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Chromosomal Complementation Using Tn*7* Transposon Vectors in *Enterobacteriaceae*

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Genetic complementation in many bacteria is commonly achieved by reintroducing functional copies of the mutated or deleted genes on a recombinant plasmid. Chromosomal integration systems using the Tn*7* transposon have the advantage of providing a stable single-copy integration that does not require selective pressure. Previous Tn*7* systems have been developed, although none have been shown to work effectively in a variety of enterobacteria. We have developed several mini-Tn*7* and transposase vectors to provide a more versatile system. Transposition of Tn*7* at the chromosomal *att*Tn*7* site was achieved by a classical conjugation approach, wherein the donor strain harbored the mini-Tn*7* vector and the recipient strain possessed the transposase vector. This approach was efficient for five different pathogenic enterobacterial species. Thus, this system provides a useful tool for single-copy complementation at an episomal site for research in bacterial genetics and microbial pathogenesis. Furthermore, these vectors could also be used for the introduction of foreign genes for use in biotechnology applications, vaccine development, or gene expression and gene fusion constructs.

Determining the potential function of a gene is achieved through targeted or random mutagenesis approaches. A well-established principle known as "Molecular Koch's postulates" was described by Stanley Falkow in the 1980s (18). These postulates stipulate that (i) a virulence trait should be associated with gene function(s), (ii) specific inactivation of the associated gene(s) with the assumed virulence trait should attenuate virulence, and (iii) reintroduction of the wild-type (WT) gene(s) into the mutant strain should lead to the restoration of pathogenicity to the WT level (18).

Cloning genes on plasmids may often provide an effective means of genetic complementation. Plasmids used for genetic complementation are valuable tools, particularly for *in vitro* studies (5, 19, 27, 28, 44, 47). However, plasmids may easily be lost in the absence of selection in conditions such as natural environments and animal models or in industries such as food production. Further, when complementation is to be tested, plasmid copy number and increased gene dosage exceed the chromosomal number. This could lead to aberrant phenotypes and limits the use of plasmids for the successful demonstration of complementation.

The use of the Tn*7* transposon is an elegant alternative. The Tn*7* transposon integrates at the site-specific *att*Tn*7*, located downstream of the highly conserved *glmS* gene, which encodes an essential glucosamine-fructose-6-phosphate aminotransferase (36). Tn*7* integration at *att*Tn*7* is mediated by the *tnsABCD* transposases (42), where TnsAB proteins recognize and excise the Tn*7* fragment from the donor element, whereas TnsCD proteins promote the integration of Tn*7* into the *glmS* transcriptional terminator (for reviews, see references 11 and 36). Since Tn*7* represents a "homing" transposon that recognizes a specific and conserved sequence in many bacteria, the Tn*7* system has been developed as a tool to integrate DNA sequences into the chromosomes of different Gram-negative bacteria (6, 7, 9, 10, 30, 32), e.g., *Pseudomonas*, *Burkholderia*, and *Yersinia* spp. Although this system provides many applications for a variety of bacteria, it comprises the use of ColE1-based suicide plasmids that can replicate readily in many enterobacteria such as *Escherichia coli* and *Salmonella enterica*.

In this report, we validated, designed, and demonstrated the efficacy of a Tn*7*-based cloning and delivery system that is optimized for use in enterobacterial species, including *E. coli* and *Salmonella*. This system was shown to be very effective for the integration of recombinant genes in five different enterobacterial species, including four pathogenic *E. coli* strains, *Salmonella enterica* serovars Typhimurium and Typhi, *Klebsiella pneumoniae*, *Cronobacter sakazakii*, and *Citrobacter rodentium*. We also generated a number of vectors with different antibiotic resistance markers to provide a more versatile system that could readily be used for genetic manipulations in strains that are naturally resistant to different antibiotics.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The strains and plasmids used in the present study are listed in Table 1, and the primers are listed in Table 2. Bacteria were grown in Luria-Bertani (LB) broth at 30 or 37°C. Antibiotics and supplements were added as required at the following concentrations: kanamycin, 40 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 15 μg/ml; gentamicin, 15 μg/ml; trimethoprim, 10 μg/ml; diaminopimelic acid (DAP), 50 μg/ml; and BCIP (5-bromo-4-chloro-3-indolylphosphate), 40 μg/ml.

