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tfoX* (sxy)-dependent transformation of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans

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Abstract

tfoX (sxy) is a regulatory gene needed to turn on competence genes. *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* has a *tfoX* gene that is important for transformation. We cloned this gene on an IncQ plasmid downstream of the inducible *tac* promoter. When this plasmid was resident in cells of *A. actinomycetemcomitans* and *tfoX* was induced, the cells became competent for transformation. Several strains of *A. actinomycetemcomitans*, including different serotypes, as well as rough (adherent) and isogenic smooth (nonadherent) forms were tested. Only our two serotype f strains failed to be transformed. With the other strains, we could easily get transformants with extrachromosomal plasmid DNA when closed circular, replicative plasmid carrying an uptake signal sequence (USS) was used. When a replicative plasmid carrying a USS and cloned DNA from the chromosome of *A. actinomycetemcomitans* was linearized by digestion with a restriction endonuclease or when genomic DNA was used directly, the outcome was allelic exchange. To facilitate allelic exchange, we constructed a suicide plasmid (pMB78) that does not replicate in *A. actinomycetemcomitans* and carries a region with two inverted copies of a USS. This vector gave allelic exchange in the presence of cloned and induced *tfoX* easily and without digestion. Using transposon insertions in cloned *kata* DNA, we found that as little as 78 bp of homology at one of the ends was sufficient for that end to participate in allelic exchange. The cloning and induction of *tfoX* makes it possible to transform nearly any strain of *A. actinomycetemcomitans*, and allelic exchange has proven to be important for site-directed mutagenesis.

Keywords

periodontitis; pathogen; USS; allelic exchange; site-directed mutagenesis; competence

1. INTRODUCTION

Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans* is a Gram-negative, facultative anaerobe of the Pasteurellaceae family that is found in the oral cavity (Henderson et al.,

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2003; Fine et al., 2006; Norskov-Lauritsen and Kilian, 2006). It is believed to cause localized aggressive periodontitis (LAP), a severe form of periodontal disease of adolescents, and several types of extraoral infections, including infective endocarditis, abscesses, meningitis, pneumonia, septicemia, urinary tract infections, and vertebral osteomyelitis (Zambon, 1985; Haffajee and Socransky, 1994; Slots, 1999; Henderson et al., 2003; Paturel et al., 2004; Fine et al., 2006). Fresh clinical isolates show a small, wrinkled (rough) colony form (Fine et al., 1999; Henderson et al., 2003; Kachlany et al., 2001a). The bacteria are known to produce several virulence factors, including a leukotoxin and a cytolethal distending toxin, and they adhere to solid surfaces and cells (Taichman and Wilton, 1981; Kolodrubetz et al., 1989; Lally et al., 1989; Eastcott et al., 1990; Mangan et al., 1991; Fives-Taylor et al., 1999; van Winkelhoff and Slots, 1999; Kachlany et al., 2001a; Narayanan et al., 2002; Henderson et al., 2003; Rose et al., 2003; Paturel et al., 2004; Rhodes et al., 2005; Diaz et al., 2006; Fine et al., 2005, 2006; Smith and Bayles, 2006). Clinical isolates autoaggregate and secrete long, bundled pili (Flp fibrils) (Holt et al., 1980; Scannapieco et al., 1987; Rosan et al., 1988; Inoue et al., 1998; Kachlany et al., 2001b; Henderson et al., 2003; Fine et al., 2006). *In vitro* *A. actinomycetemcomitans* can produce rare, stable, nonadherent, and nonaggregative genetic variants that form smooth, large colonies and fail to produce Flp fibrils (Wyss, 1989; Inouye et al., 1990; Fine et al., 1999, 2006; Kachlany et al., 2000, 2001a; Henderson et al., 2003).

Powerful genetic tools have contributed to the study of *A. actinomycetemcomitans*. The three natural methods for gene transfer (transformation, conjugation, and transduction) have all been observed in *A. actinomycetemcomitans* (Tønnum et al., 1990; Goncharoff et al., 1993; Willi et al., 1997; Galli et al., 2001). We previously developed an efficient method for conjugation-based transposon-insertion mutagenesis (Thomson et al., 1999) that allowed us to isolate three independent mutations in the catalase gene (*katA*) of *A. actinomycetemcomitans* (Thomson et al., 1999) and in genes involved in non-specific adherence (Kachlany et al., 2000, 2001b; Planet et al., 2003). For the latter, we identified the *tad* (tight adherence) locus, which encodes a novel secretion system responsible for the synthesis, assembly, and secretion of bundled type IVb pili, termed Flp fibrils (Kachlany et al., 2001a).

In this report we are concerned with transformation in *A. actinomycetemcomitans* and its utility as a genetic tool. *Haemophilus influenzae* is a Gram-negative bacterium that, like *A. actinomycetemcomitans*, is in the Pasteurellaceae family, and transformation has been well-studied in this organism (Smith et al., 1981; Lorenz and Wackernagel, 1994; Dubnau, 1999; Chen and Dubnau, 2004; Chen et al., 2005; Cameron and Redfield, 2006). Transformation probably involves binding of DNA to the cell surface and the formation of linear DNA in the periplasm that is then transported into the cytoplasm and recombined with the host genome. In *H. influenzae* there is evidence that double-stranded DNA is first taken up into structures known as “transformasomes” and that a single strand of DNA is transported across the inner membrane into the cytoplasm in a 3' to 5' direction, while the other strand is degraded (Concino and Goodgal, 1982; Barany et al., 1983; Kahn, et al., 1983; Dubnau, 1999).

Several genes are known to affect transformation in *H. influenzae* (Caster et al., 1970; Caster and Smith, 1972; Notani et al., 1972; Concino and Goodgal, 1982; Kooistra et al., 1983; Barouki and Smith, 1985, 1986; Tomb et al., 1989, 1991; Larson and Goodgal, 1991; Chandler, 1992; Dorocicz et al., 1993; Clifton et al., 1994; Gwinn et al., 1996, 1997, 1998; Chandler and Smith, 1996; Karudapuram and Barcak, 1997; Dougherty and Smith, 1999; Dubnau, 1999; Ma and Redfield, 2000; Chen and Dubnau, 2004; VanWagoner et al., 2004; Redfield et al., 2005, 2006; Cameron and Redfield, 2006; Smeets et al., 2006). The cAMP-CRP (cyclic-AMP receptor protein) complex helps to promote their expression and induce competence (the ability to take up and incorporate DNA) (Chandler, 1992; Redfield et al., 2005; Cameron and Redfield, 2006). Induction of competence in *H. influenzae* also requires the product of the *tfoX* (*sxy*) gene (Redfield, 1991; Williams et al., 1994; Zulty and Barcak, 1995; Redfield et al., 2005).

