new vectors, pJT4 and pJT5, contain only the sequences required for plasmid replication and selection and eliminate over 1,600 bp of pYGK that are not necessary for these essential functions. This will facilitate the cloning of larger inserts and may increase the stability and transformation efficiency of these constructs.

We were also interested in developing news tools to facilitate gene replacement or gene deletion mutagenesis approaches in *A. actinomycetemcomitans*. The pJT1 suicide vector was designed for the generation of chromosomal non-polar, scarless and markerless gene deletions or gene modifications in *A. actinomycetemcomitans*. pJT1 carries strong selectable markers including a spectinomycin-resistance marker that is efficiently expressed in *A. actinomycetemcomitans* to select for plasmid insertion into the target locus and the *sacB* gene under the control of the P_{trc} promoter, which provides a strong counter-selectable marker that facilitates the positive selection of mutants that have undergone the replacement of the wild type allele by the mutated allele, and have lost the integrated plasmid. Using an approach that has been used successfully in *Salmonella* to generate markerless deletion mutations (Kang et al., 2002) we have used pJT1 to generate several deletion mutants and in frame deletions that target the removal of specific functional domains of the protein encoded by the target gene. The mutant strains that were obtained are free of antibiotic markers and scars and thus can readily be subjected to additional rounds of mutagenesis without encountering the limitations of current approaches for generating multiple mutations in *A. actinomycetemcomitans*. Furthermore, pJT1 deletion mutagenesis was carried out using a serotype c strain which is not naturally competent, suggesting that the pJT1 vector will be useful for mutagenesis in both naturally and non-naturally competent strains of *A. actinomycetemcomitans*.

In summary, characterizing the *A. actinomycetemcomitans-E. coli* shuttle vector pYGK has allowed us to design new cloning, *lacZ* reporter and suicide vectors that should facilitate genetic studies and mutagenesis approaches in *A. actinomycetemcomitans*. Given the similarity of the pYGK origin of replication with other plasmids from *Mannheimia* and *Haemophilus* species, these vectors may be more generally applicable for genetic studies in other HACEK organisms as well.

Acknowledgments

This research was supported by the Public Health Service grant RO1DE14605 from the NIDCR.

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Highlights

- The nucleotide sequence of the shuttle vector pYGK facilitated contruction of new pYGK-derived plasmids.
- The minimal origin of replication of the shuttle vector pYGK was identified.
- The *mob* genes region is unnecessary for pYGK replication.
- A new scarless-markerless suicide plasmid, pJT1 was constructed.
- The effectiveness of pJT1 was demonstrated by the construction of Δ*qseB* and Δ*lsrRK* mutants.

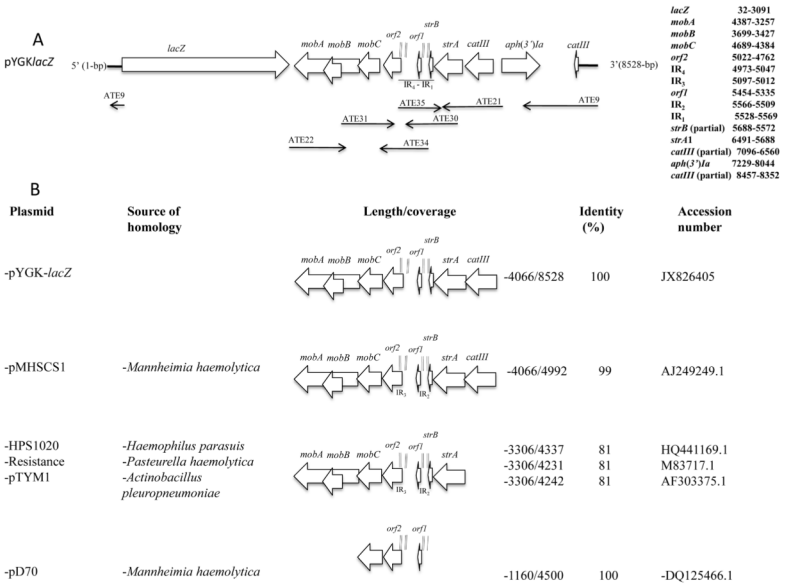

Figure 1.

Schematic representation of plasmid pYGK-*lacZ* and its comparative analysis. A) The transcriptional orientation of open reading frames in pYGK*lacZ* is indicated by open arrows. Thin arrows below the gene name show the sequence revealed and localization of the primers. B) A BLAST search using a 4066-bp DNA fragment from pYGK-*lacZ* identified related plasmid sequences in *Mannheimia haemolytica, Haemophilus parasuis, Pasteurella haemolytica and Actinobacillus pleuropneumoniae*. The orientation of gene transcription is indicated by open arrows and percent nucleotide identity is indicated on the right. The inverted repeat sequences (IR) are indicated with double smaller vertical lines.

