Identification of a New Transposon Tn*5403* in a *Klebsiella pneumoniae* Strain Isolated from a Polluted Aquatic Environment

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Abstract. A *Klebsiella pneumoniae* strain having mobilization "helper" potential has been isolated from the river Rhine. Analysis of the transconjugants resulting from the mobilization of nonconjugative pBR-type plasmids and RSF1010 derivatives showed that the transfer-helper capacity of the *K. pneumoniae* strain is related to the presence of a Tn*3*-like transposable element, Tn*5403*. This element has been identified and localized in a plasmid.

Current interest in the use of genetically engineered microorganisms (GEMs) is tempered by our poor understanding of the gene flux within the natural microbial community. In order to prevent their dispersal, genetically engineered DNAs usually lack conjugation functions, such as *tra* genes, *mob* genes, and/or *ori*T sequence. Nevertheless, transfer may occur via mobilization by a self-transmissible plasmid either by trans-complementation, or by transposon-mediated fusion [4]. The first of these mechanisms, called "donation," is the process whereby the transfer of the nonconjugative plasmid, lacking the *tra* and *mob* genes but containing an *ori*T sequence, is mediated by the products of a trans-acting conjugative plasmid, without fusion between the two plasmids. The second mechanism, called "conduction," involves the formation of a cointegrate between the conjugative and the nonconjugative plasmid. Such plasmid-plasmid fusion is characteristic of nonconjugative bacterial transposable elements, which use a replicative pathway for transposition. They belong either to the insertion sequences (IS) class or to the Tn*3* class [25]. The simultaneous occurrence of both transient cointegration and conjugation events coded by the conjugative plasmid results in the transfer of nonconjugative replicons, even if their *ori*T has been deleted [7, 11].

Several recent studies have demonstrated that indigenous bacteria are capable of plasmid mobilization in different ecosystems [13, 14, 16]. However,

little is known about the transfer mechanisms or about the naturally occurring transfer elements involved. In a previous study we demonstrated the presence of transposons in bacterial strains isolated from aquatic ecosystems [20]. One of the transposon-harboring strains was able to mobilize derivatives of pBR325 and pBR328. This mobilizing strain, isolated from the Rhine river, was identified as *Klebsiella pneumoniae ozenae*. The present study identifies Tn*5403*, a Tn*3*-like element that provides a helper function in the mobilization of nonconjugative plasmids that are harbored by the indigenous strain.

Materials and Methods

Bacterial strains and plasmids. The Gram-negative bacterial strains and plasmids used are listed in Table 1. The strain of *Klebsiella pneumoniae ozenae* was isolated from the Rhine river shortly after it was contaminated with mercury at Basel in November 1986 [17, 18]. This strain has transfer-helper capabilities for pBR-type plasmids [20].

Plasmids MR212 and MR233 are derived from the nonconjugative plasmid RSF1010 [10]. Both of them harbor a tracer-sequence corresponding to a 400-bp fragment of the vaccinia virus thymidine kinase gene [28] and lack mobilization functions (*ori*T⁻, *mob*⁻). They confer antibiotic resistance to chloramphenicol (MR212) and kanamycin (MR233). The structures of MR212 and MR233 are shown in Figs. 1 and 2. MR101 is derived from RSF1010 with deletion of the *ori*T region between the nucleotides 3097 and 3220 [23]. Plasmids were introduced into host strains by CaCl₂ transformation [6] or electroporation [8].

Mating experiments. Bacteria harboring MR212 or MR233 or MR101 were used as donor strains in triparental mating experiments; the protocol has been previously described [20]. *E. coli* transconjugants were selected with Mueller-Hinton (MH) plates including appropriate antibiotics at the following concentrations:

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Table 1. Bacterial strains and plasmids used