TfoX helps CRP bind a non-canonical CRP site (CRP-S) to turn on the genes of the competence regulon (Redfield et al., 2005; Cameron and Redfield, 2006). The TfoX protein of *H. influenzae* itself does not seem to bind DNA, but to interact with CRP (Macfadyen, 2000; Redfield et al., 2005). Plasmids containing the *tfoX* gene make *H. influenzae* cells constitutively competent (Williams et al., 1994; Zulty and Barcak, 1995).

Many species of bacteria are known to develop competence in nature (Lorenz and Wackernagel, 1994; Dubnau, 1999), including *A. actinomycetemcomitans* (Tønnum et al., 1990), whose natural transformation is not understood. Currently natural transformation in *A. actinomycetemcomitans* appears to be strain-specific and is too low to be useful in many genetic studies (Wang et al., 2002; unpublished results). In some naturally transformable bacteria, competence is induced transiently during growth, whereas in others, it seems to be constitutive. Gram-positive bacteria, like the well-studied bacteria *Bacillus subtilis* and *Streptococcus pneumoniae*, and some Gram-negative bacteria, like *Acinetobacter* species (Dubnau, 1999; Chen and Dubnau, 2004) and *Legionella pneumophila* (X Charpentier and HA Shuman, personal communication), are able to take up DNA non-specifically. In contrast, some Gram-negative bacteria, such as *H. influenzae*, *Actinobacillus pleuropneumoniae*, *Neisseria meningitidis*, and *N. gonorrhoeae*, take up their own DNA preferentially (Sisco and Smith, 1979; Goodman and Scocca, 1988; Smith et al., 1999; Bakkali et al., 2004; Hamilton and Dillard, 2006; Redfield et al., 2006). This specificity arises from the presence of Uptake Signal Sequences (USSs), also called DNA Uptake Sequences (DUSs), that occur frequently in their genomes (Smith et al., 1999; Dubnau, 1999; Chen and Dubnau, 2004; Redfield et al., 2006). In *H. influenzae*, the core USS is a 9-bp sequence, whereas in *A. pleuropneumoniae*, also in the Pasteurellaceae, the 9-bp sequence is different (Bakkali et al., 2004; Redfield et al., 2006). In *Neisseria* spp, the core USS is a 10-bp sequence that is distinct (Kroll et al., 1998). We were the first to report that the *A. actinomycetemcomitans* genome has numerous copies of a sequence identical to the 9-bp core USS of *H. influenzae*, and we predicted that the sequence is part of a USS, important to transformation in *A. actinomycetemcomitans* (Thomson et al., 1999). Experimental evidence indicates that the function of the sequence is indeed in transformation, as predicted (Wang et al., 2002, 2006; this work). Other studies have been concerned with the presence of USSs in the genomes of the Pasteurellaceae and the functional structure, origin, lineage, and evolutionary significance of the USSs (Bakkali et al., 2004; Redfield et al., 2006; Wang et al., 2006).

H. influenzae-like transformation genes occur in the genome of *A. actinomycetemcomitans* (Wang et al., 2006; Cameron and Redfield, 2006; MK Bhattacharjee and DH Figurski, unpublished results), which is consistent with the ability of this organism to undergo natural transformation. In *A. actinomycetemcomitans*, genes also involved in the formation of a type IV pilus-like system, distinct from those that are responsible for FliP fibrils, are thought to be required for transformation (Wang et al., 2003). Although transformation of *A. actinomycetemcomitans* has been used for genetic studies (Tønnum et al., 1990; Sreenivasan et al., 1991; Sato et al., 1992; Kolodrubetz and Kraig, 1994; Wang et al., 2002; Fujise et al., 2004), transformation is not frequent enough or it requires specific strains. Importantly, the methods do not work on most of the strains that we have tested (data not shown).

We report here a general and efficient method for the transformation and site-directed mutagenesis of *A. actinomycetemcomitans* based on its *tfoX* gene. We show that nearly all strains of *A. actinomycetemcomitans*, including those with different serotypes, can be transformed. Transformation occurs with circular or linear DNA containing a USS when the cloned *tfoX* gene of *A. actinomycetemcomitans* is *in trans* on a plasmid and induced. Without the cloned *tfoX*, no transformants are recovered. Transformation with genomic DNA from *A. actinomycetemcomitans* results in allelic exchange with chromosomal DNA. Because there are more copies per µg of DNA, it is easier to get allelic exchange with cloned *A.*

actinomycetemcomitans DNA either on a replicative plasmid that has been linearized or on a suicide plasmid. For best results, the plasmid should also contain a region with a USS. We also show that circular replicative plasmids containing a USS readily give transformants in which the plasmid replicates extrachromosomally.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

For our experiments we used nalidixic acid-resistant (Nal^r) versions of the strains, which are listed in footnote 1 of Table 1. *A. actinomycetemcomitans* was grown in AAGM (*A. actinomycetemcomitans* Growth Medium) with added bicarbonate in screw cap tubes (broth) or in a 10% CO₂ atmosphere (agar), as described (Fine et al., 1999). Nal^r mutants of *A. actinomycetemcomitans* strains were obtained by selection on AAGM plates containing 20 µg/ml nalidixic acid (Nal). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth (Ausubel et al., 1989) with aeration or on LB agar. For *A. actinomycetemcomitans*, kanamycin (Km) was used at a final concentration of 40 µg/ml; streptomycin, at 20 µg/ml; and chloramphenicol (Cm), at 2 µg/ml; for *E. coli*, Km was used at 50 µg/ml; and Cm, at 50 µg/ml. Where indicated, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at the specified concentration. All strains used are listed in Table 1.

2.2 DNA Procedures

Genomic DNA was prepared according to a published procedure (Ausubel et al., 1989). Plasmid DNA was isolated using plasmid Miniprep kits (Qiagen). Amplification of DNA by PCR was done with *Taq* DNA polymerase, according to the manufacturer's (Qiagen) recommendations. Oligonucleotide primers were synthesized by Sigma. Restriction endonucleases and phage T4 DNA ligase were used as directed by the manufacturer (New England Biolabs). Separation of DNA by electrophoresis in gels has been described (Ausubel et al., 1989). Nucleotide sequences were determined by the Columbia University DNA sequencing facility using a Perkin-Elmer Applied Biosystems Automated DNA Sequencer 373A.

