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Mini-D3112 Bacteriophage Transposable Elements for Genetic Analysis of *Pseudomonas aeruginosa*

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Small bacteriophage D3112 transposable elements deleted for most of the phage-lytic functions while retaining the sites required for transposition and packaging were constructed to facilitate genetic studies in *Pseudomonas aeruginosa*. These mini-D derivatives were constructed with the terminal 1.85 kilobases (kb) of the phage left end and 1.4 kb of the phage right end and either the Tn*5* kanamycin resistance or the pSC101 (pBR322) tetracycline resistance determinant. Thermally induced lysates of strains lysogenic for both a mini-D element and D3112 *cts* (temperature-sensitive repressor) transduced *P. aeruginosa* PAO recipients to drug resistance at frequencies of between 10^{-4} and 10^{-5}/PFU of the helper phage. As for the parent plaque-forming D3112 phage, the mini-D171 element could insert itself into many different sites in the chromosome but the frequency of insertion into particular genes varied widely. Among 1,000 insertions, none resulted in auxotrophy but 10 resulted in pigment production. Insertions were also selected in a cloning plasmid with a transduction scheme. At least eight different insertion sites were found to have been used among 10 individual insertions. Transductants harboring these mini-D elements were immune to infection by D3112, since they contained the D3112 repressor gene in the left 1.85-kb terminal fragment. Chromosomal genes were transduced in a generalized fashion 100 to 1,000 times more frequently by the mini-D–D3112 *cts* lysates than by the D3112 *cts* phage alone. Mini-D171–D3112 *cts* lysates also yielded some transductants that retained the drug resistance marker of the mini-D element and which were unstable for the chromosomal transduced marker. This is consistent with the miniduction properties of Mu whereby transduced genes are flanked by two mini-D elements in the same orientation.

D3112 and related temperate bacteriophages of *Pseudomonas aeruginosa* (1, 29, 30) have a DNA structure similar to that of the transposable *Escherichia coli* bacteriophage Mu (13). Restriction endonuclease digestion and agarose gel electrophoresis of these phage DNAs produce DNA fragments from the interior of the phage genome that form sharp bands and diffuse, heterogeneous-length terminal fragments that produce fuzzy bands (29). These heterogeneous DNA sequences represent variable amounts of host DNA covalently attached to each end, with the right end being considerably more variable in size. Sequencing analysis of the D3112 termini revealed that, like Mu, D3112 generates a five-base-pair duplication upon insertion and has 5′ TG 3′ as the two most terminal base pairs (N. E. Kent, A. Darzins, and M. J. Casadaban, manuscript in preparation).

Bacteriophage D3112 is also capable of acting as an insertional mutagen. Lysogens of the phage have insertions in many different locations, and occasionally these insertions are inside particular genes, although auxotrophic mutations are formed hundreds of times less frequently by D3112 than by Mu. As for Mu, insertions in a particular gene map to many different sites (43).

Induction of a D3112 prophage results in transposition of the phage whereby the D3112 termini recombine with multiple chromosomal regions and amplify the internal phage sequences (43). The transposable property of D3112 give it the potential of being developed into a powerful tool for studying gene function and regulation in *P. aeruginosa* and perhaps other nonenteric bacteria in which Mu replicates poorly (40).

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The termini of transposable elements contain the *cis*-reactive sequences involved in the transposition reaction (20). In an attempt to separate the integrative functions of D3112 from its viral properties, we constructed two mini-D3112 elements which contain short sequences from both ends of the phage. During the course of this work, Yanenko et al. (55) have also constructed mini-D elements without convenient drug resistance markers. Here, we present the construction and physical characterization of these D3112 derivatives and describe their properties of transposition, increased generalized transduction, and specialized miniduction.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The strains, phages, and plasmids used in this study are described in Table 1. The *E. coli* host used in the cloning experiments was JM83 (51).

Media. *E. coli* and *P. aeruginosa* strains were routinely grown in LB rich medium (38) or minimal medium as described by Brammer and Clarke (7), except that the trace element solution was omitted. Amino acids were added to a final concentration of 1 mM. Glucose was added to a final concentration of 50 mM. For solid media, agar (Difco Laboratories) was added to 1.5%. The antibiotic concentrations for *E. coli* were as follows: ampicillin, 50 μg/ml; kanamycin, 40 μg/ml; tetracycline, 25 μg/ml. For selection of *P. aeruginosa* transconjugants after triparental mating, Pseudomonas Isolation Agar (Difco) was supplemented with carbenicillin (1 mg/ml), kanamycin (100 μg/ml), or tetracycline (300 μg/ml). The concentration of tetracycline in minimal media was 50 μg/ml. The β-lactamase (*bla*) gene was selected in *E. coli* as ampicillin resistance (Ap^r) and in *P. aeruginosa* as carbenicillin resistance (Cb^r).