Strain or plasmid	Characteristics[a]	Source or reference
Strain		
E. coli K12 UB281	pro met Nal[r]	[2]
E. coli K12 UB1832	his try lys lac rpoB Sm[r] Rif[r]	[2]
E. coli K12 UB1636	his try lys lac rpoB Sm[r]	[3]
P. putida mt-2 KT2440	hsdM[+] hdsR[-] Fa[r]	[1]
K. pneumoniae ozonae KIIIA	Hg[r]	[20]
Plasmid		
RSF1010	IncQ tra- mob+ oriT + Sm[r] Su[r]	[10]
MR101	RSF1010 ΔoriT Sm[r] Su[r]	this work
pSa	IncW tra+ mob+ oriT+ Kn[r] Cm[r] Su[r] Sm[r]	[27]
pKT231	RSF1010 derivative Kn[r]	[1]
MR212	RSF1010 ΔoriT Δmob Cm[r]	this work
MR233	RSF1010 ΔoriT Δmob Kn[r]	this work
p81.2	MR212::Tn5403	this work
p81.3	MR212::Tn5403	this work
p81.4	MR212::Tn5403	this work
p81.5	MR212::Tn5403	this work
p81.6	MR212::Tn5403	this work
p74.3	MR212::Tn5403	this work
pA328-1	pCE328::Tn5403	[20]
pB328-1	pCE328::Tn5403	[20]

[a] Phenotype abbreviations: resistance to nalidixic acid (Nal[r]), streptomycin (Sm[r]), rifampicin (Rif[r]), fusidic acid (Fa[r]), mercury chloride (Hg[r]), sulfonamide (Su[r]), kanamycin (Kn[r]), and chloramphenicol (Cm[r]).

nalidixic acid, 40 μg/ml; streptomycin, 100 μg/ml; rifampicin, 50 μg/ml; kanamycin, 25 μg/ml; and chloramphenicol, 25 μg/ml. The *P. putida* strain was obtained with selective succinate medium [12] containing 150 μg/ml kanamycin antibiotic. Transconjugants were tested for the presence of the tracer sequence by the colony-blot hybridization test [9].

DNA biochemistry. Plasmid DNA was isolated from *E. coli* strains by a clear lysate technique [24]. Large-scale preparations of plasmid DNA used the cesium chloride–ethidium bromide equilibrium centrifugation method of Clewell [5]. *P. putida* strain plasmid DNA was extracted by the rapid boiling method [15]. Very large plasmids were tested by the technique of Rosenberg [19]. Other standard DNA manipulations, including restriction endonuclease digestion, agarose gel electrophoresis, DNA fragment isolation, ligation, and Southern transfer to Hybond-N nylon filters (Amersham) were performed in accordance with manufacturer's instructions or as described by Sambrook et al. [21]. The Tn3 subgroup probe was derived from ColE1::Tn3 as a 1.3-kb SacI-AccI fragment internal to the tnpA gene. The Tn21 probe was a 1.2-kb KpnI-SphI fragment obtained by digestion of plasmid R100 [20]. A specific probe for Tn5403 was prepared from the 800-bp SmaI-SmaI fragment. The DNA was labeled by a random-priming, nonradioac-
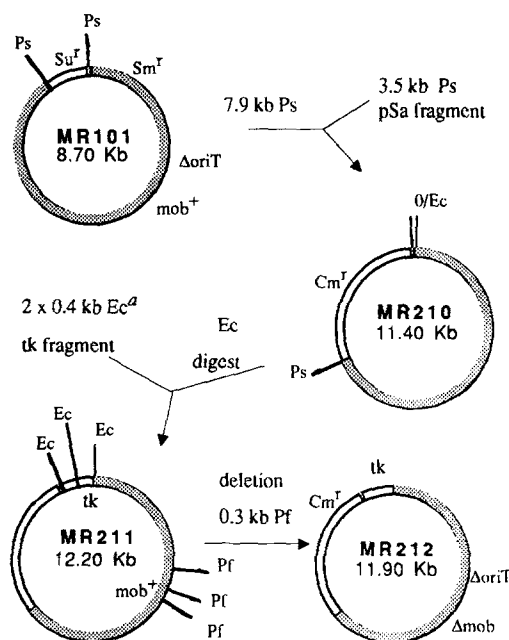