2.3 Plasmids

The broad-host-range, mobilizable IncQ plasmids, pJAK12, pJAK13, and pJAK17 (a gift from J. Kornacki) contain *lacI^q* and *tacp* and are based on pMMB67HE and pMMB67EH (Fürste et al., 1986) where the selective markers interrupt the ampicillin-resistance gene. They and their derivatives were introduced into strains of *A. actinomycetemcomitans* by conjugative transfer with an *oriT* mutant of the IncP plasmid RK2, as described previously (Thomson et al., 1999). All plasmids are listed in Table 1. Constructed plasmids were made as follows. *pMB7* and *pMB8*. The *tfoX* gene (Fig. 1) was amplified from *A. actinomycetemcomitans* Y4N and CU1000N by PCR, respectively, using primers TfoX up (5'-TAAAAAGCTTCCTGAAGGATTTAATTATGACAACGG-3'), which overlaps the first translational start, and TfoX dn (5'-GGTGGGATCCAAATGAAAAACGCTCAATAGAGCGG-3'). The primers included the sequences for *Hind*III and *Bam*HI restriction sites, respectively, at their 5'-ends. The PCR product was digested with *Hind*III and *Bam*HI, then cloned into *Hind*III- and *Bam*HI-cleaved pJAK17. When circular IncQ plasmids were used to transform cells, the resident *tfoX⁺* plasmid was not selected and was lost by incompatibility. When digested plasmid or non-IncQ DNA (e.g., genomic DNA) was used, the resident *tfoX⁺* plasmid and any incoming IncQ plasmid, was readily lost after one overnight because IncQ plasmids are unstable in *A. actinomycetemcomitans* in the absence of selection (unpublished results). *pMB22*. The mutated catalase gene (*kata2::IS903φkan*) of Aa1394 (Thomson et al., 1999) was amplified by PCR using primers Cat5 up (5'-ATCGCCGGTTTCGTGAAAGTC-3') and Cat10 dn (5'-

TGACCTGCTGCAAGTGTCTGA-3'). The PCR product was digested with *Hind*III, which cuts internally at two *Hind*III sites flanking the *katA* gene in the chromosome, and then cloned at the *Hind*III site of pJAK13. Clones were screened for the orientation in which the *tac* promoter of pJAK13 is present upstream of the *katA* promoter. The cloned DNA contains a mutated *katA* structural gene with its intact promoter, one copy of the USS upstream, and two inverted copies of the USS downstream (Fig. 2A). *pMB40*. The region containing two inverted copies of the USS downstream of *katA* was amplified by PCR using primers CatPac1 up (5'-CCTTAATTAATACGGAATTGGCAAACGCC-3') and CatPac2 dn (5'-CCTTAATTAACACTATAATTTGGTGAATTTTC-3'), which include sequences for restriction sites for *Pac*I, and then cloned into pCR2.1-TOPO, following the manufacturer's (Invitrogen) recommendations. The PCR product contained one *Hind*III site close to the USS region. Clones were screened for the orientation of the insert, in which the USS region was flanked by *Hind*III sites, one from the PCR product and one from the vector. A *Hind*III-*Kpn*I fragment was cloned into *Hind*III- and *Kpn*I-cleaved pJAK12 to give *pMB40*. Its polylinker region contains the following sites in sequence: *Eco*RI, *Sac*I, *Kpn*I, *Sac*I, *Bam*HI, *Spe*I, *Bst*XI, *Eco*RI, *Pac*I, inverted USS repeats, and *Hind*III (Fig. 2B). *pMB78*. The *Hind*III-*Kpn*I fragment of *pMB40* that contains the inverted USS repeats from *katA* was cloned into *Hind*III- and *Kpn*I-cleaved pUC19 to give *pMB78*, which contains the same order of restriction sites and USSs in the polylinker region as *pMB40* (Fig. 2B). *pMB79*. A promoterless *katA* gene from Y4N *katA3::IS903 ϕ kan* (Aa1395) (Thomson et al., 1999) was amplified using primers Cat17 up (5'-CTTTTGTCTGGTACCTATAATTTCTGTC-3') and Cat18 dn (5'-GGTTGTGACTAGTAATCTTCGTCG-3') (Fig. 2A), which contain sequences for *Kpn*I and *Spe*I sites, respectively. After digestion, the resulting *Kpn*I-*Spe*I fragment was cloned into *Kpn*I- and *Spe*I-digested *pMB78*. *pMB88*. A promoterless *katA* gene from Y4N was amplified using primers Cat17 up and Cat18 dn and cloned into *pMB78*, following the same procedure as described for the construction of *pMB79*. *pMB89-pMB94*. Isolated *pMB88* DNA was subjected to *in vitro* transposon mutagenesis with EZ::TN<KAN-2> (Epicenter), using the manufacturer's recommendations. *E. coli* Top10 competent cells were transformed with the resulting library of *pMB88* EZ::TN<KAN-2> mutants, all of which contained an expressed Km-resistance gene (*neo*) that occurs in the transposon. Km-resistant (Km^r) colonies were screened for those that contained the transposon inserted in the cloned *katA* gene. The positions and orientations of the insertions (Fig. 2C) were determined by PCR using two sets of primers, each of which contained one primer at one of the two ends of the *neo* gene (from Epicenter) and one in the vector.

2.4 Transformations

E. coli Top10 cells (Invitrogen) were transformed according to the manufacturer. Competent cells of *A. actinomycetemcomitans* smooth (nonadherent) strains were made as follows. *A. actinomycetemcomitans* strains containing the cloned *tfoX* gene of Y4N or CU1000N were streaked onto plates supplemented with Cm and incubated at 37°C for 3-4 days. Individual colonies were then inoculated into 5-10 ml of AAGM broth containing 2 µg/ml Cm and grown at 37°C overnight. We added Cm to the growth medium to select for the *tfoX*⁺ plasmid. The overnight culture was used to inoculate (1:100) 10 ml of fresh AAGM broth containing Cm. The culture was grown for 3-4 hr at 37°C or until an A₆₀₀ reading of 0.1-0.2. *TfoX* expression was induced by adding 1 mM IPTG, and the culture was grown for another 1 hr at 37°C. Although the optima (Table 2) were found to be 0.2 mM IPTG and an A₆₀₀ of 0.6, the conditions used routinely were adequate and more convenient. The A₆₀₀ was measured and the cells were pelleted at 7K rpm for 5 min in a Sorvall SS34 rotor. The cells were resuspended in the appropriate volume of the same medium with IPTG to give a final A₆₀₀ reading of 1.0.