Construction of Tn*7* and transposase vectors. The procedure was adapted for *E. coli* based on the system developed by Choi et al. (7). First, the mini-Tn*7*, containing a multiple cloning site and a gentamicin resistance (Gm^r) cassette flanked by the two Tn*7* ends, was amplified from pUC18-mini-Tn*7*-Gm with the primers CMD1067 and CMD1068. The

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
Citrobacter rodentium		
ICC168 strain	Attaching and effacing mice pathogen	1
QT2787	*C. rodentium* ICC168 + pSTNSK; Km^r	This study
Cronobacter sakazakii		
BAA-894 strain	Isolated from a powdered formula used during a neonatal intensive care unit outbreak	23
QT2765	*C. sakazakii* + pSTNSK-Tp; Km^r Tp^r	This study
Escherichia coli		
DH5 αΠ	λ*pir* lysogen of DH5 α; Tc^r	37
MGN-617	*thi thr leu tonA lacY glnV supE* Δ*asdA4 recA*::RP4 2-Tc::Mu(*pir*); Km^r	17
S17-1(λ*pir*)	λ*pir* lysogen of S17-1 (Tp^r Sm^r *thi pro* Δ*hsdR hsdM*$^+$ *recA* RP4::2-Tc::Mu-*km*::Tn*7*)	41
QT2085	MGN-617 + pGP-Tn*7*-*pst*; Ap^r Gm^r	13
QT2740	MGN-617 + pGP-Tn*7*-Gm-*xylE*; Ap^r Gm^r	This study
Pathogenic strains		
536	UPEC WT pyelonephritis strain	22
QT2732	536 + pSTNSK; Km^r	This study
CFT073	UPEC WT pyelonephritis strain	34, 46
QT2496	CFT073 + pSTNSK; Km^r	This study
QT1911	CFT073 Δ*pstSCA*::*FRT*	13
QT2207	QT1911 + pSTNSK; Km^r	13
QT2651	QT1911 + pSTNSK-Cm; Km^r Cm^r	This study
χ7122	Avian pathogenic strain, O78:K80:H9 *gyrA*; Nal^r	39
QT2707	χ7122 + pSTNSK; Nal^r Km^r	This study
EDL933	Enterohemorrhagic *E. coli* (EHEC) O157:H7	40
QT2705	EDL933 + pSTNSK-Tp; Km^r Tp^r	This study
Klebsiella pneumoniae		
subsp. *pneumoniae* KPPR1 strain		ATCC 43816
QT2710	*K. pneumoniae* strain KPPR1 + pSTNSK-Tp; Km^r Tp^r	This study
Salmonella enterica serovars		
Typhi Ty2a	Vaccine strain of *S.* Typhi Ty2 strain	24
QT2774	*S.* Typhi Ty2a + pSTNSK; Km^r	This study
Typhimurium SL1344	*S. enterica* subsp. *enterica* serovar Typhimurium	21
QT2706	SL1344 + pSTNSK-Tp; Km^r Tp^r	This study
Plasmids[b]		
pCP20	FLP helper plasmid Ts replicon; Ap^r Cm^r	15
pFCM1 (AY597271)	Chloramphenicol resistance FRT vector pFCM1; Ap^r Cm^r	7
pFTP1 (AY712951)	Trimethoprim resistance FRT vector pFTP1; Ap^r Tp^r	7
pGP704	*oriR6K mobRP4*; Ap^r	33
pGP-Tn*7*-Cm (JQ429759)	pGP-Tn*7*-FRT::Cm; Ap^r Cm^r	This study
pGP-Tn*7*-FRT	pGP-Tn*7*-Gm::FRT; Ap^r	This study
pGP-Tn*7*-Gm (JQ429758)	pGP704::Tn*7*-Gm; Ap^r Gm^r	13
pGP-Tn*7*-Gm-*xylE*	pGP-Tn*7*-Gm::*xylE*; Ap^r Gm^r	This study
pGP-Tn*7*-*pst*	pGP-Tn*7*-Gm::*pstSCA*; Ap^r Gm^r	13
pGP-Tn*7*-Tp (JQ429760)	pGP-Tn*7*-Cm::Tp; Ap^r Tp^r	This study
pMEG685	*xylE* cassette vector; Ap^r	16; Megan Health (St. Louis, MO)
pST76-K (Y09897.1)	ori*SC101*(Ts); Km^r	38
pSTNSK (JQ436536)	pST76-K::*tnsABCD*; Km^r	13
pSTNSK-Cm (JQ436537)	pSTNSK::Cm; Km^r Cm^r	This study
pSTNSK-Tp (JQ436538)	pSTNSK::Tp; Km^r Tp^r	This study
pTNS2 (AY884833)	T7 transposase expression vector, ori*R6K*; Ap^r	7
pTP223[c]	λ-red IPTG-inducible vector; Tc^r	35
pUC18-mini-Tn*7*-Gm (AY619004)	pUC18-mini-Tn*7*-Gm (Gm^r on mini-Tn*7*T; for gene insertion in Gm^s bacteria); Ap^r Gm^r	7

[a] Ap^r, resistance to ampicillin; Km^r, resistance to kanamycin; Sm^r, resistance to streptomycin; Tp^r, resistance to trimethoprim; Gm^r, resistance to gentamicin, Tc^r, resistance to tetracycline; Cm^r, resistance to chloramphenicol.
[b] GenBank accession numbers are indicated in parentheses where applicable.
[c] Addgene plasmid 13263.

amplified product was digested with MfeI and PspxI (New England Biolabs) and then ligated into the suicide vector pGP704 previously digested with EcoRI and SalI, creating plasmid pGP-Tn*7*-Gm (Fig. 1A). Second, the Tn*7* transposase-encoding genes *tnsABCD* were excised from plasmid pTNS2 by SphI and XmaI digestion and ligated into the same sites of the temperature-sensitive plasmid pST76-K, resulting in plasmid pSTNSK (Fig. 1B).