Fig. 1. Construction of the MR212 plasmid. [a]A tandem of the 0.4-kb fragment situated within the *tk* gene was inserted in the MR211 plasmid. Abbreviations: Ec, EcoRI; Pf, PflMI; Ps, PstI; oriT and mob, elements essential for mobilization.
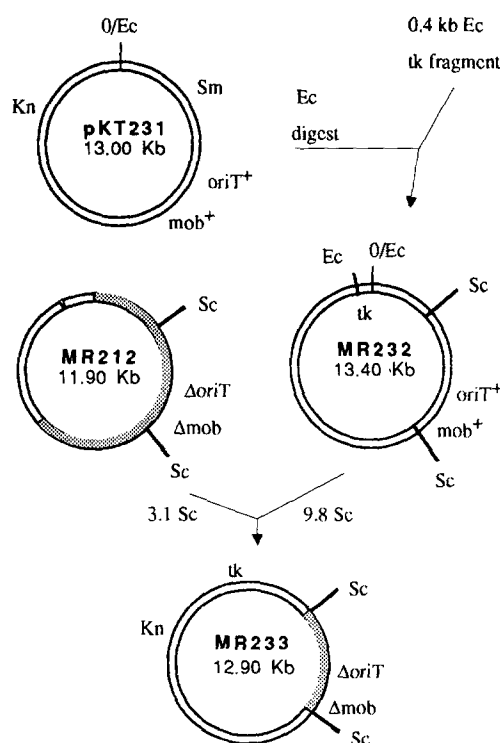

Fig. 2. Construction of the MR233 plasmid. Abbreviation: Ec, EcoRI; Sc, ScaI; oriT and mob, elements essential for mobilization.

tive technique based on dig-UTP incorporation (Boehringer-Mannheim "DNA labeling and nonradioactive detection" kit). The size of native plasmids was determined electrophoretically with a supercoiled DNA ladder (Gibco BRL) as reference.

DNA was sequenced on both strands by the dideoxy chain termination method [22] with the T7 sequencing kit (Pharmacia). Synthetic oligonucleotide primers and [^{35}S] dATP (NEN) were used. The extremities of Tn*5403*, at the junctions with the target plasmid MR212, were sequenced in the p81.6 plasmid with two synthetic oligonucleotide primers referring to the nucleotide sequence of RSF1010 [23]. One hybridized at positions 1840–1857, and the other hybridized at positions 3049–3033. The plasmid–transposon junction areas of the other MR212::Tn5403 derivatives and the pCE328::Tn5403 derivatives were sequenced with two primers homologous to a sequence situated about 100 nucleotides within the transposon and directed toward the borders: 5' GATC-GCACGGGAGCTCG 3' and 5' TGGGCGCACATTCTACT 3'. A computer-assisted homology search was performed by a system connected to the sequence data banks GenBank and EMBL.

Results

Mobilization of RSF1010 derivatives lacking *mob* and *ori*T functions.

The "helper" potential of the *Klebsiella* strain for mobilizing RSF1010-type plasmids was tested in triparental mating experiments with the *K. pneumoniae* environmental helper strain (H), a donor strain (D) harboring the *ori*T$^-$ *mob*$^-$ derivatives of RSF1010: MR212 or MR233 and a recipient strain (R). Inter-species transfer of the RSF1010 derivatives was investigated in mating experiments with *E. coli* and *P. putida* strains used as donor or as recipient cells (Table 2, exp. 1, 2, 3). No mobilization of RSF1010 derivatives was detected. Intra-species (*E. coli*-*E. coli*) transfer of the RSF1010 derivatives was detected (Table 2, exp. 4, 5, 6). The MR212 markers were transferred from a donor *E. coli* strain to a recipient *E. coli* strain in the presence of the environmental *Klebsiella* strain, suggesting that the RSF1010 derivatives were mobilized. One transconjugant (Tc74.3) was isolated in experiment 4, and five transconjugants (Tc81.2, Tc81.3, Tc81.4, Tc81.5, Tc81.6) were found in experiment 5. The control experiments without the helper strain produced no transconjugants of the MR212 or MR233 plasmids (not shown). The six transconjugants obtained in experiments 4 and 5 were used for further analysis.