TABLE 1. Strains, phages, and plasmids

Strain, phage, or plasmid	Description or genotype[a]	Source or reference
P. aeruginosa		
PAO1	Prototroph, FP$^-$	23
PAO25	*leu-10 argF10* FP$^-$	22
PAO222	*ilv-226 his-4 lys-12 proA82 met-28 trp-6* FP$^-$	22
PAO4141	*met-9020 pro-9024 blaP9202 blaJ9111 aph-9001* FP$^-$	H. Matsumoto
ADD222	PAO222::D3112 *c*ts	This study
RM265	*leu-10 recA102* FP$^-$	27
CD10	PAO4141::D3112 *c*ts	This study
Phages		
D3112 *c*ts	*c*ts, temperature-sensitive repressor (38 kb)	43
Mini-D163	*c*ts, defective phage (3.3 kb)	This study
Mini-D165	*c*ts, defective phage Km^r (4.9 kb)	Fig. 1
Mini-D171	*c*ts, defective phage Tc^r (4.7 kb)	Fig. 1
Plasmids		
pBR322	rep_{pMB1} Ap^r Tc^r (4.4 kb)	48
pKT240	rep_{R300B} Ap^r Km^r (12.9 kb)	2
pLAFR1	rep_{RK2} $oriT_{RK2}$ *cos* Tc^r (21 kb)	18
pTJS140	rep_{RK2} $oriT_{RK2}$ rep_{pMB1} *lac'IPOZ* Ap^r (8.0 kb)	D. Helinski and T. Schmidhauser
pUC12-pUC19	rep_{pMB1} *lac'IPOZ* Ap^r (2.7 kb)	51
pUC4-KIXX	rep_{pMB1} *lac'IPOZ* Ap^r Km^r (4.2 kb)	Pharmacia-LKB
pADD161	pUC12 (Ap^r): *Hin*cII, *Hin*dIII D3112 *c*ts left end (4.6 kb)	Darzins and Casadaban, unpublished data
pADD163	pUC (Ap^r) with mini-D163 (6.5 kb)	Fig. 1
pADD165	pUC (Ap^r) with mini-D165 (Km^r) (7.1 kb)	Fig. 1
pADD165.1	pLAFR1 (Tc^r) with mini-D165 (Km^r) (25.5 kb)	This study
pADD171	pUC (Ap^r) with mini-D171 (Tc^r) (7.7 kb)	Fig. 1
pADD171.1	pKT240 (Ap^r Km^r) with mini-D171 (Tc^r) (17.6 kb)	This study
pNEK172	pUC19 with D3112 right end (4.7 kb)	Kent and Casadaban, unpublished data

[a] The *P. aeruginosa* gene designations used have been described previously (44). *oriT*, RK2 origin of transfer (21); *cos*, cohesive-end site; rep_{pMB1}, replicon from pMB1; rep_{R300B}, replicon from R300B; rep_{RK2}, replicon from RK2; FP$^-$, absence of the chromosome-mobilizing plasmid FP2.

DNA methods. Restriction enzymes, DNA polymerase (Klenow enzyme), and T4 DNA ligase, were purchased from New England BioLabs and used as recommended by the supplier. Rapid small-scale (26) and large-scale (32) isolations of plasmid DNA in *E. coli* and small-scale isolation of plasmid DNA in *P. aeruginosa* (6) were done as previously described. *P. aeruginosa* chromosomal DNA was isolated by the method of Marmur (35). DNA restriction fragments were purified from agarose gels after electrophoretic separation by the method of Vogelstein and Gillespie (52). *E. coli* cells were transformed by the method of Mandel and Higa (33) as modified by Morrison (39). Southern hybridization (46) of *P. aeruginosa* DNA was performed as previously described (11), except that DNA fragments were transferred to 0.45-μm-pore-size nylon membranes (Micron Separations Inc.) The other procedures used were those of Maniatis et al. (34).

Construction of mini-D3112 elements. Clones of the left and right ends of D3112 in pUC vectors were combined in vitro to place the termini in the wild-type phage orientation (Fig. 1). The left-end clone, pADD161 (N. E. Kent, A. Darzins, and M. J. Casadaban, manuscript in preparation), contained 1.85 kilobases (kb) of D3112 *c*ts phage DNA, up to the *Hin*dIII site inserted between the blunt *Hin*cII site and the *Hin*dIII site of pUC12. The right-end clone, pNEK172, contained 1.4 kb of phage DNA, from the rightmost *Hin*dIII site of D3112 to an *Sma*I site outside the phage in the tetracycline resistance gene (31) from an RP4 plasmid with a D3112 insertion in the tetracycline resistance gene. This fragment was inserted between the *Hin*dIII and *Sma*I sites of pUC19. Plasmids pADD161 and pNEK172 were digested with *Xmn*I and *Hin*dIII, and the resulting DNA fragments were resolved on a 0.7% agarose gel. The largest fragments in each digest were isolated, ligated, and used to transform JM83 to ampicillin resistance. The resulting plasmid, designated pADD163, contained the mini-D3112 element D163 (Fig. 1) with no selectable drug resistance between its termini.

To conveniently select for mini-D elements, antibiotic resistance markers were inserted into pADD163. A *Hin*dIII fragment containing the kanamycin resistance gene from Tn*5* on plasmid pUC4-KIXX (Pharmacia Biotechnology) was inserted into the *Hin*dIII site of pADD163 to form pADD165 with mini-D165 (Fig. 1). Likewise, an *Eco*RI-*Ava*I fragment containing the tetracycline resistance determinant from plasmid pBR322 was made blunt with the DNA polymerase Klenow fragment and ligated into the similarly filled in *Hin*dIII site in pADD163 to form pADD171 with mini-D171 (Fig. 1).