Competent cells of *A. actinomycetemcomitans* rough (adherent) strains were transformed as follows. Ten ml AAGM broth containing Cm was inoculated with a fresh colony from an

AAGM plate (with Cm) and grown overnight at 37°C. The overnight culture of *A. actinomycetemcomitans* was supplemented with 1 mM IPTG and incubated at 37°C for 1 hr. (See the above note about the optima from Table 2.) The cells were scraped from the walls of the tube with a sterile wooden dowel (Fisher) and pelleted at 7K rpm for 5 min in a Sorvall SS34 rotor. One ml of the supernatant was saved for later use. Clumps of cells were resuspended in 20-50 µl of the saved broth and disrupted with a disposable mortar and pestle (ISC Bioexpress). The cells were then resuspended in the remainder of the saved supernatant. To obtain single cells, the suspension was centrifuged in an Eppendorf microfuge for 5 sec to remove debris and remaining clumps, and the supernatant was filtered through a 5 µm filter (Fisher). The filtrate contained mostly single cells, as seen under a microscope. This filtration step to obtain single cells is only required for calculating transformation efficiencies.

For transforming both adherent and nonadherent strains of *A. actinomycetemcomitans*, 20 µg genomic DNA or 0.1 µg of DNA from pUC19-based plasmids (pUC19, pMB78, pMB79, pMB89-93) or 0.5 µg of DNA from pJAK13-based plasmids (pJAK13, pMB22, pMB40) in a small volume of buffer (1-5 µl) was added to 100 µl of competent cells (nonadherent or adherent) and incubated at 37°C for 2-4 hr. Transformants were selected by spreading transformed cells on AAGM plates containing the appropriate antibiotic.

3. RESULTS

3.1 The *tfoX* (*sxy*) gene of *A. actinomycetemcomitans*

A search of the *A. actinomycetemcomitans* genome database (Cameron and Redfield, 2006; Roe et al., 2006; MK Bhattacharjee and DH Figurski, data not shown) revealed the presence of a *tfoX*-like gene (coordinates 385359-384727), whose product has 41% amino acid identity with that from *H. influenzae* (Fig. 1). The *tfoX* gene of *A. actinomycetemcomitans* shares many properties with the *tfoX* gene from *H. influenzae* (Zulty and Barcak, 1995). It has two potential ribosome-binding sites and can give a protein of 24.0 kDa, which is similar to what is observed in *H. influenzae*. The pIs of the predicted TfoX proteins are 9.5 for *H. influenzae* and 9.8 for *A. actinomycetemcomitans*. The *tfoX* gene of *H. influenzae* is regulated by CRP. Based on the *E. coli* consensus sequence for CRP-binding (Harrison and Aggarwal, 1990), we identified two potential CRP-binding sites in the upstream region of *tfoX* from *A. actinomycetemcomitans*. We do not know if either is functional, but there is evidence that cAMP can affect transformation in *A. actinomycetemcomitans* (Wang et al., 2002). Also upstream of the *tfoX*-coding sequence in *A. actinomycetemcomitans* is a σ^{70} -like promoter sequence. Downstream, as in *H. influenzae*, is an inverted repeat sequence with the potential to form a stem-loop structure followed by several uridine residues, a characteristic feature of factor-independent transcription terminators (Platt, 1981).

3.2 *tfoX*-dependent transformation of *A. actinomycetemcomitans* Y4N

The *tfoX* gene from *A. actinomycetemcomitans* Y4N was cloned onto an IncQ *tacp* expression plasmid to test production of competence in Y4N after induction of the cloned gene with IPTG. Transformations were done with genomic DNA from the *katA* insertion mutant strain Y4N *katA3::IS903 ϕ kan* (Aa1395, Fig. 2A) (Thomson et al., 1999). Km^r transformants were selected and screened for their inability to show catalase activity (Thomson et al., 1999). When *tfoX* was *in trans* and induced, Y4N could be transformed easily (Tables 2 and 3). No transformants were seen in the absence of cloned *tfoX*. The low level of transformation in the absence of inducer, IPTG, (Table 2) is probably due to the leaky nature of the *tac* promoter. The number of transformants increased upon induction of the cloned *tfoX* gene with a low level of IPTG (0.02 mM), but showed a slight decrease at higher levels of IPTG (0.1 mM and above), the reason for which is not known. In terms of absorbance, maximum competence was reached at A₆₀₀ of 0.6 and decreased significantly thereafter (Table 2). The transformation efficiency also

increased with increasing amounts of transforming DNA and reached a maximum at 20 µg of total genomic DNA (Table 2). For routine transformations, we used about 20 µg of genomic DNA.

3.3 Transformation of recent clinical isolates

To transform adherent strains of *A. actinomycetemcomitans*, we tested the competence of NaI^{r} derivatives of several recent clinical isolates (Table 1). Nearly all of the isolates, of varying serotypes, could be transformed when the cloned *tfoX* gene of *A. actinomycetemcomitans* strains Y4N or CU1000N was present in the cells (Table 3; data not shown). Although strains DF2200N (serotype a) and IDH781N (serotype d) transformed at about 20-times higher efficiency than Y4N, some gave lower efficiencies. There was no apparent correlation of transformation efficiency with serotype (except possibly for serotype f, see below). Rough (adherent) cells were transformed at higher efficiency than their isogenic smooth (nonadherent) variants (compare the rough and smooth strains of IDH1705N, Table 3). We have shown before that rough *A. actinomycetemcomitans* can form large clumps, each of which can contain millions of cells (Kachlany et al., 2000). Consistent with that observation, we found that the transformation efficiencies of the rough strains can be even higher with single cells. When single cells of rough *A. actinomycetemcomitans* IDH781N were prepared and used, a 20-fold increase in transformation efficiency was observed (Table 3). We postulated that either all cells in a clump may not be accessible to the transforming DNA or that each clump may contain several transformants, but only give rise to a single colony.

Serotype f strains (CU1000N and NJ9100N) did not transform (Table 3), not even when single cells were used (data not shown), while strains of all other serotypes gave transformants. One possible reason for the inability to yield transformants is restriction-modification. To test this possibility, we used genomic DNA from a random library of *IS903*ϕ*kan* insertion mutants made in the serotype f strain CU1000N (Thomson et al., 1999). Because the mutants came directly from a serotype f strain, they should all be modified and not be subject to restriction by that strain. While we could obtain transformants in strains of other serotypes, we were never able to obtain transformants in our serotype f strains. This result shows that the lack of transformation in serotype f strains is not due to restriction-modification.