Analysis of the transconjugants obtained by mobilizing pBR-type plasmids and RSF1010 derivatives.

The plasmid contents of the transconjugants TcA328-1 and TcB328-1 obtained by mobilizing a pBR328 derivative [20] and the transconjugants Tc74.3, Tc81.2, Tc81.3, Tc81.4, Tc81.5, Tc81.6 from RSF1010 derivatives were determined. Only one plasmid larger than the nonconjugative plasmid pCE328 or MR212 was detected. The plasmids harbored by the transconjugants were named pA328-1, pB328-1, p74.3, p81.2, p81.3, p81.4, p81.5, and p81.6 respectively. Comparison of their restriction profiles with those of the *ori*T-deleted parent plasmid indicated that all these

Table 2. Results of mating experiments

Experiment no.	Strains involved in mating[a]	CFU recipient[b]	CFU transconjugant[b]
1	D: UUB281[MR233] H: UKIIIA R: UKT2440	8.3 10^8	0
2	D: UUB1832[MR233] H: UKIIIA R: UKT2440	3.9 10^8	0
3	D: UKT2440[MR233] H: UKIIIA R: UUB1832	7.5 10^8	0
4	D: UUB281[MR212] H: UKIIIA R: UUB1636	7.5 10^8	1
5	D: UUB281[MR212] H: UKIIIA R: UUB1832	2.0 10^8	5
6[c]	D: UTc 74.3 R: UUB281	1.3 10^9	713
7	D: UUB1832 [p81.6] R: UUB281	1.9 10^9	0
8	D: UUB1832 [pA328-1] R: UUB281	2.1 10^9	0

[a] D, donor strain; H, helper strain; R, recipient strain.
[b] The number of CFU of transconjugants and CFU of recipient cells was measured at the end of the experiment.
[c] Results of the mating experiment performed at 30°C.

transconjugants plasmids contained the same 3.6-kb insert, designated Tn*5403*. However, the insertion point in the target plasmid and its orientation were different in each case. The restriction map of Tn*5403* shows that the insertion element contained single restriction sites for *Hind*III, *Hind*II, *Bst*EII, *Acc*I, and two *Sma*I sites. There were no restriction sites for *Eco*RI, *Sca*I, and *Pst*I (Fig. 3A).

The transconjugant Tc74.3 was used as donor strain for secondary mating experiments with *E. coli* UB281 as recipient strain at 30°C and at 37°C. Mobilization was detected only at 30°C with a frequency of 5 × 10^{-7} transconjugants per recipient cell (Table 2, exp. 6). Only one plasmid having the same restriction profile as p74.3 was detected in the new transconjugants. Plasmid p81.6 or pA328-1 was introduced into *E. coli* UB1832 strain by transformation. The transformed strains were used as donors in crosses with the *E. coli* strain UB281. No transconjugants were detected in any of these mating experiments (Table 2, exp. 7 and 8).

Identification of the insertion element.

The sequences of the extremities of Tn*5403* in p81.6 contain 38-bp perfect inverted repeated sequences (IRs) on each side of Tn*5403* (Fig. 3B). The sequence of the

A

B

Tn*5403* L-end: 5'-3' <u>GGGG</u>TCGGTTCCGGCTGAGGG<u>CGAAA</u>TGACACCC<u>TAAG</u>