Genetic procedures. Recombinant plasmids in *E. coli* were introduced into *P. aeruginosa* by using triparental matings with pRK2013 as the helper mobilizing plasmid (12, 17). D3112 *c*ts lysogens were isolated from confluent zones of lysis made by placing a drop of a D3112 *c*ts lysate onto a bacterial lawn spread on LB agar media containing 1 mM $MgSO_4$ and incubating it overnight at 30°C. Potential lysogenic survivors of D3112 infection were purified from the turbid zone by being streaked to single colonies. Lysogens were confirmed by testing for temperature sensitivity, ability to release phage at 42°C, and immunity to superinfection by D3112.

Transfer of mini-D3112 elements in *P. aeruginosa*. To introduce mini-D165 and mini-D171 into *P. aeruginosa*, fragments containing these elements were cloned onto broad

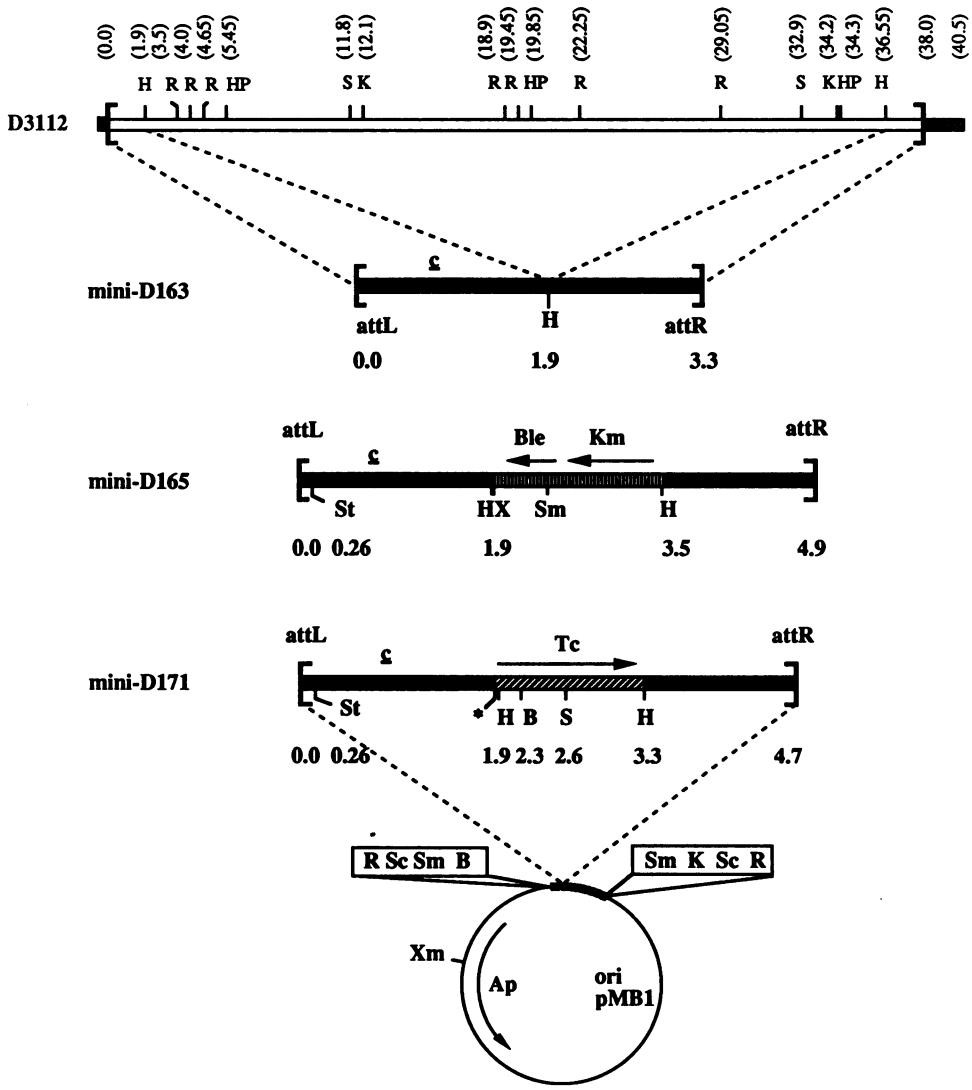

FIG. 1. Construction of mini-D elements D163, D165, and D171. The restriction map of D3112 phage DNA is shown above (43). The ends of D3112 are shown as brackets. The cross-hatched area adjacent to the D3112 left and right ends represents packaged host sequences. Positions are given in kilobases from the left side of each genetic element. The horizontally lined region in mini-D165 represents Tn*5* sequences (5, 37), and the diagonally lined area in mini-D171 represents pBR322 sequences (48). The asterisk in mini-D171 denotes an *Hin*dIII-*Eco*RI junction which does not recreate either parent site. A *Hin*dIII site at the *Ava*I-*Hin*dIII junction in mini-D171 is recreated. The location of each mini-D element on plasmids pADD163, pADD165, and pADD171 with flanking restriction sites is shown at the bottom. The thin lines in pADD163, pADD165, and pADD171 represent pUC sequences, the cross-hatched region adjacent to the D3112 left end represents 30 bp of host chromosome, and the stippled region adjacent to the D3112 right end represents approximately 280 bp of the RP4 *tetA* sequence (53). Construction details are given in Materials and Methods. Abbreviations: attL and attR, D3112 left and right termini, respectively; Ap, ampicillin resistance; Ble, bleomycin resistance; Km, kanamycin resistance; Tc, tetracycline resistance; B, *Bam*HI; H, *Hin*dIII; HP, *Hpa*I; K, *Kpn*I; R, *Eco*RI; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; St, *Stu*I; X, *Xho*I; Xm, *Xmn*I; *c*, D3112 repressor.