3.4 Transformation is helped by a USS

Most of the *A. actinomycetemcomitans* strains that could be easily transformed with genomic DNA for *katA::IS903*ϕ*kan* could rarely be transformed for *tadE::IS903*ϕ*kan*. *katA* has four flanking USSs (Fig. 2A). *tadE* is a gene well within the *tad* locus (Kachlany et al. 2000); no USSs were found in a search of the 14-kb *tad* locus sequence from strains HK1651 (Roe et al., 2006) or CU1000 (Planet et al., 2003).

Only the two highly competent strains, DF2200N and IDH781N, gave *tadE::IS903*ϕ*kan* transformants, but at a 100-fold lower efficiency than *katA::IS903*ϕ*kan*. The few *tadE::IS903*ϕ*kan* transformants that we obtained may have arisen by using a USS present outside the *tad* locus. The closest USS is 3.0 kb downstream of the Km-resistance gene (*kan*) from the *IS903*ϕ*kan* insertion in *tadE*.

3.5 Transformation with plasmid DNA

We tested if plasmid DNA can be used to transform *A. actinomycetemcomitans* and replicate. An IncQ broad host-range plasmid is known to replicate in *A. actinomycetemcomitans* (Goncharoff et al., 1993; Table 4). We found that extrachromosomal pMB22 plasmid DNA could be recovered after transformation (data not shown) and that the presence of a USS was important (e.g., compare pJAK13 with pMB40, Table 4), although occasionally one or two transformants were seen with pJAK13, which does not have a USS. pMB22 is a derivative of

pJAK13 that has *katA*::IS903 ϕ *kan* and three USSs (Table 1; Fig. 2A). Circular pMB22 plasmid DNA transformed DF2200N(pMB7) at high efficiency (Table 4). The transformants were both Km- and streptomycin-resistant, as expected for the complete plasmid. pMB22 extrachromosomal DNA could be isolated from the transformants (data not shown).

3.6 Allelic exchange

pMB22 DNA digested with *Pst*I gave a similar number of transformants as did the corresponding circular DNA. This result is similar to what was observed for circular and linear plasmid DNA in *H. influenzae* by Gromkova and Goodgal (1981), but different from transformation of chemically-competent *E. coli*, in which transformation with linear plasmid DNA is about 1000-fold less efficient than transformation with circular plasmid DNA (Shigekawa and Dower, 1988).

The different forms of plasmid gave different products. When the transformation was done with digested (linear) *katA*-mutant plasmid pMB22, all the resulting *A. actinomycetemcomitans* transformants tested were catalase-negative, while those obtained with undigested (circular) pMB22 DNA were all catalase-positive. This can happen if, after entering the cell, the linear DNA does not recircularize but undergoes allelic exchange with the chromosome. In contrast, circular pMB22 DNA probably replicates as an extrachromosomal plasmid, and the chromosomal *katA* remains intact. As evidence for this hypothesis, circular pMB22 plasmids could be isolated from the catalase-positive (circular plasmid DNA) transformants (data not shown). Allelic exchange with the chromosome in the catalase-negative (linearized plasmid DNA) transformants was confirmed by PCR, using primers Cat13 up and Cat14 dn that flank the IS903 ϕ *kan* insertion (Fig. 2A). Transformants arising from undigested (circular) plasmid DNA gave two bands that corresponded to the wild-type *katA* gene present in the chromosome and the mutated *katA* gene on the plasmid. Transformants arising from digested (linearized) plasmid DNA gave only one PCR product that corresponded to *katA*::IS903 ϕ *kan*. These results indicate allelic exchange with the chromosome from transformation with linear plasmid DNA.

Transformation with IncQ plasmids is useful for studies in which a replicating plasmid is needed. To get allelic exchange easily, we constructed a suicide vector, a pUC19 derivative (pMB78) that cannot replicate in *A. actinomycetemcomitans* (Galli et al., 1996) and has two inverted copies of the USS in the polylinker (Table 1; Fig. 2B).

A promoterless mutant *katA* from Y4N *katA*3::IS903 ϕ *kan* (Aa1395) was cloned into pMB78 to give pMB79 (Table 1). Both undigested (circular) and digested (linear) pMB79 gave Km^r transformants (Table 5). Because the cloned *katA* DNA lacks the *katA* promoter (Fig. 2A), expression of Km^r indicated that *kan* was expressed from the chromosomal *katA* promoter after homologous recombination with the chromosome. This arrangement was confirmed by PCR. All 30 transformants tested in each experiment were catalase-negative and 10/10 tested gave only one PCR product that corresponded to the mutant *katA* (data not shown). The absence of a PCR product that corresponded to the wild type *katA* gene indicated that double homologous recombination took place to replace the intact chromosomal *katA* gene.

When pMB79 was digested with *Kpn*I, the cloned *katA* DNA remained adjacent to the USSs. When the plasmid was digested with *Spe*I, the cloned *katA* DNA and the USS region were at the two ends of the linear DNA. Both digested plasmid DNAs could transform and undergo allelic exchange indistinguishably (Table 5). On a molar basis for a particular gene, transformation and allelic exchange with genomic DNA was about as efficient as that with digested plasmid DNA carrying the cloned gene (Table 5).

3.7 Homology sufficient for allelic exchange

The plasmids pMB89, pMB90, pMB91, pMB92, and pMB93 were made by *in vitro* insertions of EZ::TN<KAN-2> (a derivative of Tn5) at different positions in *katA* in pMB88 (Table 1; Fig. 2C), which contains a promoterless *katA* gene. The plasmids were digested with *KpnI* or *SpeI* and used to transform *A. actinomycetemcomitans* DF2200N(pMB7). Transformants were selected by the Km^r from the transposon insertions, then screened for loss of catalase activity to indicate allelic exchange with the chromosome. We observed an orientation effect. When the *neo* gene of the EZ::TN<KAN-2> insertion mutants was in the same direction as *katA*, transformants were abundant (Table 6), but there were fewer transformants when the insertion was in the opposite orientation. The different insertions also allowed us to test how little homologous sequence at an end of the transposon allowed allelic exchange to occur. We found that 78 bp of homology at one end still allowed that end to participate in allelic exchange (see pMB89, Table 6). Whether 78 bp of homology is sufficient at both ends of the transforming DNA could not be ascertained from this experiment.