Derivatives of the mini-Tn*7* pGP-Tn*7*-Gm vector were also constructed. The Gm^r cassette of pGP-Tn*7*-Gm was replaced by a *cat* (chloramphenicol resistance [Cm^r]) or a *dhfrII* (trimethoprim resistance [Tp^r]) cassette. The Cm^r resistance cassette was amplified from pFCM1 with primers CMD1466 and CMD1467. The replacement of the Gm^r cassette by a Cm^r *cat* cassette was achieved by the procedure described by Murphy and Campellone (35) using the pTP223 vector, creating the pGP-Tn*7*-Cm vector. Thereafter, the Cm^r cassette of pGP-Tn*7*-Cm was replaced by the Tp^r cassette. The Cm^r cassette was removed by digesting the pGP-Tn*7*-Cm vector with Bpu10I and BstBI. After the unpaired ends were filled in with the Klenow fragment, the Tp^r cassette, obtained from the digestion

TABLE 2 Primers used in this study

Strain or source	Gene (primer sequence [5′–3′])	
	Forward	Reverse
Strains		
Citrobacter rodentium		
ICC168	*glmS* (ACATCATTGAGATGCCGCACGTTG)	*rod_40121* (ACTGAGAAGCCGGAAGGTTGAGTT)
Cronobacter sakazakii		
BAA-894	*glmS* (TTGAAGAGGTTATCGCGCCGATCT)	*ESA_04000* (AAACGCGCTGAAGAGAAACAGCTG)
Escherichia coli		
536	*glmS* (CACCAATCTTCTACACCGTTCCGC)	*pstS* (AGATCAGTTTGGTGTACGCCAGGT)
CFT073	*glmS* (CACCAATCTTCTACACCGTTCCGC)	*pstS* (AGATCAGTTTGGTGTACGCCAGGT)
χ7122	*glmS* (GATCTTCTACACCGTTCCGC)	*stgA* (TTATTTCTTATATTCGACAGTAAAT)
EHEC EDL933	*glmS* (CACCAATCTTCTACACCGTTCCGC)	Intergenic region between *glmS* and *z5225* (TCCACAACTATGAATTCGCGTAGA)
Klebsiella pneumoniae subsp. *pneumoniae* KPPR1 strain	*glmS* (ACATGCACATCATTGAGATGCCGC)	*pstS* (ATCTGCTTAACGCCACCAGAGGAA
Salmonella enterica serovars		
Typhi Ty2a	*glmS* (ACATGCACATCATTGAGATGCCGC)	*stgA* (GTCAGGTCGATATGGAACTCGGTA
Typhimurium SL1344	*glmS* (GGAGATTGTGGTGGCGCCGA)	*sl3827* (CCACGCCATCAGTGGTGGGG)
Sources		
pUC18-mini-Tn*7*-Gm	CMD1067 (TGCGGTCAATTGTACCGCACAGATGCGT AAGGAGAA)	CMD1068 (AACGCCGCTCGAGTTTATAGTCCTGTCGG GTTTCGCCA)
pFCM1	CMD1466 (TCCGGCCCTAGGCGAATTAGCTTCAA)	CMD1467 (CTACTGCCTAGGGCTCGAATTGGGGA)

of the pFTP1 vector with SmaI, was cloned into the blunt end sites of pGP-Tn*7*-Cm devoid of the Cm^r cassette, creating the pGP-Tn*7*-Tp vector. Similarly, derivatives of pSTNSK transposase vectors were constructed. The Cm^r and Tp^r cassettes were excised, respectively, from vectors pFCM1 and pFTP1 (7) with a XmaI digestion. The respective cassettes were cloned into the XmaI site of pSTNSK, creating, respectively, pSTNSK-Cm and pSTNSK-Tp. The pGP-Tn*7*-Gm-*xylE* vector was constructed by cloning the *xylE* cassette, encoding the catechol 2,3-dioxygenase, previously isolated from pMEG685 by XhoI digestion, into the XhoI site of pGP-Tn*7*-Gm.