Tn*5403* R-end: 5'-3' <u>GGGG</u>TCGGTTCCGGCTGAGGG<u>CGAAA</u>TGACACCC<u>TAAG</u>
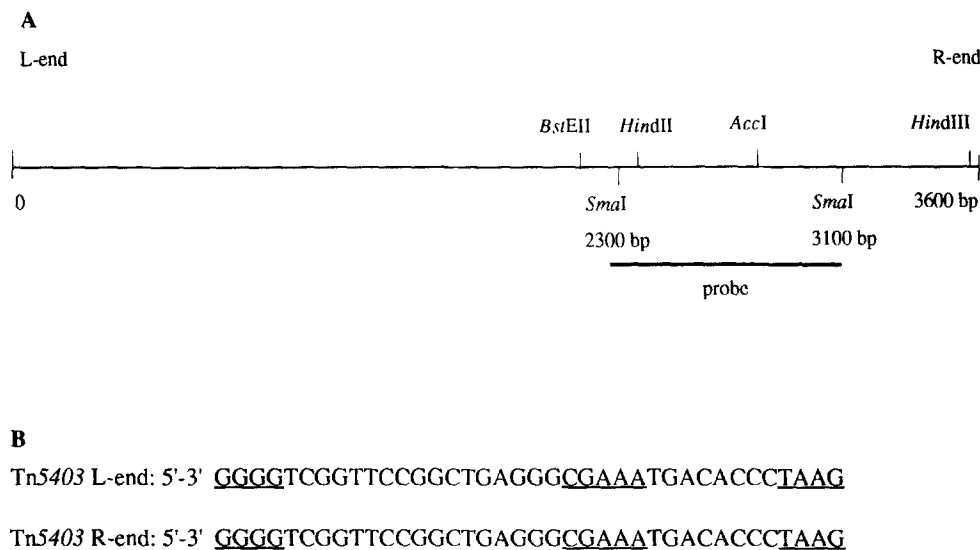
Fig. 3. Restriction map of Tn*5403* and sequence of the IRs. (A) Restriction map of the 3.6-kb insertion element. The left (L) and right (R) end were randomly chosen. (B) Sequence of the 38-bp Tn*5403* terminal inverted repeats. The underlined sequences correspond to the consensus motifs characteristic of the Tn*3* transposon family IRs.

two IRs was confirmed in the other transconjugants with two primers directed toward the borders of the transposon. The sequencing data confirmed the restriction enzyme analysis as to the orientation of Tn*5403* and clarified its exact positions in the target plasmids. Five bp of the target DNA were duplicated in each case.

The IRs of the new transposon Tn*5403* contained the same consensus sequence blocks specific to all the elements of the Tn*3* family: 5'GGGG-(N)$_{17}$-CGAAA-(N)$_8$-TAAG 3' [25]. But the rest of the sequence showed very little homology with the other elements of this group. The relationship of Tn*5403* with the two main subgroups of the Tn*3* family, subgroup Tn*3* and subgroup Tn*21* [25], was examined by hybridization with a Southern blot of the *Sma*I restriction digest of plasmids pA328-1, pB328-1, p74.3, p81.2, p81.3, p81.4, p81.5, and p81.6. The probes specific for the subgroups Tn*3* and Tn*21* [20] were used, but no hybridization was detected (results not shown).

Localization of Tn*5403* in the *K. pneumoniae* strain. The *Klebsiella pneumoniae* strain contained several plasmids (Fig. 4A). Tn*5403* was located by hybridizing *K. pneumoniae* plasmid DNA with the Tn*5403* *Sma*I-*Sma*I probe. There was a major reaction with a 9.5-kb plasmid and a faint reaction with a 20-kb plasmid (Fig. 4B).

Fig. 4. Location of the Tn*5403* element in the *K. pneumoniae* KIIIA strain. (A) Agarose gel electrophoresis of the total plasmid DNA from KIIIA. (B) Hybridization of the total plasmid DNA from KIIIA with the Tn*5403* *Sma*I-*Sma*I probe.

Discussion

The *K. pneumoniae* strain isolated from the river Rhine can, in addition to mobilizing derivatives of the narrow-host-range pBR-plasmid, mobilize deriva-
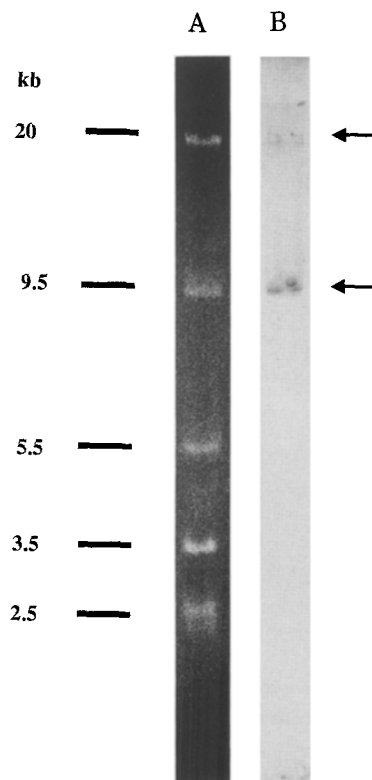
tives of the broad-host-range plasmid RSF1010. However, mobilization of these later plasmids was detected only in an *E. coli–E. coli* context. The lack of mobilization with *E. coli–Pseudomonas* system may be