4. DISCUSSION

A. actinomycetemcomitans has a *tfoX* gene, which, when cloned and induced, can make almost any strain of *A. actinomycetemcomitans* competent for transformation. Only our two serotype f strains failed to give any transformants. High transformation efficiency also requires a nearby USS, and it works in the polylinker of the plasmid suicide vector that we constructed. A USS has been shown to be important for transformation in *A. actinomycetemcomitans* (Wang et al., 2002, 2006). The use of linearized replicative plasmid DNA, circular suicide plasmid DNA, or genomic DNA readily yields transformants that have undergone allelic exchange with the chromosome. (One of the ends of the incoming DNA can have as little as 78 bp of homology.) The use of plasmid DNA to get allelic exchange has been helpful for site-directed mutagenesis of chromosomal genes (see, e.g., Kaplan et al., 2004; Fine et al., 2005; Rhodes et al., 2005; Perez et al., 2006). Transformation with undigested replicative plasmid DNA can yield *A. actinomycetemcomitans* strains that carry an extrachromosomal plasmid. Like allelic exchange, transformation to give extrachromosomal plasmid is most efficient if the plasmid carries a USS.

The maximum transformation efficiency we obtained for *A. actinomycetemcomitans* was for single cells. We disrupted clumps of cells with a disposable mortar and pestle before adding the DNA for routine transformation experiments. While transformation was improved, this method is not efficient for making single cells quantitatively.

To express TfoX in different strains of *A. actinomycetemcomitans*, we cloned *tfoX* from *A. actinomycetemcomitans* Y4N and CU1000N into an IncQ vector and put them under the control of the inducible *tac* promoter. One amino acid difference occurs at position 73, which is Thr in Y4N *tfoX* and Met in CU1000N *tfoX* (Fig. 1B). Both genes worked equally well in our transformation procedure. Because we were able to transform nearly all strains we tested (Table 3), these strains very likely have all the necessary genes for natural transformation, as has been described for *A. actinomycetemcomitans* HK1651 (Cameron and Redfield, 2006). It remains to be seen whether the induction of the resident chromosomal *tfoX* gene in strains of *A. actinomycetemcomitans* can increase the natural transformation efficiency, but we believe it would. The ability to be transformed may be a general property of *A. actinomycetemcomitans*, except maybe in serotype f strains. The failure to transform serotype f strains CU1000N and NJ9100N is not due to restriction-modification (Section 3.3). We cannot ascertain at present whether serotype f strains are not competent or whether they transform in our method at a level below what we can detect (about 1 transformant in 10⁹ cells). The presence or absence of complete sets of transformation genes in the genomes of these strains would be an important clue.

Development of competence in bacteria is usually tightly regulated (Dubnau, 1999; Chen and Dubnau, 2004; Sexton and Vogel, 2004), but it is not yet known what signals competence in *A. actinomycetemcomitans*. The environmental signal for the development of competence in *H. influenzae* is not known either, but an increase in adenosine 3',5'-cyclic monophosphate (cAMP) level triggers the expression of the early competence gene *tfoX* (Wise et al., 1973; Dorocicz et al., 1993; Redfield et al., 2005). The TfoX protein then activates other competence genes (the competence regulon) through CRP at CRP-S sites (Redfield et al., 2005; Cameron and Redfield, 2006). Competence of *A. actinomycetemcomitans* D7S can be stimulated by cAMP (Wang et al., 2002), but added cAMP did not significantly increase competence in the various *A. actinomycetemcomitans* strains we used here with cloned *tfoX* (MK Bhattacharjee, unpublished results). One possible explanation for this observation is that the cells have enough cAMP in these growth conditions to accommodate the increase in TfoX that results from induction of the cloned gene. This possibility would bypass a requirement for the addition of exogenous cAMP to increase transformation efficiency. A similar result was reported for cloned *tfoX* on a plasmid in *H. influenzae* (Zulty and Barcak, 1995).

In our method for making competent *A. actinomycetemcomitans*, we observed that both circular and digested plasmids were able to transform (Tables 4 and 5), but they led to different outcomes. Incoming circular DNA is linearized by the transformation process; but, because there is a resident IncQ plasmid (with *tfoX*), we may be selecting recombinants in which the linear incoming plasmid has recombined with the homologous resident plasmid to form a cointegrate that is resolved to give two IncQ monomeric plasmids (Gryczan et al., 1980). Regardless, the ease of obtaining allelic exchange in *A. actinomycetemcomitans* has important applications in creating site-specific mutations. We and others are currently using this approach to make mutations in several regions of interest in the chromosome.

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Abbreviations

A₆₀₀, absorbance of light at wavelength 600 nm
 AAGM, *A. actinomycetemcomitans* Growth Medium
 bp, base pairs
 C, Centigrade
 cAMP, adenosine 3',5'-cyclic monophosphate
 Cm, chloramphenicol
 CRP, cAMP receptor protein, also called the catabolite activator protein
 CRP-S, non-canonical CRP binding-site recognized by CRP in the presence of TfoX
 EZ::TN<KAN-2> derivative of Tn5 used for mutagenesis *in vitro*
 hr, hour(s)
 Inc, plasmid incompatibility group
 IPTG, isopropyl-β-D-thiogalactopyranoside
 IS903 ϕ *kan*, artificial transposon based on Tn903
 K, x 1000
 Km, kanamycin
kan, gene of Tn903 for kanamycin-resistance
kataA, catalase gene of *A. actinomycetemcomitans*
 kb, kilobase pair(s)
lacI^q, *E. coli* mutation that results in high level of LacI repressor

LAP, localized aggressive periodontitis
 LB, Luria-Bertani medium
 min, minute(s)
 ml, milliliter(s)
 mM, millimolar
 Nal, nalidixic acid
neo, gene of Tn5 for kanamycin-resistance
 ORF, open reading frame
oriT, origin of transfer
 PCR, polymerase chain reaction
 r, resistant
 rpm, rotations per minute
 s, sensitive
 sec, second(s)
tacP, *tac* promoter
tfo, transformation
tad, tight adherence
 µg, microgram(s)
 µl, microliter(s)
 µm, micrometer(s) or micron(s)
 USS, uptake signal sequence
 :: insertion

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A*A. actinomycetemcomitans*

CRP -35 -10 CRP
 CCAATTCTCGATCAAGATCACAATTTAACATTAAATAACAATATTTTCTTCATATAA
 ATTTAATTTATGATATAAATACAAAATAACAATTACCTGAAGGATTTAATTATG.....
 stop RBS start
TAAGAATCGCAAAAAGAAAAAACCGCTCTATTGAGCGTTTTTCA

H. influenzae

CRP -35 -10
 TTAAATTTACGATCTGGCTCACAAATCTGACATTTATATTTAGGTGTAGTAAAAATTTCA
 CRP
 TGATGTAAATGAAATCAGAAGTACTTCTACTGACTCTTTTAAATAATTATTCAT
 RBS start stop
 TGGAGGTTTAAATATG.....TAAATTTGTTCAATCAATAATTTTTTGC