Delivery of Tn*7* into *Enterobacteriaceae*. A classical mating (5 or 18 h) using 2×10^7 CFU ml^{-1} of donor strain *E. coli* SM10λ*pir*-derivative MGN-617, harboring the pGP-Tn*7*-Gm or derivative vectors and 1×10^7 CFU ml^{-1} of the recipient strains, carrying either pSTNSK or other Tn*7* transposase-encoding plasmids, was performed at 30°C on LB agar plates supplemented with DAP. After incubation, the mating lawn was then serially diluted, spread onto LB Gm plates, and incubated at 42°C for 4 to 5 h and then for 18 h at 37°C. Colonies were then screened for resistance to Gm and sensitivity to Ap and Km. Since the Ap^r cassette is located outside of the Tn*7* region on the vector, sensitivity to Ap denotes the proper integration of Tn*7*-Gm at *att*Tn*7* instead of incorporation of the vector into the chromosome. Also, since the transposases are encoded on a temperature-sensitive plasmid, incubation at 42°C was undertaken to promote the loss of the pSTNSK or derivative vectors from the recipient strain, which was denoted by sensitivity to Km. Furthermore, the use of LB Gm plates without DAP selected for growth of the recipient strain, since the MGN-617 donor strain is an *asd* mutant that requires DAP for growth.

Confirmation of integration of the Tn*7* transposon at the established *att*Tn*7* site located downstream of the *glmS* gene within different clones

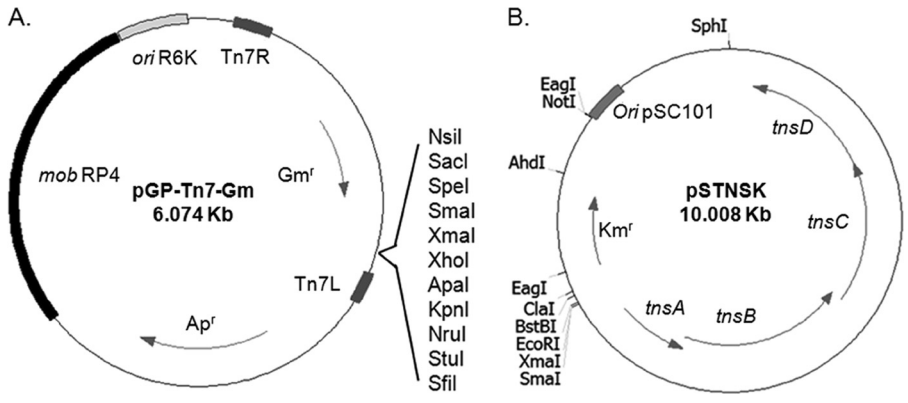

FIG 1 Features of the mini-Tn*7* and transposases vectors. (A) The mobilizable suicide vector pGP-Tn*7*-Gm contains the conjugative transfer Mob RP4 and the *ori* R6K. A multiple cloning site is integrated between the two Tn*7* ends. Derivatives were constructed by replacing the Gm^r gene by the Cm^r and Tp^r genes, respectively. (B) The thermosensitive suicide vector pSTNSK contains the pSC101 origin and transposases *tnsABCD*. Derivatives were constructed by cloning, respectively, the Cm^r and Tp^r genes into the XmaI site. Accession numbers are referenced in Materials and Methods. Tn7L and Tn7R indicate left and right ends of Tn*7*, respectively.

was verified by PCR in different enterobacterial strains using the primer pairs listed in Table 2.

Alkaline phosphatase assay. Alkaline phosphatase activity was determined as described by Crepin et al. (14). Briefly, the cells were grown in LB medium, were adjusted to an optical density at 600 nm (OD_{600}) of 1.0, and 4 μg of *p*-nitrophenyl phosphate/ml was added to cells permeabilized by 50 μl of 1% sodium dodecyl sulfate and 50 μl of chloroform. Color development was monitored at 420 nm, and PhoA activity was expressed in Miller units (MU), calculated as follows: $1{,}000 \times [OD_{420} - (1.75 \times OD_{550})]/T$ (min) $\times$ V (ml) $\times$ OD_{600}, where T represents the length of reaction time, and V represents the culture cell volume. The activity of PhoA in each strain was calculated at each hour throughout the growth curve.

Nucleotide sequence accession numbers. The vectors were sequenced, and the GenBank accession numbers are as follows: pGP-Tn*7*-Gm (JQ429758), pGP-Tn*7*-Cm (JQ429759), pGP-Tn*7*-Tp (JQ429759), pSTNSK (JQ436536), pSTNSK-Cm (JQ436537), and pSTNSK-Tp (JQ436538).

RESULTS AND DISCUSSION

Characteristics of the *tnsABCD* transposases and Tn*7* transposon vectors. Here, the chromosomal Tn*7* integration systems described by Choi et al. (6, 7, 9, 10) were modified for practical use in *Enterobacteriaceae*, including WT pathogenic *E. coli* strains, *Salmonella enterica*, *Klebsiella pneumoniae*, and *Cronobacter sakazakii*. Since the pTNS2 (7) vector containing the *tnsABCD* genes encoding the Tn*7* transposase system possesses an R6K origin of replication, which is of limited use in *Enterobacteriaceae*, we developed a more versatile vector that could be maintained in a variety of enterobacteria. Hence, the transposase *tnsABCD* genes were cloned into the thermosensitive vector pST76K (Fig. 1B). By using such a vector, bacteria can maintain the transposase system when grown at 30°C but lose it following cultivation at either 37 or 42°C.