because the mobilizing element must cross from *Klebsiella* to *E. coli* and then from *E. coli* to *Pseudomonas*, whereas in the first case it must cross from *Klebsiella* to *E. coli*. Since both *E. coli* and *K. pneumoniae* belong to the Enterobacteriaceae, this might favor mobilization by facilitating conjugation.

Analysis of the transconjugants obtained by mobilization of MR212 showed a single plasmid MR212::Tn*5403*. The presence of the 3.6-kb insert (Tn*5403*) suggests that transposon-mediated mobilization is involved [for review see 25]. The properties of Tn*5403* suggest that the new transposon is related to the Tn*3* family. These include insertion in different sites of the target plasmid, duplication of 5-bp of the target DNA at the insertion point, occurrence at each end of the element of perfect 38-bp inverted repeated sequences (IRs), the presence of the consensus sequence motifs characteristic of the Tn*3* family, and the temperature sensitivity of the mobilization process of MR212, which occurs more often at 30°C than at 37°C. The IRs of Tn*5403* show very little homology with those of any Tn*3*-like transposons identified to date, except for the disposition of the consensus motifs, which are identical with those found in all the Tn*3*-like elements. But the absence of hybridization with the Tn*3* and Tn*21* specific probes suggests that the new element does not belong to either of the two main subgroups of the Tn*3* family. Tn*5403* is, at 3.6 kb, one of the smallest element of this family. All these characteristics suggest that the new transposon is different from the previously identified Tn*3*-like elements.

The mobilization of an *ori*T-deleted nonconjugative plasmid via transposon-mediated fusion involves the formation of a cointegrate between the nonconjugative plasmid and a helper plasmid harboring conjugative functions (*tra*+) and the transfer of the fused plasmids by conjugation. The resolution of the transient cointegrate inside the transconjugant cells, after completion of the transfer, generates two plasmids, the original helper plasmid (*tra*+), still harboring a copy of the transposon, and the mobilized plasmid enlarged with a copy of the transposon. Our extraction of DNA by the "clear lysate" technique or the method of Rosenberg [19] led to the electrophoretic detection of only one extrachromosomal element, corresponding to the mobilized plasmid enlarged by a 3.6-kb insert. The apparent absence of a conjugative helper plasmid in the transconjugants is in contradiction with the fact that the progeny recovered from primary mating experiments could mobilize the MR212::Tn*5403* derivatives in secondary crosses. On the other hand, bacteria transformed with the plasmid extracted from the transconjugants (MR212::

Tn*5403* and pCE328::Tn*5403*) could not transfer their extrachromosomal DNA, showing that the techniques used for plasmid preparation did not extract the element harboring the *tra*+ functions. There may, therefore, be hidden *tra* genes in the transconjugants. These genes may be either in the chromosome or within a very large plasmid that was not detected by the techniques used. Analysis of the total genome of transconjugants by pulse-field electrophoresis should provide an answer to this question.

There are a few published reports on the mobilization of nonconjugative plasmids [10, 13, 14, 16]. Sorensen [26] demonstrated recently that pBR322-derivatives can be transferred from *E. coli* K12 to bacteria isolated from seawater in the presence of an F′ plasmid harboring a γδ transposable element, suggesting the involvement of transposons in plasmid mobilization to indigenous bacteria. Our previous studies [20] showed that transposons are present in bacteria isolated from polluted aquatic environments. We isolated a transposon-harboring strain displaying mobilization "helper" capabilities for nonconjugative plasmids. The present results demonstrate that the "helper" capabilities are actually related to the presence of a new transposable element, Tn*5403*, belonging to the Tn*3* family.

ACKNOWLEDGMENTS

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