B

Aa MTTVSQRLKNEFKQLVDGEISIKNLFTGHGIFHSG-IMFALHQNDSIFLRAED
 Hi MNIKDEHIDSVCSLLDQLV-GNVSFKNLFTGYGLFHKEETMFAIWQNKKLYLRGEG
 M
 Aa ELVPYLES LGAVAYSIDPNNITNK LALHRY YQLPHSIRKNKPLFEKTVKLSIQQAKF
 Hi VLAIQLTKLGCEPFTTNELN-KRFVLSQYYALSDQILRSNRLCRKLIILSIKQILE
 Aa KKVAEDLTKKERIKELANFSIKHERLLAKINIYTVSEFQKIGAIHSYVRLKKLGVS
 Hi QKLECTLRKLNRLKDLPNLTIKHERALIKVGITNVAMLREIGAENALVELKKSGSG
 Aa ANVELLWTLCAALKNKHASLLTEKEKSAILTKLNVLLAANGLRKVKA
 Hi ATLDIFYWKLVCALQNKNSQMLSQAERLLKKLNEVLRKNGLKGYRKLDDE

Fig 1.

Comparison of the *tfoX* regions of *A. actinomycetemcomitans* and *H. influenzae* (Section 2.3). A. Nucleotide sequences of the regions upstream and downstream of the *tfoX* structural gene, which is delimited by “start” and “stop,” from *A. actinomycetemcomitans* HK1651 (Roe et al., 2006) and *H. influenzae* Rd (Zulty and Barcak, 1995). Potential ribosome-binding sites (RBS) and CRP-binding sites (CRP) are indicated. σ^{70} -like promoter sequences are marked by “-35” and “-10.” The inverted repeat sequences that can serve as potential intrinsic terminators are shown by convergent arrows. They are followed by six T's shown in italics. The potential CRP and terminator sequences are in bold. Potential RBS, -35, -10, start, and stop sequences are underlined. B. Alignment of the predicted amino acid sequences of the TfoX proteins. Aa is *A. actinomycetemcomitans*; Hi, *H. influenzae*. Identical (:) and similar (.) amino acids are

indicated. At position 73 the bold 'M' (CU1000) above the bold 'T' (Y4 and HK1651) indicates the amino acid difference in TfoX from CU1000.

Fig 2.
Molecular genetic maps (Section 2.3). A. Map of the *katA* region of *A. actinomycetemcomitans* Y4N (not to scale) (Thomson et al., 1999) showing uptake signal sequences (USS), positions of transposon IS903 ϕ *kan* (Tn) insertions in strains Aa1394 and Aa1395, and the names, positions, and orientations of the primers used for PCR (arrows). B. Relative positions of the *tacp* promoter (*tacp*), restriction sites, and the USSs in pMB40. The order of restriction sites and the USSs is the same in pMB78. C. Positions and orientations of the EZ::TN<KAN-2> transposon in pMB89, pMB90, pMB91, pMB92 and pMB93. The arrow below each indicates the direction of *neo*, which arbitrarily orients the transposon.

TABLE 1
Strains and plasmids (Sections 2.1, 2.3).

Strain or plasmid	Relevant characteristics	Reference or source
<i>A. actinomycetemcomitans</i> ¹		
Aa307	Rough clinical isolate, serotype c	Haubek et al., 1995
Aa1394	Y4N <i>katA2::IS903</i> ϕ kan, serotype b; Km ^r , Nal ^r	Thomson et al., 1999
Aa1395	Y4N <i>katA3::IS903</i> ϕ kan, serotype b; Km ^r , Nal ^r	Thomson et al., 1999
CU1000	Rough clinical isolate, serotype f	Fine et al., 1999
CU1060	Spontaneous smooth variant of CU1000, serotype f	Fine et al., 1999
DF2200	Rough clinical isolate, serotype a	Kaplan et al., 2002
IDH781	Rough clinical isolate, serotype d	Haubek et al., 1995
IDH1705	Rough clinical isolate, serotype e	Haubek et al., 1995
NJ9100	Rough clinical isolate, serotype f	Kaplan et al., 2002
Y4	Smooth strain, serotype b	ATCC ²
<i>E. coli</i>		
Top10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i> (ϕ 80 <i>lacZ</i> Δ M15)	Invitrogen
Plasmids		
pCR2.1-TOPO	Cloning vector with 3'-T overhangs for ligation of <i>Taq</i> -amplified PCR products; Km ^r , Ap ^r	Invitrogen
pJAK12	Broad-host-range IncQ expression vector, derivative of pMMB67HE with Ω fragment insertion in <i>bla</i> ; <i>lacI</i> ^q , <i>tacp</i> , Mob ⁺ ³ ; Sm ^r	J.A. Kornacki
pJAK13	Same as pJAK12 with polylinker in opposite orientation	J.A. Kornacki
pJAK17	Broad-host-range IncQ expression vector, derivative of pMMB67HE with Ω cat insertion in <i>bla</i> ; <i>lacI</i> ^q , <i>tacp</i> , Mob ⁺ ; Cm ^r	J.A. Kornacki
pMB7	<i>tfoX</i> from <i>A. actinomycetemcomitans</i> Y4 cloned into pJAK17; Cm ^r	This work
pMB8	<i>tfoX</i> from <i>A. actinomycetemcomitans</i> CU1000 cloned into pJAK17; Cm ^r	This work
pMB22	Y4 <i>katA2::IS903</i> ϕ kan cloned into pJAK13 (Aa1394; Fig. 2A); Km ^r ; Sm ^r	This work
pMB40	<i>katA</i> region with two inverted copies of the USS in pJAK12 (Fig. 2B); Sm ^r	This work
pMB78	<i>katA</i> region with two inverted copies of the USS in pUC19 (Fig. 2B); Ap ^r	This work
pMB79	Y4 <i>katA3::IS903</i> ϕ kan cloned into pMB78; Km ^r ; Ap ^r	This work
pMB88	Promoterless <i>katA</i> from Y4 cloned into pMB78; Ap ^r	This work
pMB89 - 93	EZ::TN<KAN-2> at various positions in <i>katA</i> of pMB88 (Fig. 2C); Km ^r ; Ap ^r	This work
pUC19	Cloning vector; ColE1 origin of replication; Ap ^r	New England Biolabs

¹ A spontaneous Nal^r mutant of each strain was isolated and used for all transformation studies. These strains are indicated with 'N' at the end and have the formal names shown in parenthesis: Aa307N (MB1230), CU1000N (CU1000N), CU1060N (CU1060N), DF2200N (MB1228), IDH781N (MB1220), IDH1705N (MB1221), NJ9100N (MB1259) and Y4N (Y4N).