The Tn*7*-containing vectors developed by Choi et al. (7) possess a pUC18 (ColE1) origin of replication. However, the ColE1 replicon is functional and gives plasmids of high-copy number in many enterobacteria, such as *E. coli* and *Salmonella* (31). To provide an efficient system that is amenable to the use of Tn*7* for the single-copy integration of recombinant genes at the *att*Tn*7* site in a variety of enterobacteria, the modified mini-Tn*7* transposon described in Materials and Methods was cloned into the λ*pir*-dependent suicide vector pGP704, creating the pGP-Tn*7*-Gm plasmid. Furthermore, the Gm^r cassette is flanked by the flippase recognition target (FRT) sites. These sites are recognized by the flippase recombination enzyme (FLP), which can be introduced on vectors such as pCP20 (15). By promoting reciprocal recombination across the inverted repeats (FRT), the resistance cassette can be excised from the chromosome by the FLP recombinase (data not shown). The pGP704 suicide plasmid was selected since it possesses the *mobRP4* region and the R6K origin of replication, and it is highly mobilizable and is an excellent suicide vector for the introduction of DNA into a variety of bacterial species in a nonreplicating form (33).

Since certain bacterial strains have innate or naturally acquired resistance to a variety of antibiotics, we also generated Tn*7* system vector derivatives of pGP-Tn*7*-Gm in which the Gm^r cassette is replaced by a Cm^r and Tp^r cassette (Table 1). Further transposase-encoding derivatives of pSTNSK were constructed by incorporating the Cm^r and Tp^r cassettes into this vector (Table 1).

The development of a two-plasmid system using a temperature-sensitive replicon to encode the transposase system and a mobilizable *pir*-dependent suicide vector for the introduction of the Tn*7* transposon system provides a simple means for introducing recombinant genes into the chromosomes of a variety of enterobacterial strains through a simple biparental mating without the need of a helper plasmid, as previously described (6–10).

TABLE 3 Integration of Tn*7*::*xylE* at the *att*Tn*7* site[a]

Strain or vector combination	CFU[b]	% integration[c]
Strains		
Citrobacter rodentium ICC168	10^7	68
Cronobacter sakazakii BAA-894	10^7	96
Escherichia coli		
536	10^6	97
CFT073	10^7	86
χ7122	10^6	96
EDL933	10^7	80
Klebsiella pneumoniae subsp. *pneumoniae* KPPR1	10^8	80
Salmonella enterica serovars		
Typhi Ty2a	10^5	89
Typhimurium SL1344	10^6	96
Vector combinations		
pGP-Tn*7*-Gm with:		
pSTNSK	10^7	86
pSTNSK-Tp	10^7	94
pSTNSK-Cm	10^6	91
pSTNSK with:		
pGP-Tn*7*-Gm	10^7	86
pGP-Tn*7*-Cm	10^7	98
pGP-Tn*7*-Tp	10^2	15

[a] The vectors used were pGP-Tn*7*-Gm-*xylE* and pSTNK for the Tn*7* and transposase vectors, respectively.
[b] Following the overnight conjugation, the number of CFU resistant to Gm was calculated by spreading the conjugation layer onto Gm plates.
[c] Integration of Tn*7* at *att*Tn*7* was evaluated by patching colonies onto Gm, Ap, and Km plates. The numbers represent the percentages of colonies out of at least 100 that were exclusively resistant to Gm. Integration of Tn*7*::*xylE* to *att*Tn*7* was PCR validated. All of the exclusively Gm^r clones tested were positive.

Transposition of Tn*7* in *Enterobacteriaceae*. To determine the efficacy of the Tn*7* system in *Enterobacteriaceae*, the *xylE* gene was cloned into pGP-Tn*7*-Gm, creating the pGP-Tn*7*-Gm-*xylE* vector. Introduction of the *xylE* gene provided a practical phenotypic reporter for screening, since colonies expressing *xylE* turn yellow after exposure to a solution of catechol (26). The uropathogenic *E. coli* (UPEC) strains CFT073 and 536, the enterohemorrhagic O157:H7 *E. coli* strain EDL933, the avian pathogenic *E. coli* strain χ7122, *Salmonella enterica* serovar Typhimurium (strain SL1344) and serovar Typhi (strain Ty2a), *Klebsiella pneumoniae* strain KPPR1, and *Cronobacter sakazakii* strain BAA-894 were among strains tested for integration of the *xylE* gene at the *att*Tn*7* site. Mating and screening methodologies were performed as described in Materials and Methods. After conjugation, 10^7 Gm^r CFU from the bacterial lawn were obtained (Table 3). Of these, 100 colonies were separately plated onto Gm, Ap, and Km plates as described in Materials and Methods.