host range plasmids pLAFR1 (Tc^r) and pKT240 (Ap^r Km^r), respectively. Plasmids pADD165 and pADD171 were digested with *Eco*RI, and the fragments carrying mini-D165 and mini-D171 were ligated with *Eco*RI-cleaved pLAFR1 and pKT240, respectively. Following transformation, tetracycline- and kanamycin-resistant colonies were screened to find the recombinant clones pADD165.1 and pADD171.1 with the kanamycin-resistant mini-D165 and tetracycline-resistant mini-D171 elements, respectively.

Plasmids pADD165.1 and pADD171.1 were mobilized by the Tra^+ helper plasmid pRK2013 into *P. aeruginosa* CD10, which is lysogenic for D3112 *c*ts. CD10 contains the *aph-9001* mutation in the chromosomal aminoglycoside 3′-phosphotransferase II (36, 42), which renders cells sensitive to kanamycin. The mating mixture of cells was plated onto Pseudomonas Isolation Agar selective plates containing either kanamycin (for mini-D165) or tetracycline (for mini-D171). All 10 of the kanamycin-resistant transconjugants tested were also tetracycline resistant, which suggested that mini-D165 was present on the original pADD165.1 plasmid. This was verified for two of these by restriction analysis of plasmid DNA (data not shown). These CD10(pADD165.1) transconjugants were also temperature sensitive (42°C) and released phage. Five tetracycline-resistant transconjugant colonies for pADD171.1 were also isolated, but all were ampicillin and kanamycin sensitive, which indicated the

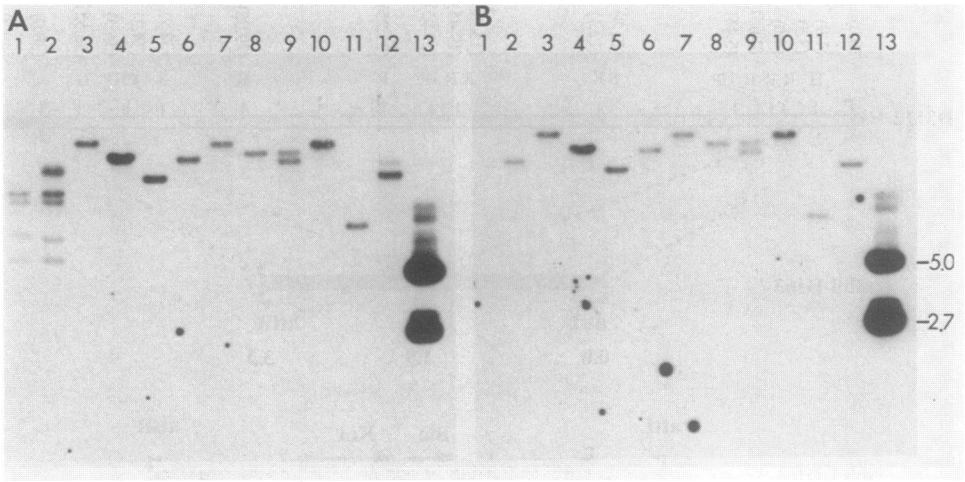

FIG. 2. Southern hybridization of mini-D171 transductants. Chromosomal and plasmid DNAs were digested with *Eco*RI. Lanes: 1, CD10 (a double D3112 lysogen); 2, CD10::mini-D171; 3 to 12, PAO1::mini-D171 transductants 1 to 10; 13, pADD171. A, pADD171 probe, B, pBR322 probe. Molecular size markers in kilobases are denoted at the right.

likely loss of pKT240 vector sequences by transposition of the mini-D171 element from pADD171.1 to the chromosome. All of the CD10 transconjugants were temperature sensitive (42°C) and released phage, as did the parental CD10 lysogen strain.

Preparation and use of D3112 *cts* lysates. D3112 *c*ts and mini-D171–D3112 *c*ts lysates were prepared by thermoinduction. An overnight culture was grown at 30°C in LB with the drug to which resistance was encoded by the mini-D element and diluted 1:100 in 25 ml of LB broth without drugs. This culture was grown for approximately 3 h to mid-log phase at 30°C and shifted to 42°C for 2 h or until lysis occurred. Chloroform to 1% of the volume, $MgSO_4$ to 2 mM, and $CaCl_2$ to 0.2 mM were added to the lysates, and cell debris was removed by centrifugation. The titers of the resulting lysates were determined, and the lysates were stored at 4°C until use. Virtually no loss in phage titer was detected in a D3112 phage lysate stored at 4°C for over 1 year.