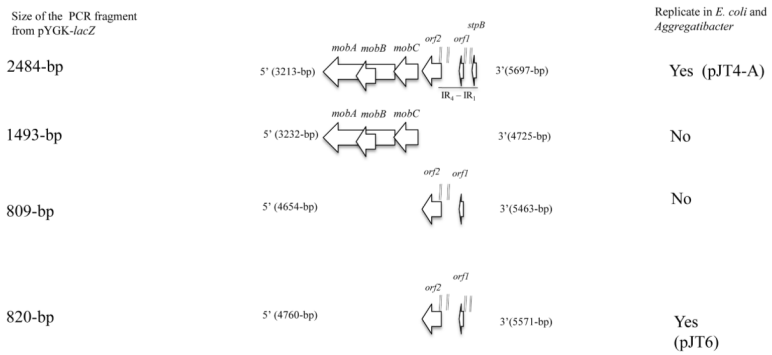

Figure 2.

Mapping the pYGK origin of replication. A) Schematic representation of DNA fragments harboring different regions of the pYGK origin of replication and mapping of the minimal region required for plasmid replication in *A. actinomycetemcomitans* and *E. coli*.

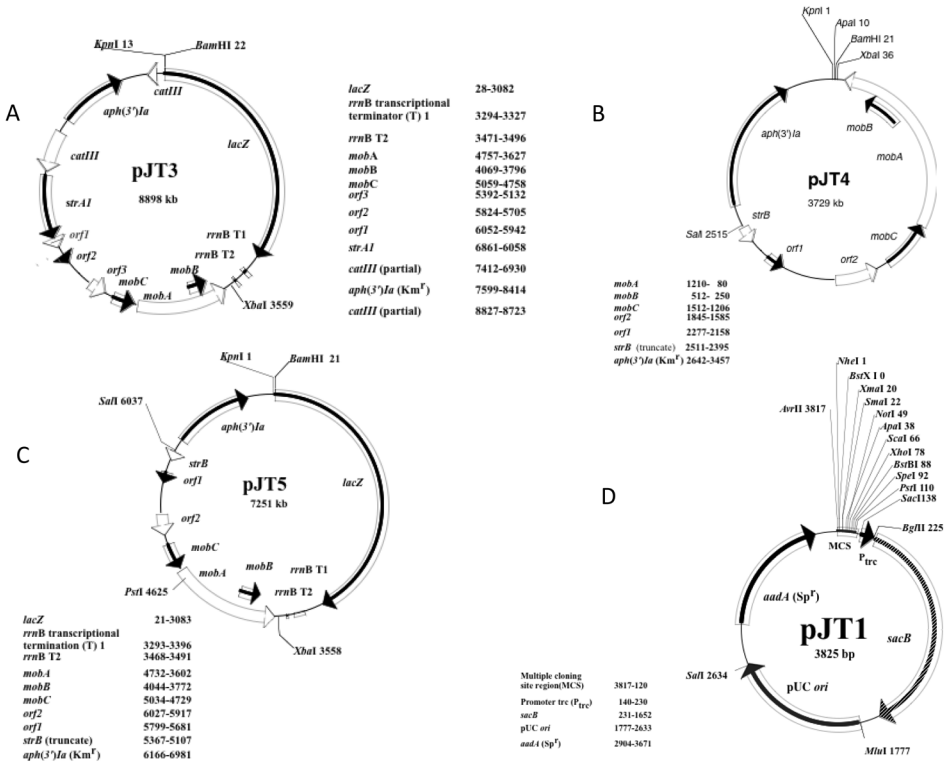

Figure 3.

Physical maps of transcriptional/translation reporter plasmid pJT3 (A), the cloning vector pJT4 (B), the transcriptional/translation reporter plasmid pJT5 (C) and the suicide vector pJT1 (D).