Thereafter, clones were screened for their proper expression of *xylE*. By spraying a solution of catechol, strains harboring the Tn*7*-Gm-*xylE* fragment at the *att*Tn*7* site turned yellow, whereas the WT strains remained white (Fig. 2A). Proper integration of Tn*7*-Gm-*xylE* at *att*Tn*7* was verified by PCR with the primer pairs listed in Table 2. All of the yellow clones correctly integrated the Tn*7*-Gm-*xylE* fragment at *att*Tn*7*, which is denoted by the presence of

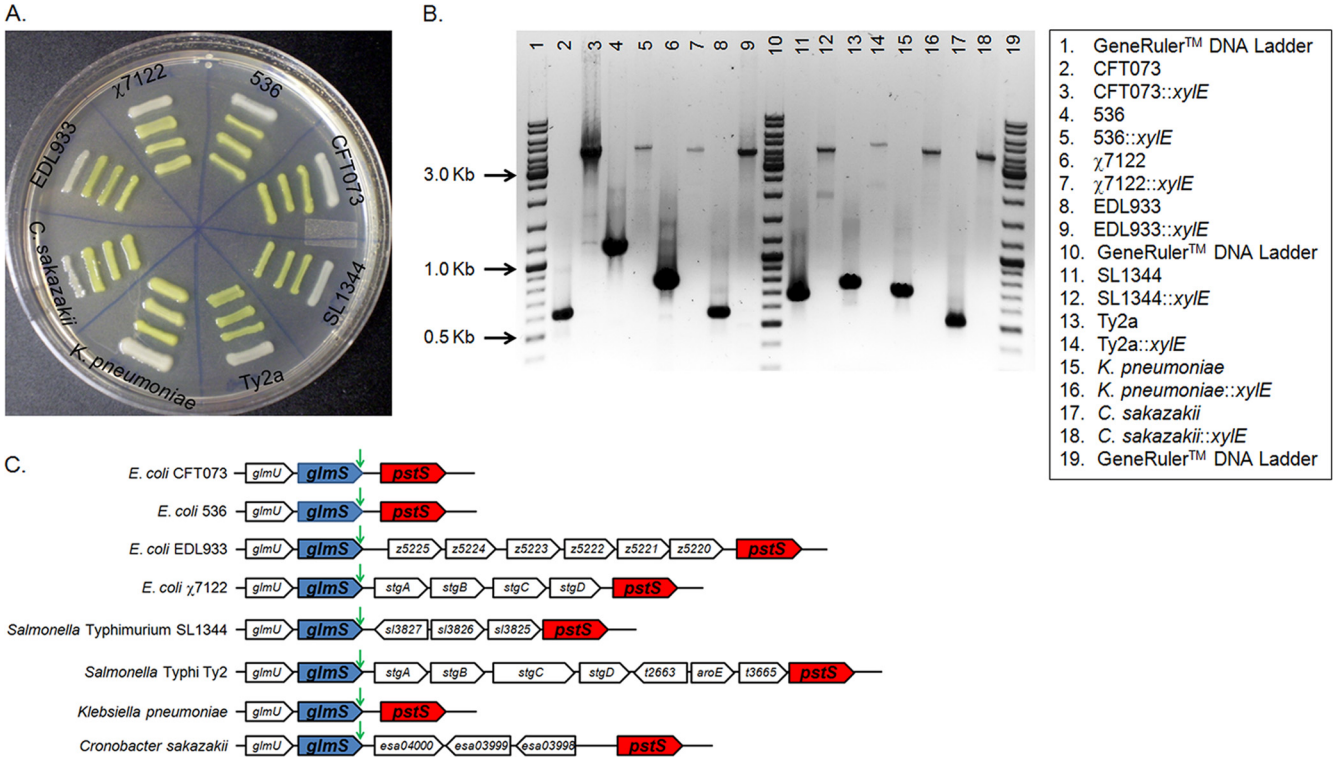

FIG 2 Functionality of genes integration at *att*Tn*7* with the transposon Tn*7*. (A) Expression of *xylE* gene following transposition at *att*Tn*7*. After reaction with a solution of catechol, the WT strains remained white, whereas clones in which *xylE* was integrated at *att*Tn*7* appeared yellow. Strains: *Cronobacter sakazakii* (BAA-894), *Escherichia coli* (536, CFT073, χ7122, and EDL933), *Klebsiella pneumoniae* (*K. pneumoniae* subsp. *pneumoniae* KPPR1), *Salmonella* Typhi Ty2a and *Salmonella* Typhimurium SL1344 (*S. enterica* serovars Typhi and Typhimurium, respectively). (B) Integration of *xylE* at *att*Tn*7* was verified by PCR using the primer-pairs listed in Table 2. Strains are as described for panel A. (C) Chromosomal visualization of the *glmS-pstS* region in different strains tested. In *S.* Typhi Ty2, *stgC* has been previously annotated as a pseudogene. However, it may encode the usher of the Stg fimbriae (20). The green arrow represents the *att*Tn*7* site. The lengths of open reading frames and intergenic regions are not drawn to scale.

amplification products observed between 4 and 5 kb (Fig. 2B). Based upon the resistance, coloration, and PCR screening results, these strains incorporated the *xylE* gene at the *att*Tn*7* site at an efficiency ranging from 80 to 96% (Table 3).