Infections were performed on solid plates because of the low infectivity of *P. aeruginosa* cells when grown in liquid media (54; A. Darzins, C. Roncero, and M. J. Casadaban, unpublished data). Equal volumes (0.15 ml) of an overnight culture of recipient cells and lysate were mixed (multiplicity of infection, approximately 0.1 to 1), spread onto a LB agar plate, and incubated at 30°C for 3 h. Following incubation, the cells were removed from the plates, washed with 0.85% saline, and suspended in 2 ml of saline. Drug-resistant transductants were selected on Pseudomonas Isolation Agar media supplemented with tetracycline or kanamycin.

RESULTS

Transposition of mini-D3112 elements in *P. aeruginosa*. Mini-D3112 elements were constructed by combining the D3112 left and right ends in their normal orientation with respect to each other on a pUC replicon and inserting either a Km^r gene or a Tc^r gene. These drug-resistant mini-D elements were cloned into broad host range plasmids and introduced by mobilization into a strain of PAO1 lysogenic for D3112 *c*ts (CD10). Lysates were made from several CD10 transconjugants with mini-D165 (Km^r) and mini-D171 (Tc^r), as described in Materials and Methods, and used to infect PAO4141. Km^r and Tc^r transductants arose at frequencies between 10^{-4} and 10^{-5}/PFU of the helper phage. Ten Tc^r (mini-D171) transductants were selected and tested for lysogenic functions. Only one transductant released phage and was temperature sensitive and indicated that a helper phage was most likely present. All 10 of the transductants were immune to infection by D3112 at 30°C, since no plaques were seen, even with 10^9 infecting D3112 phage. This implied that the immunity gene near the left end was present.

The 10 transductants were next examined by Southern hybridization. *Eco*RI-digested chromosomal DNAs of each transductant and its parental strain were probed initially with pADD171 nick translated with ^{32}P. The hybridization pattern of the parental lysogen strain CD10 had four bands, implying that two copies of the phage were inserted in the chromosome (Fig. 2A, lane 1). The probe recognized pairs of bands for each insert of D3112, one for each end. The hybridization pattern in lane 2 was consistent with the hypothesis that upon transfer of pADD171.1 into *P. aeruginosa* the mini-D171 element transposed into the chromosome with a concomitant loss of the pKT240 plasmid sequences and the Ap^r and Km^r markers. In addition to the four bands representing the resident D3112 prophage termini, another *Eco*RI fragment was present which was larger than 5 kb, which is the size of the mini-D171 *Eco*RI fragment on pADD171.1. The larger size was consistent with an insertion into the chromosome.

Figure 2A, lanes 3 to 12, represents the hybridization profile of the 10 Tc^r transductants. Eight of these transductants appeared to contain single copies of the mini-D element, as evidenced by the presence of single hybridizing bands, although the possibility exists that two elements inserted themselves into *Eco*RI fragments of similar sizes. The different sizes of the *Eco*RI bands for the individual Tc^r transductants demonstrated that the mini-D element transposed into many (at least six) different places of the PAO chromosome. Two of the transductants (no. 7 and 10; Fig. 2A, lanes 9 and 12) contained at least two different insertions which hybridized to the probe. One of these (no. 10) was the transductant which released phage.

To distinguish between insertions of the mini-D171 element and the helper D3112 *c*ts, the blot was stripped of the labeled pADD171 probe and rehybridized with nick-trans-

lated ^{32}P-labeled pBR322 DNA, which contained the Tc^r gene of mini-D171 (Fig. 2B). Eight of the transductants contained single insertions of mini-D171 as evidenced by the single hybridizing band. Of the transductants which contained more than one hybridizing band with pADD171 as a probe (Fig. 2A), it appeared that transductant 7 (lane 9) contained two integrated copies of the mini-D element and transductant 10 (lane 12) contained single copies of mini-D171 and D3112 *c*ts. Since single copies of mini-D171 and D3112 *c*ts should have generated three hybridizing bands in Fig. 2A (lane 12), it is likely that the lower, more intense band actually represents two superimposed fragments, one recognizing the *Eco*RI fragment containing the mini-D171 element and the other recognizing one end of D3112 *c*ts.

To test for randomness of insertion, approximately 1,000 Tc^r PAO1 transductant colonies from a single transduction experiment were patched to minimal-glucose plates to ascertain the frequency of auxotrophs generated by transposition of mini-D171 to the *P. aeruginosa* chromosome. All of the Tc^r colonies grew on minimal-glucose plates, and no auxotrophs were found. Ten of the transductants, however, produced an unidentified *P. aeruginosa* pigment.