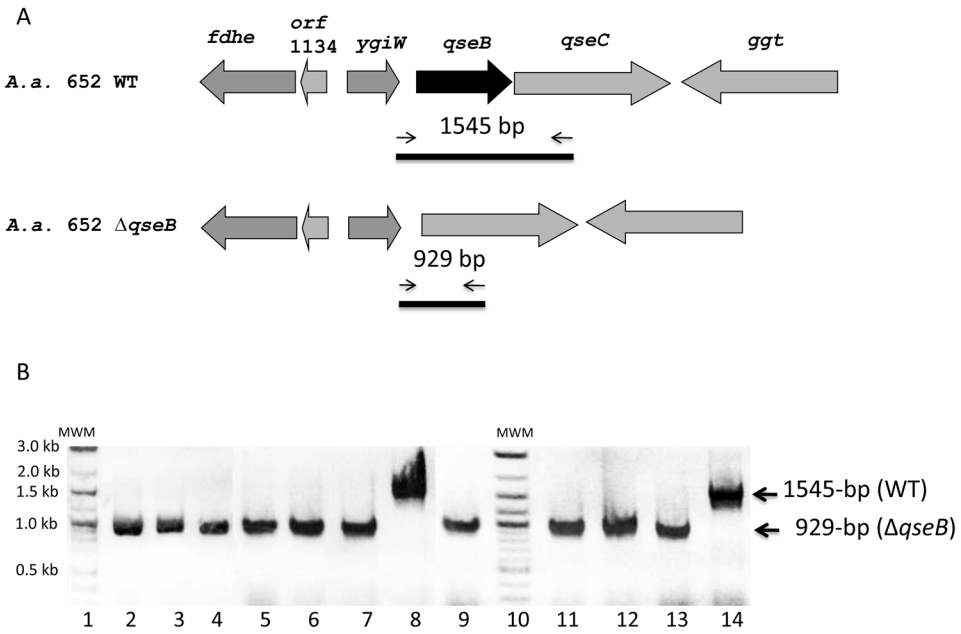

Figure 4.

Deletion of the *qseB* gene in *A. actinomycetemcomitans* 652. (A). Schematic representation of the *ygiW-qseB-qseC* locus. The transcriptional orientation of genes is indicated by open arrows. Thin arrows represent primers used for PCR confirmation of the deletion of *qseB*. (B). PCR products obtained with the *A. actinomycetemcomitans* Δ*qseB* mutant strains. Deletion of the *qseB* gene was confirmed by PCR amplification of a 929-bp fragment with primer set MDJR-63F and MDJR-61R in ten randomly selected Suc^R and Sp^S colonies obtained with pDJR37 suicide vector as described in Material and Methods. The amplification profile used was: 94°C for 2 min for 1 cycle and then 94°C for 30 s, 58°C for 30 s, and 68°C for 2 min for 35 cycles. MWM, molecular weight marker.

Table 1

Strains and plasmids used in this work

Strain or plasmid	Derived, relevant genotype or characteristics[a]	Source or reference
Escherichia coli		
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB. lacI*^{q}ZΔ*M15* Tn10 (Tc^{r})]	Stratagene
Aggregatibacter actinomycetemcomitans		
652	Wild type, serotype c,	Laboratory stock
652-JR37	652 Δ*qseB*	This study
652-TE47	652 Δ*lsrRK*	This study
Plasmids		
pUC18	Amp^{r}; cloning vector	(Yanisch-Perron et al., 1985)
pBAD-HA-*lacZ*	Amp^{r}, expression vector	Invitrogen
pYGK	Km^{r}, cloning shuttle vector	Brogan et al., 1996
pYGK-*lacZ*	Km^{r}, promoterless *lacZ* reporter vector	James et al., 2006
pUC4K	Km^{r}, cloning shuttle vector	(Viera and Messing, 1982)
Suicide vectors		
pVT1461	Sp^{r}; template for amplifying the spectinomycin gene	(Mintz et al., 2002)
pRE112	Cm^{r}: template for amplifying the *sacB*	(Edwards et al., 1998)
pJT1	Sp^{r}; suicide vector	This study
Derived from suicide vector pJT1		
pDJR37	Sp^{r}; flanking region to *qseB*	This study
pATE47	Sp^{r}; flanking region to *lsrRK*	This study
Derived from reporter vector pYGK-*lacZ*		
pJT3	Km^{r}, promoterless *lacZ* reporter vector	This study
pJT4	Km^{r}, cloning vector	This study
pJT5	Km^{r}, promoterless *lacZ* reporter vector	This study
pJT6	Km^{r}, cloning vector	This study
pJT12	Km^{r}, promoter *lsrR* (255-bp)-*lacZ*	This study
Derived from pJT3		
pATE23	Km^{r}, promoter *lsrR* (255-bp)-*lacZ*	Torres-Escobar et al., 2013
Derived from pJT5		
pATE65	Km^{r}, promoter *lsrR* (255-bp)-*lacZ*	This study

[a] Amp^{r} ampicillin resistance, Km^{r} kanamycin resistance, Tc^{r} tetracyclin resistance, Sp^{r} Spectinomycin resistance.