Interestingly, although *E. coli* EDL933 and χ7122, *S.* Typhimurium (SL1344) and *S.* Typhi (Ty2a), and *Cronobacter sakazakii* contained modified sites at *att*Tn*7*, due to differences in the *glmS* terminator loop or presence of additional genes such as fimbrial operons between *glmS* and *pstS* (Fig. 2C), the Tn*7* transposon was still efficiently targeted to the *att*Tn*7* site at the 3′ end of the *glmS* gene (Fig. 2B). In these strains, we screened the Tn*7* integration at *att*Tn*7* site with primers homologous to the first gene found downstream of *glmS*. Proper amplification showed that these genes adjacent to *glmS* in the WT strains were still present after integration at the *att*Tn*7* sites.

Derivatives of the pSTNSK and pGP-Tn*7*-Gm vectors were also tested in UPEC strain CFT073. By using pSTNSK-Tp and pSTNSK-Cm transposase-encoding vectors with pGP-Tn*7*-Gm, we found that the transposition of Tn*7*-Gm at the *att*Tn*7* site occurred at a rate superior to 90% (Table 3). The combination of pSTNSK with pGP-Tn*7*-Cm produced similar results. Although the pGP-Tn*7*-Tp transposon vector was functional, its efficacy was considerably reduced, since only 10^2 Tp^r colonies were obtained and, among them, 15% integrated Tn*7* at *att*Tn*7* compared to 10^7 CFU and >90% for pGP-Tn*7*-Cm or pGP-Tn*7*-Gm (Table 3). Although the pGP-Tn*7*-Tp vector is not as efficient as pGP-Tn*7*-Gm and pGP-Tn*7*-Cm, it can be a suitable alternative for use in strains that are resistant to both Gm and Cm.

Integration of Tn*7* was also assayed in *Citrobacter rodentium*, a natural pathogen of mice used as a model of infections for enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (2). Using the pGP-Tn*7*-Gm and pSTNSK vectors, 10^7 CFU were determined to be resistant to Gm and, among them, 68% correctly integrated the Tn*7*-Gm fragment at *att*Tn*7* (Table 3).

These results validate that the Tn*7* system we developed can be applied to a variety of bacterial species. Compared to previously described procedures (6, 7, 9, 10, 30, 43), our method is simpler since transposition to *att*Tn*7* does not require the use of a helper plasmid. Indeed, it can be easily achieved by a classical conjugation procedure. Previously, a simple and convenient procedure using single vectors containing both the mini-Tn*7* and transposases was described previously (32). However, although this method was shown to be efficient in some enterobacterial strains such as *E. coli* K-12, it did not work well in our prototypical CFT073 and χ7122 pathogenic *E. coli* strains (data not shown). Furthermore, since this vector only possesses the Ap^r cassette, its use is limited because many clinical and environmental enterobacterial strains are increasingly resistant to beta-lactams and other antibiotics (4).

Due to the presence of different combinations of antibiotic markers, the vectors discussed here can be useful with a large