Chromosomal transduction by D3112 *c*ts–mini-D171 lysates. Bacteriophage D3112 is a generalized transducing phage and can transduce various chromosomal markers at frequencies of 8.0×10^{-8} to 2.0×10^{-9} (29). Mini-D171, which is only 4.7 kb, should be capable of packaging up to 35 kb of an adjacent host sequence if it uses a Mu-like DNA-packaging mechanism. Upon injection into a recipient cell, this host DNA can replace resident DNA in a *recA*-mediated generalized transduction. To determine whether such a D3112 structure could be used to transduce *P. aeruginosa*, we compared the transduction frequencies of D3112 *c*ts and mini-D171–D3112 *c*ts lysates. A mini-D171–D3112 *c*ts mixed lysate was able to transduce all seven of the PAO chromosomal markers tested in *recA*$^+$ strains as high as 700 times more efficiently than was a D3112 *c*ts lysate alone (Table 2). For the *recA* strain, however, no transductants were detected with D3112 *c*ts and they were detected at a 240-fold-reduced frequency with mini-D171–D3112 *c*ts. Different genes were transduced at slightly different frequencies, which might reflect the presence of nearby sequences in the chromosome similar to the phage-packaging site, for generalized transduction, or hot spots for mini-D3112 insertions for transductions mediated by the mini-D element. A PAO D3112 lysogenic recipient was also tested for its ability to be transduced with a mini-D171–D3112 *c*ts lysate. The lysogen was transduced at approximately the same frequency as the nonlysogen, and this transduction occurred 100 to 500 times more efficiently with mini-D than with the D3112 *c*ts lysate alone (Table 2).

Another way in which these mini-D elements might be able to convey DNA to recipient cells is if the host DNA becomes bracketed by two copies of the mini-D element in the same orientation. It is possible that the entire mini-D–bacterial DNA–mini-D complex can be inserted into the genome of the recipient by a *recA*-independent transposition event, a process which was first described for Mu and known as mini-Mu-duction (15, 16). To distinguish between generalized transduction (as described above) and possible specialized transduction as by mini-D-duction, transductants (recombinant colonies prototrophic for a specific marker) were tested for tetracycline resistance. It was determined that between 2 and 10% of the chromosomal gene transductants were Tc^r. Three of the PAO222 Ilv$^+$ Tc^r transductants (Table 2) were tested for stability of the Ilv$^+$ and Tc^r markers. The transductants were grown overnight in LB broth without drugs and plated for single colonies on LB plates. Over 100 colonies from each transductant were patched to minimal-glucose–tetracycline plates lacking isoleucine and valine and LB-tetracycline plates. Only one of the three transductants gave rise to Ilv$^-$ segregants (13% of the colonies) which were still D3112 immune and Tc^r. All of the other colonies were Ilv$^+$ and Tc^r. This one transductant thus has the properties of a mini-D-ductant with the chromosomal *ilv-226* region integrated by a transposition process with flanking mini-D171 elements as found with mini-Mu's (16). The other two Ilv$^+$ transductants presumably contained separate mini-D171 insertions which had also undergone recombination in a generalized fashion.

TABLE 2. Transduction frequencies of different recipients by D3112 *c*ts and D3112 *c*ts–mini-D171 lysates

Recipient	Selected marker	Transduction frequency[a]	
		D3112 *c*ts	mini-D171–D3112*c*ts
PAO222	Ilv	1.8×10^{-8}	2.5×10^{-6}
	Lys	1.3×10^{-8}	2.7×10^{-6}
	Met	5.3×10^{-8}	8.3×10^{-6}
	Trp	8.3×10^{-9}	4.2×10^{-6}
	His	2.7×10^{-8}	4.7×10^{-6}
	Pro	6.7×10^{-9}	3.3×10^{-6}
ADD222 (PAO222:: D3112)	Ilv	9.0×10^{-9}	1.3×10^{-6}
	Lys	$<3.3 \times 10^{-9}$	1.6×10^{-6}
	Met	2.3×10^{-8}	3.3×10^{-6}
PAO25	Leu	1.0×10^{-9}	7.0×10^{-7}
RM265 (*recA*)	Leu	$<1.0 \times 10^{-9}$	2.9×10^{-9}

[a] A 100-μl portion of an overnight culture of each recipient was plated onto an LB agar plate, spread to dryness, and incubated at 30°C for 8 h. Following incubation, the cells were removed from the LB agar plate in 5 ml of LB-Mg^{2+} and used directly for infection. A 1-ml culture volume (2×10^{10} bacteria per ml) was mixed with a D3112 *c*ts or D3112 *c*ts–mini-D171 lysate at a multiplicity of infection of 0.1. After 30 min at 37°C, the cells were washed twice in 0.85% saline, suspended in 2 ml of saline, and plated on selective medium. The plates were incubated at 30°C for 3 to 4 days. Transduction frequencies were expressed as the number of transductants per PFU of the infecting lysate. The positions of chromosomal markers on the *P. aeruginosa* PAO map have been previously described (41, 44).

Plasmid transduction and insertion with mini-D171. To test the ability of this minielement to integrate into a small genetic region, we used a transduction scheme which was developed to localize mini-Mu insertions into plasmids (8). We chose to use a composite plasmid designated pTJS140, which contains replicons from both plasmids RK2 and pUC9. This plasmid is 8.0 kb and replicates with a high copy number in *E. coli* and with a copy number similar to that of plasmid RK2 in *P. aeruginosa*. Plasmid pTJS140 was mobilized into AD222 from *E. coli* with selection for carbenicillin resistance. Mini-D171 was introduced into this AD222 transconjugant by infection with a CD10::mini-D171 lysate with selection on Pseudomonas Isolation Agar-tetracycline media. The colonies obtained were tested for temperature sensitivity and phage release. The presence of an intact pTJS140 plasmid was tested by restriction analysis of plasmid DNA. One of these strains was heat induced to make a phage lysate with a titer of 1.8×10^9 PFU/ml. Infection of an overnight culture of PAO1 yielded Cb^r PAO1 transductants at a frequency of 10^{-6} per helper PFU. All 50 of the Cb^r transductants tested also had the mini-D171 Tc^r marker. The plasmid DNAs of 10 of these Cb^r Tc^r transductants were analyzed by digestion with *Eco*RI and agarose gel electro-