Table 2

Oligonucleotides used in this work

Related product and oligonucletide	Sequence (5′ →3′)
MCS assembly	
MDJR-16F	AGCTTGTGCTAGCATCTGGTACCCCACCCGGGTGGAAATTGGGCCCAATTAATGGCGGCCGCTACGTCGTAGTA
MDJR-17R	CTGGAAGTACTACGACGTAGCGGCCGCCATTAATTGGGCCCAATTTCCACCCGGGTGGGGTACCAGATGCTAGCACA
MDJR-18F	CTTCCAGCTACTCGAGTTATTCGAACTAGTATTCGTTACTGCAGCCAATATTGCGCGCTTATTGCTGAGCTCCAGCTTCGT
MDJR-19R	CAAGAGATCTACGAAGCTGGAGCTCAGCAATAAGCGCGCAATATTGGCTGCAGTAACGAATACTAGTTCGAATAACTCGAGTAG
MDJR-20F	AGATCTCTTGTTACACGCGTCTTATTCTGTCGACCTTCCTTTAGGATCCATTGAGTTACCTAGGGGTTATGCTAGCTAG
MDJR-21R	AATTCTAGCTAGCATAACCCCTAGGTAACTCAATGGATCCTAAAGGAAGGTCGACAGAATAAGACGCGTGTAA
P_{trc} promoter	
MDJR-22F	CATTCTGAAATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACA
MDJR-23R	GATCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACATTATACGAGCCGGATGATTAATTGTCAACAGCTCATTTCAGAATGAGCT
***sacB* gene**	
MDJR-24F	AAGGAGACAAGATCTATGAACATCAAAAAGTTTGCAAAACAAGC
MDJR-27R	AACTAGCGAACGCGTTCAAAAGGTTAGGAATACGGTTAGC
pUC *ori*	
MDJR-34F	GCGGTAATACGCGTATCCACAGAATCAGGGGATAACG
MDJR-35R	TAAGCATTGTCGACTGTCAGACCAAGTTTACTCATATATAC
Spectinomycin gene	
MDJR-28F	GTCGTACGGTCGACTTTCGTTCGTGAATACATGTTATAATAAC
MDJR-29R	GTAATCTGGCTAGCCCTAGGCAAGGGTTTATTGTTTTCTAAAATC
***qseB* deletion**	
MDJR-49F *Not*I	
MDJR-82R *Xho*I	GCCGTTACCCTCGAGAGGATCATCTTCGATTAATAAAATCCGC
MDJR-83F *Xho*I	GTTCACGGCCTCGAGTATGCACTGGGAAAAAATGATGAAACTG
MDJR-84R *Pst*I	GTCCGCTTTCCTGCAGGACACCAACTTTAGTCATGAAAGAC
PCR verification	
MDJR-63F	CCAAGTGGAAATCGAACGCAAAGCCTGGC
MDJR-61R	CGTACGTTTTGCCACTTGTTCACCGATTTGTTTCAGG
***lsrRK* double deletion**	

Related product and oligonucleotide	Sequence (5′ →3′)
ATE-86F *Not*I	CGTCAACATAATTATTCCCTCCGCGGCCGCTTATTTCGACCGC
ATE-87R *Xho*I	CGTTTCTTGATTATCCGTCTCGAGCATTAATTCGTTC
ATE-88F *Xho*I	GGCACCAGGGTTGTAGCTCGAGCGAACGATAAAAAATCG
ATE-89R *Pst*I	GATGCAATTTTTGTCTGCAGCATATATACCTC
PCR-verification	
ATE-62F	GAGCTGATGGGCTTTATTCG
ATE-102R	CAAGATCTCAGCCGTTTCATCACC
Upstream region *lsrR*	
lsrR255 f-3	GTGAGTTTGGGTACCCATCTTTTACCTGCCG
lsrR255 r-6	CTTGATTATCCG TGGATCCCATTAATTCGTTC
***lsrRK* operon**	
lsrR F-109	CCGGCGATAATTTTCATGGATCCCAGCGTGGATTTCC
lsrK R-110	GGAACCGGTGGCATAATCTAGAAAGTGCGGTGGG
***crp* gene**	
crp F-151	GCCGAATCAGGGTACCAACCAGCAATGGC
crp R-152	CCCGACAGGTTCTCTAGAGTTTGTAACGATGC
Nucleotide sequence of pYGK-*lacZ*	
ATE9R	CGGAAACCAGGCAAAGCGCCATTC
ATE21R	CCCCTTGTATTACTGTTTATGTAAGC
ATE22F	GATTGGTGGCGACGACTCCTGGA
ATE30R	CGAAAATCGCACCTGCTTCC
ATE31F	GCCTGTTTTTTGTCGGTTGG
ATE34F	GGCGGTAGGTTTTTAGTGTTGG
ATE35R	CCTATTCAATGTATTGGGGATC
Mapping *ori*T region	
ATE-38F	GTCGACCTGCAGGGGACTAGTGGTACCGGGCCCGGAAAGCC
ATE-39R	CGCTGAGGTCTGCTAGCCTCGTGAAGAAGG
ATE-40F	GGTTGAAACTAGTAAGCCTATTATCTAGATTTG
ATE-41R	CTTGACCCCCGACCGCTAGCTTTCAGCGGTC
ATE-107F	GAGTCGACCTGCAGGCATGCTCTAGATGGTTGAAAC
ATE-108R	CCCTCTGACTTGGGGATCCTTGATGTTCATGCCGCC