² ATCC, American Type Culture Collection.

³ Mob⁺, The plasmid is not self-transmissible in conjugation, but can be mobilized.

TABLE 2

Conditions for transformation^{1,2}

<i>Induction</i>									
mM IPTG	0 (1.8) ³	0.02 (10.4)	0.10 (8.0)	1.0 (7.0)					
<i>Absorbance</i>									
A ₆₀₀	0.071 (0.72)	0.205 (6.6)	0.393 (7.1)	0.628 (25.2)	0.830 (10.7)	1.017 (4.9)			
<i>Amount of transforming DNA</i>									
µg genomic DNA	0 (0)	5 (1.3)	10 (1.2)	20 (7.1)	30 (6.1)	50 (3.1)			

¹ Transformation was done as described in Materials and Methods (Section 2.4), except for one variable in each experiment as indicated. An equal number of cells was used for each transformation. DNA was genomic DNA from Y4N *katA3::IS903Δkan*.

² Y4N(pMB7) was transformed; Y4N without pMB7 gave no transformants.

³ Numbers in parentheses indicate transformants/µg DNA.

TABLE 3
Transformation of various strains of *A. actinomycetemcomitans* with genomic DNA.

Strain	Colony Phenotype ²	Serotype	Resident Plasmid	Transformants/ μ g DNA ¹	
				<i>katA3::IS903ϕkan</i> ³	<i>tadE::IS903ϕkan</i> ⁴
DF2200N	R	a	pMB7	111.8	1.8
Y4N	S	b	vector ⁵	0	0
Y4N	S	b	pMB7	5.9	0
Aa307N	R	c	pMB7	7.6	ND ⁶
IDH78IN	R	d	pMB7	120.9	0.8
IDH78IN (Single Cells)	R	d	pMB7	2573.3	ND
IDH1705N	R ₇	e	pMB7	23.8	0
IDH1705N	S ⁷	e	pMB7	0.16	ND
CU1000N ⁸	R	f	pMB7	0	0
CU1060N ⁸	S	f	pMB7	0	0
NJ9100N	R	f	pMB7	0	ND

¹ Transformation was done as described in Materials and Methods (Section 2.4). Transformants were selected on AAGM plates containing kanamycin.

² S: smooth; R: rough.

³ Genomic DNA from Y4N *katA3::IS903 ϕ kan*.

⁴ Genomic DNA from CU1000N *tadE::IS903 ϕ kan*.

⁵ Vector (pJAK17) control for all other strains gave no transformants.

⁶ ND, not determined.

⁷ A spontaneous smooth variant of IDH1705N with the formal name MB1221.

⁸ The same results were obtained with pMB8 as the resident plasmid.

TABLE 4
Allelic exchange after transformation with IncQ Plasmid DNA.

Strain ¹	Colony Phenotype ²	Transforming DNA ³	Relevant Features	Transformants ⁴ /μg DNA	Catalase Phenotype ⁵
DF2200N	R	pJAK13	Vector, circular	0	ND
DF2200N	R	pMB40	2 USS, circular	1.7 × 10 ⁴	+
DF2200N	R	pMB22	<i>katA3::IS903φkan</i>	5.1 × 10 ²	+
DF2200N	R	pMB22 + PstI	3 USS, circular <i>katA3::IS903φkan</i>	1.7 × 10 ²	–
Y4N	S	pJAK13	3 USS, linear	0	ND
Y4N	S	pMB22	Vector, circular <i>katA3::IS903φkan</i>	3.0	+
Y4N	S	pMB22 + PstI	3 USS, circular <i>katA3::IS903φkan</i>	6.0	–

¹ Both strains contained the plasmid pMB7 and were transformed as described in Materials & Methods (Section 2.4).

² S, smooth; R, rough

³ 0.5 μg plasmid DNA was used for each transformation.

⁴ Transformants were selected on AAGM plates containing kanamycin for pMB22 or streptomycin for pJAK13 and pMB40.

⁵ + indicates that all transformants tested showed catalase activity; – indicates that all transformants tested did not show catalase activity; ND, not done. All Y4N transformants were tested; 30 DF2200N transformants were tested for each different DNA. Allelic exchange was confirmed by PCR (see text).

TABLE 5

Transformation with a suicide vector.¹

Transforming DNA ²	Relevant Features	Transformants/μg DNA
pUC19	Vector	0
pMB78	circular plasmid DNA 2 USS	0
pMB79	circular plasmid DNA <i>katA3::IS903φkan</i> , 2 USS,	2.9×10^4
pMB79 + SpeI	circular plasmid DNA <i>katA3::IS903φkan</i> , 2 USS,	1.1×10^5
pMB79 + KpnI	linear plasmid DNA <i>katA3::IS903φkan</i> , 2 USS,	2.3×10^5
Y4N <i>katA3::IS903φkan</i>	linear plasmid DNA <i>katA3::IS903φkan</i> genomic DNA	5.0×10^2

¹ DF2200N(pMB7) was transformed as described in Materials and Methods (Section 2.4). Transformants were selected on AAGM plates containing kanamycin.

² 0.1 μg DNA for plasmids; 20 μg DNA for genomic DNA.

TABLE 6

Homology sufficient at one end for allelic exchange.

Plasmid	Homology (bp) beyond the two ends of the transposon ¹	Relative orientation ²	Transformants ³ /μg DNA	
			Circular DNA	
			Linear DNA	
			<i>Kpn</i> I cut	<i>Spe</i> I cut
pMB89	1318, 78	+	1.3 × 10 ⁵	6.6 × 10 ⁴
pMB90	170, 1226	–	2.2 × 10 ³	3.2 × 10 ³
pMB91	288, 1108	+	6.2 × 10 ⁴	1.2 × 10 ⁵
pMB92	481, 915	–	5.7 × 10 ³	1.5 × 10 ³
pMB93	275, 1121	–	9.0 × 10 ²	7.0 × 10 ²
				3.0 × 10 ⁴
				5.0 × 10 ²
				4.0 × 10 ⁴
				5.0 × 10 ²
				1.0 × 10 ³

¹ Homology beyond the two ends of the transposon was determined by sequencing outwards from the ends of the transposon.

² + indicates *katA* and *neo* are oriented in the same direction; – indicates they are oriented in opposite directions. The orientations were determined by sequencing outwards from either end of the transposon.

³ DF2200N(pMB7) was transformed as described in Materials and Methods (Section 2.4). All transformants tested had replaced the wild type *katA* gene (chromosomal) with a mutant gene (plasmid-borne), as determined by PCR.