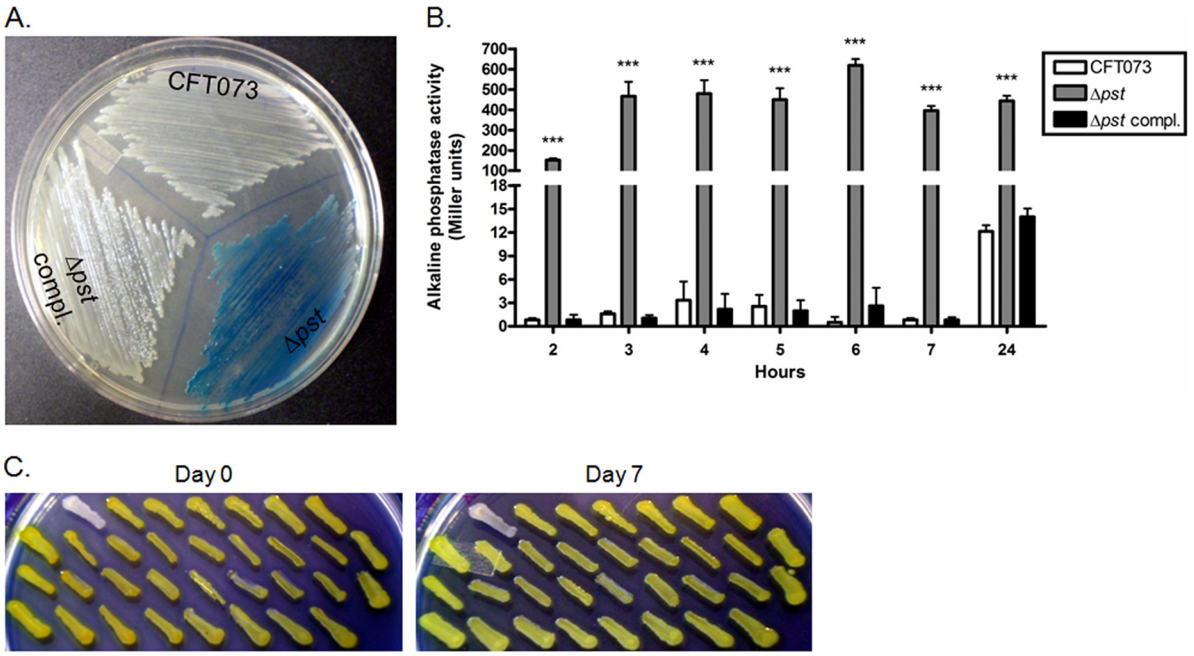

FIG 3 Chromosomal complementation of *pst* mutant restores native production levels of PhoA. (A) On LB BCIP agar plates, the Δ*pst* strain was blue, whereas the WT and the complemented strains were white. (B) Production of PhoA was quantified by an alkaline phosphatase assay. As in panel A, the *pst* mutant massively produced PhoA, whereas in the WT and Δ*pst* complemented strains the production of PhoA was at the basal level. (C) Production of *xylE* from *att*Tn*7* in strain CFT073 after passages without selection pressure over a 7-day period. The white patches represent the WT strain, whereas the yellow ones represent those producing *xylE* from the *att*Tn*7* site.

spectrum of species. They therefore provide an efficient means for introducing recombinant genes encoding reporter fusions and epitope-tagged or chimeric proteins or for complementation of specific mutations in strains by the reintroduction of functional gene(s) at an episomal site.

Chromosomal complementation using Tn*7* in UPEC strain CFT073. The UPEC CFT073 strain is an archetypal strain that has been used in a number of laboratories to investigate the pathogenesis of *E. coli* urinary tract infections (34). This strain does not contain any native plasmids (46), and transcomplementation by plasmids has been shown to be difficult in the absence of antibiotic selection, since plasmids may be rapidly lost without selective pressure.

The *pstSCAB-phoU* gene cluster encodes the phosphate-specific transport system (Pst) and belongs to the Pho regulon. This regulon is controlled by the two-component regulatory system PhoBR, which activates genes involved in the acquisition and metabolism of different kinds of phosphate groups in phosphate starvation conditions (25, 45). The Pst system and the alkaline phosphatase PhoA are among the Pho regulon members. In addition to being involved in phosphate transport, the Pst system negatively regulates the Pho regulon, since its disruption constitutively activates PhoBR (25, 45). Furthermore, the Pst system is also required for virulence since its inactivation attenuated the virulence of pathogenic strains (12, 29). Using the Tn*7* transposon system we describe here, we have successfully complemented the virulence of a *pst* mutant in UPEC CFT073 strain by introducing these genes in a single copy at the *att*Tn*7* site (13).

By activating the PhoBR regulon through disruption of the Pst system, the alkaline phosphatase PhoA becomes constitutively expressed. The production of PhoA can be visualized by plating strains onto LB agar plates supplemented with BCIP (3). Strains producing or not producing PhoA will appear blue or white, respectively. As observed in Fig. 3A, the Pst mutant cultures appeared blue, whereas the WT CFT073 strain remained white. Complementation of the *pst* mutant at *att*Tn*7* restored the white phenotype of the *pst* mutant (Fig. 3A). Thereafter, quantification of PhoA in the WT, Δ*pst*, and complemented strains was evaluated at various time points by an alkaline phosphatase assay. As shown in Fig. 3B, the WT and complemented strains produced PhoA at a basal level, whereas PhoA production was considerably higher in the *pst* mutant.

Stability of genes introduced in single copy using the Tn*7* system. The stability of integration of *xylE* at *att*Tn*7* was evaluated after 14 passages, over a period of 7 days, in LB broth without selective pressure in strain CFT073. As shown in Fig. 3C, at 7 days postinoculation, 100% of the colonies expressed *xylE* (yellow patches). The stability of the *pstSCA* genes inserted at the *att*Tn*7*, in the *pst* mutant, was also evaluated after passage in the murine model of urinary tract infection. As for *xylE*, 100% of colonies contain the *pstSCA* genes at *att*Tn*7* (13; data not shown).

Conclusion. In this report, we developed a series of practical vectors for the integration of Tn*7* at the *att*Tn*7* site that were shown to be effective in a variety of enterobacterial species. This procedure is also versatile since several vectors with different selection markers have been constructed. Furthermore, integration of Tn*7* at *att*Tn*7* was shown to be efficient in a variety of *Enterobacteriaceae*, including pathogenic *E. coli*, *Salmonella*, *Klebsiella*, *Cronobacter*, and *Citrobacter* strains. In addition to serving as a chromosomal complementation method, integration of Tn*7* at *att*Tn*7* can be useful in biotechnology applications, in vaccine development, and in gene expression and gene fusion constructs.

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