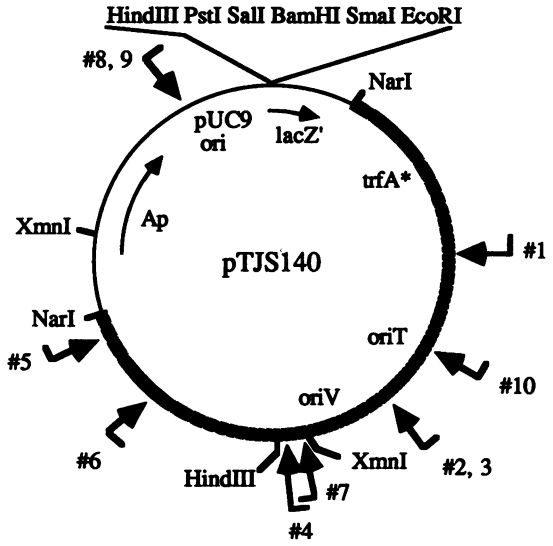

FIG. 3. Localization of mini-D171 insertions in pTJS140. Thick diagonal and thin black lines represent RK2 and pUC9 sequences in pTJS140, respectively. Insertion sites are denoted by large arrows. The direction of the perpendicular line adjacent to these arrows represents the orientation of the mini-D171 left end. oriT, Plasmid RK2 transfer origin (21); oriV, origin of vegatative replication (47); trfA*, replication protein of plasmid RK2 (45).

phoresis, which revealed that all contained a single linear band of approximately 13 kb, which is the size of mini-D171 plus pTJS140. The positions of the mini-D171 insertions were determined by gel analysis after digestion with *Eco*RI, *Stu*I, and *Xmn*I (Fig. 3). Plasmid pTJS140 contains a unique *Eco*RI site in the multiple cloning site of pUC9 and two *Xmn*I sites. Mini-D171 contains a unique *Stu*I site approximately 300 base pairs (bp) from the D3112 left end (Kent et al., unpublished data). For the 10 mini-D171 insertions examined, at least eight different insertion sites in the plasmid were found. Two pairs of insertions (no. 8 and 9 and no. 2 and 3) could not be separated by this agarose gel analysis. Eight of the ten insertions occurred within the RK2 portion of the pTJS140 plasmid. Insertions in both orientations were found at nearly the same frequency. None of the 10 insertions examined had lost or gained any restriction endonuclease sites, as expected for insertions of mini-D171 in pTJS140 with no detectable deletions or rearrangements.

DISCUSSION

We have described the construction of selectable D3112 derivatives which are useful for genetic analysis of *P. aeruginosa*. These mini-D3112 elements lack the genes essential for phage growth but retain the terminal sequences required in *cis* for transposition. The terminal 1.85 kb from the D3112 left end and the terminal 1.4 kb from the right end, in the presence of a helper D3112 phage, were sufficient to allow transposition of the mini-D elements. The sequences essential for transposition of the *E. coli* bacteriophage Mu have been shown to be within the terminal 163 bp on the left end and the terminal 52 bp on the right end (20). These regions include the terminal site-specific binding of the Mu A transposase protein (9). Further subcloning and deletion analysis of the D3112 termini will be needed to determine the DNA sequences essential for transposition.

P. aeruginosa PAO strains harboring either a clone of the terminal 1.85 kb of the D3112 left end or the mini-D elements themselves were immune to infection by D3112 phage, suggesting that the D3112 repressor gene (*c*) is located near the left end. This result is consistent with the work of Yanenko et al. (54), which localized the D3112 repressor (*c*) within this region.

By themselves, the mini-D elements described here are not lethal at elevated temperatures and do not show a reduced frequency of transfer into nonlysogenic strains. This is consistent with their lack of D3112 replication genes and any other killing genes, as in mini-Mu elements which do not kill without the replication-transposition genes and the Mu *kil* gene. The possibility that the mini-D phage lack the gene(s) required for transposition is consistent with the location of three early genes, possibly involved with replication, in the map interval of 1.3 to 14.5 kb (54). Those genes were mapped by recombination and complementation analysis of temperature-sensitive mutants with spontaneous deletions of a D3112 prophage. This proposed location of the D3112 replication genes near the phage left end is a striking parallel to *E. coli* bacteriophage Mu (14, 50). The proposed location of the transposition genes near the left end provides an explanation for the transposition-insertion of these mini-elements in nonlysogenic recipient cells. As is thought to be the case for Mu (8), the minielements would frequently be packaged along with a large segment of host DNA which would frequently contain an insertion of a helper prophage, the left end of which might then be packaged to contain the transposition genes. Those mini-D elements that are packaged along with only host chromosomal DNA would not be able to transpose unless they were supplied with transposase in *trans* by a coinfecting phage.