Related product and oligonucletide	Sequence (5′ →3′)
ATE-115	CAGGCGGCATGAACATCAAGTCGACGGGGGGGGGGGAAAGCCACGTTGTG
ATE-116	CACAACGTGGCTTTCCCCCCCCCCCGTCGACTTGATGTTCATGCCGCCTG
ATE-117	CTTCACGAGGTACCAGAGGGCCCTCAGCGGATCCCCCCCCCCCTCTAGATGGTTGAAACTAAA
ATE-118	TTTAGTTTCAACCATCTAGAGGGGGGGGGGATCCGCTGAGGGCCCTCTGG*TA*CCTCGTGAAG
ATE-177F	GCCCTTTTGC AATCTAGAGGTCAAAA AGGAAACTCG
ATE-178R	CCAATGCTTTAGGTCGACAAAGAAAAAGGGATCG
Kanamycin marker	
ATE-36F	GATACTAGTTTTCTAGAGGTGAGCCTG
ATE-37R	GCAAAGGCTAGCTAGTAATGCAAGAGATTGCG
ATE-105F	TAACGTCGTGATTCGACGGATCCGGGGGG
ATE-106R	GCAAAATTGATTCGACTCTAGAGGGGGGG
ATE-179F	TAACGTCGTGATTCGACGTCGACGGGGGG

Table 3

Invert, direct and putative *ori*T sequences of pYGK.

Invert repeat 1	5′ TCTTTTTCCCTAGCTT-N_9-AAGCTAGGGAAAAAGA 3′ 3′ AGAAAAAGGGATCGAA-N_9-TTCGATCCCTTTTTCT 5′
Invert repeat 2	5′ GAAACGATTCTAT-N_{18}-ATAGAATCGTTTC 5′ 3′ CTTTGCTAAGATA-N_{18}-TATCTTAGCAAAG 3′
Invert repeat 3	5′ TTTTT-N_6-AAAAA 3′ 3′ AAAAA-N_6-TTTTT 3′
Imperfect Invert repeat 4	5′ CACAACCTTTCGACAACCTTTCGAGCACCCATTGAC-N_3- CTCAATAGGTAGTCGAAAAGTTACCGAAAGGTTATG 3′ 3′ GTGTTGGAAAGCTGTTGGAAAGCTCGTGGGTAACTG-N_3- GAGTTATCCATCAGCTTTTCAATGGCTTTCCAATAC 5′
Direct repeat 1	5′ CGAAA-N_6-CGAAA 3′ 3′ GCTTT-N_6-GCTTT 3′
Direct repeat 2	5′ ACAACCTTTCGACAACCTTTCGA 3′ 3′ TGTTGGAAAGCTGTTGGAAAGCT 5′
oriT 1	5′ CCGAAAG 3′ 3′ GGCTTTC 5′
oriT 2	5′ CCGAAAG 3′ 3′ GGCTTTC 5′

Table 4

Strain/plasmid	Derived from	Miller units
Aa 652		0
Aa 652/pYGK-*lacZ*		0
Aa 652/pATE12($P_{lsrR255}$-*lacZ*)	pYGK-*lacZ*	916±31
Aa 652/pJT3		0
Aa 652/pATE23($P_{lsrR255}$-*lacZ*)	pJT3	930±36
Aa 652/pJT5		0
Aa 652/pATE65($P_{lsrR255}$-*lacZ*)	pJT5	928±28