Upon introduction of mini-D171-containing plasmid pADD171.1 into D3112 lysogen CD10, it was observed that transconjugants carrying the Km^r marker of pKT240 were not obtained. Instead, only transconjugants carrying the Tc^r marker of mini-D171 could be found. This was in contrast to CD10(pADD165.1) transconjugants, in which both pLAFR1 (Tc^r) and mini-D165 (Km^r) antibiotic resistance markers were found together. One possible explanation that could account for the apparent instability of pADD171.1 in *P. aeruginosa* is based on the high copy number of the pKT240 vector. It is possible that the presence of the D3112 termini at high copy numbers could titrate out or bind up enough repressor molecules to partially derepress the transposition functions of the resident prophage. Since the copy number of pLAFR1, which is essentially the same as the copy number of IncP plasmid RP4 (five to seven per chromosome), is lower than that of the IncQ-based plasmid pKT240 (2, 4), the amount of repressor in the cell is sufficient to keep the replication functions from being expressed.

Since bacteriophage Mu exhibits little target specificity upon transposition and has limited hot spots for insertion, it integrates at nearly random locations in the host chromosome. From 1 to 2% of Mu lysogens interrupt chromosomal genes, giving rise to auxotrophic mutations (49). This is in stark contrast to the low frequency of auxotrophic mutations 0.013% (29) from D3112 lysogens and our finding of no auxotrophs (<0.1%) arising from mini-D171 chromosomal insertions. However, 1% of mini-D transductants were pigmented.

From the low frequency of insertional mutations created by D3112 one might infer that upon integration the phage has a marked preference for particular sequences or regions. However, the generalized and plasmid-transducing abilities of mini-D171–D3112 *c*ts mixed lysates described in this study and our recent mini-D3112 replicon in vivo cloning

studies (10) strongly suggest that D3112 can insert itself in many places around the *P. aeruginosa* chromosome, at least during its lytic phase of growth. The hypothesis that D3112 has a broad target specificity is also supported by the fact that insertions in a particular gene, *amiE* (amidase), could readily be isolated by selection for inactivation of the gene (43). In addition, our recent sequence analysis has revealed that transposition of D3112 into a tetracycline resistance gene created a 5-bp duplication of the host sequence contiguous to the phage genome (Kent et al., in preparation). Furthermore, examination of the leftmost terminal sequences of DNA isolated from the phage showed that host sequences are packaged along with phage DNA at the left end, and inspection of the adjacent 5 bp produced in four different phage-host junction fragments failed to demonstrate any type of insertion site consensus beyond the preference for a high G+C content upstream of the 5-bp duplication in the host sequence. Does D3112 have a preference for intergenic or nonessential regions? Does D3112 display two different insertion specificities, one during lysogeny and another during lytic growth? The availability of stable, selectable mini-D3112 elements will be useful in probing these questions regarding target specificity.

We have also shown that mini-D3112 derivatives can transduce host genes much more efficiently than can D3112 by itself. This is consistent with a correspondingly larger amount of host DNA which can be packaged by the minielement and injected into a recipient cell, where it can replace resident DNA by *recA*-mediated homologous recombination (generalized transduction). *P. aeruginosa* phages F116L (28) and G101 (25) are generalized transducing phages of 63 and 58 kb, respectively, which transduce markers at a frequency of 1×10^{-7} to 5×10^{-7}/PFU (24). Generalized transduction frequencies generated by the mini-D system described in this study were, at best, 20-fold greater (Table 2) than the frequencies reported for F116L and G101. Mini-D3112-mediated generalized transduction, therefore, is potentially more useful than generalized transduction by large phages, such as F116L or G101, for fine structure mapping because it involves smaller host DNA segments (<35 kb).

In this study, a PAO *recA* recipient was transduced 240 times less efficiently than was a Rec^+ strain (Table 2), indicating that any mini-D-duction process occurred at a low frequency, if at all. However, examination of three Ilv^+ Tc^r transductants in a Rec^+ strain for the stability of these markers revealed one transductant that frequently lost the Ilv^+ marker while always retaining Tc^r and immunity to D3112. This suggests that the mini-D elements can mediate the *recA*-independent process of miniduction, but because these elements lack the transposition genes the frequency of this event is low.

Introduction of new genetic material into bacteria is an important step in constructing organisms with novel and useful activities and usually involves introducing recombinant plasmids containing foreign genes which encode desirable gene products. However, plasmids in the absence of selective pressure may be unstable with respect to inheritance of the cloned gene or continued expression of that gene. An alternative method to introducing genes on extrachromosomal elements is the use of transposable elements as vehicles to introduce new or altered genetic material directly into the bacterial chromosome (3, 19). The mini-D3112 elements described here can be used as excellent vehicles to introduce genetic material into the chromosome of *P. aeruginosa* and, for that matter, into any other organisms in which D3112 can transpose. We have demonstrated that mini-D elements can be used to introduce drug resistance markers into *P. aeruginosa* strains. Furthermore, Southern hybridization analysis of several mini-D171 transductants showed that most of the Tc^r colonies contained single insertions (Fig. 2). The mini-D insertions, once established, should not excise themselves from the chromosome because they lack the D3112 replication functions and contain a functional repressor gene to repress the transposition of any incoming D3112 helper phage.

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