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Derepressed Transfer Properties Leading to the Efficient Spread of the Plasmid Encoding Carbapenemase OXA-48

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The current emergence of the carbapenemase OXA-48 among *Enterobacteriaceae* is related to the spread of a single IncL/M-type plasmid, pOXA-48a. This plasmid harbors the bla_{OXA-48} gene within a composite transposon, Tn*1999*, which is inserted into the *tir* gene, encoding a transfer inhibition protein. We showed that the insertion of Tn*1999* into the *tir* gene was involved in a higher transfer frequency of plasmid pOXA-48a. This may likely be the key factor for the successful dissemination of this plasmid.

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Plasmids belonging to the IncL/M incompatibility group are commonly identified among environmental and clinical enterobacterial isolates (1–3). They have been increasingly identified as a source of multidrug resistance. Indeed, IncL/M-type plasmids are the main plasmids responsible for the dissemination of specific extended-spectrum β-lactamase genes such as the $bla_{CTX-M-3}$ gene and have also been shown to harbor carbapenemase genes such as the bla_{NDM-1} and bla_{OXA-48} genes (4–6). OXA-48 carbapenemase is an Ambler class D β-lactamase that was first identified in 2001 from a multidrug-resistant *Klebsiella pneumoniae* isolate from Turkey (7). Many sporadic cases have been reported, first in Turkey and then in many other European countries and Israel (8–13). OXA-48 is now considered an endemic carbapenemase in *Enterobacteriaceae*, at least in Turkey and in North African countries such as Morocco, Algeria, and Tunisia, and a source of outbreak situations in France, Belgium, Spain, and the Netherlands (14–20). The spread of OXA-48 producers and of KPC producers represents the most important source of multidrug resistance in Europe and the United States. The spread of the bla_{OXA-48} gene is linked mostly to the dissemination of the single 62-kb IncL/M-type plasmid pOXA-48a (20). Most of the OXA-48-positive *Enterobacteriaceae* harbor this specific plasmid, which is spreading among various enterobacterial species (20). Sequence analysis of plasmid pOXA-48a showed that the bla_{OXA-48} gene is bracketed by two copies of IS*1999*, giving rise to a composite transposon named Tn*1999* (8, 21). Tn*1999* is inserted into the *tir* gene, encoding a transfer inhibition protein, which is therefore not functional (6). Interestingly, a gene similar to this *tir* gene has been shown to inhibit plasmid RP4 conjugal transfer (22). Therefore, the aim of this study was to evaluate whether the disruption of the *tir* gene consecutive to the Tn*1999* insertion in pOXA-48a could play a role in the dissemination of this plasmid and, consequently, of the bla_{OXA-48} gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Clinical strains *K. pneumoniae* 11978 (bla_{OXA-48}) and *K. pneumoniae* 601 (bla_{NDM-1}) were previously described (7, 23). *Escherichia coli* TOP10(pOXA-48a) and *E. coli* TOP10(pNDM-OM), harboring the natural plasmids of *K. pneumoniae* 11978 and *K. pneumoniae* 601, respectively, were obtained by mating-out assays. Nalidixic acid-resistant *E. coli* JM109, nalidixic acid-resistant *Enterobacter cloacae* SB, and rifampin-resistant *E. coli* TOP10 were used for mating-out assays. The kanamycin-resistant plasmid pCRBluntII-TOPO (Invitrogen, Cergy-Pontoise, France) was used as a cloning vector. Bacterial strains were grown in Luria-Bertani (LB) broth at 37°C. When necessary, antibiotics were added at the following concentrations: ticarcillin at 50 $\mu g \cdot ml^{-1}$, temocillin at 50 $\mu g \cdot ml^{-1}$, kanamycin at 30 $\mu g \cdot ml^{-1}$, rifampin at 60 $\mu g \cdot ml^{-1}$, and nalidixic acid at 20 $\mu g \cdot ml^{-1}$.

PCR assays. Phusion DNA polymerase (Thermo Fisher Scientific, Villebon-sur-Yvette, France) was used for PCR experiments according to the manufacturer's instructions. PCR primers are indicated in Table 1. On plasmid pOXA-48a, the *tir* gene is truncated by transposon Tn*1999* harboring the bla_{OXA-48} gene (6). In order to construct a DNA fragment encompassing the entire *tir* gene, DNA fragments of 530 bp and 326 bp were amplified by PCR with primer pairs preTir-For/TirEnd-Rev and TirEnd-For/preTir-Rev, respectively (Table 1). Primer preTir-For was designed in order to replace the σ^{70} −35 promoter sequence (TCGGCA) of the *tir* gene by a −35 promoter sequence, TTGGCA, closer to the *E. coli* σ^{70} −35 promoter consensus sequence TTGACA in the corresponding amplicon (Fig. 1) (24). Primer TirEnd-For was designed as follows: the first 20 nucleotides of primer TirEnd-For were specific to the 3′ extremity of the first truncated fragment of *tir*, and the last 20 nucleotides were specific of the 5′ extremity of the second truncated fragment of *tir* (Fig. 1). Primer TirEnd-Rev was the reverse complement of primer TirEnd-For (Table 1). These DNA fragments were subsequently used as templates for a PCR experiment with primers preTir-For and preTir-Rev, giving rise to an 856-bp DNA fragment corresponding to the entire reconstructed sequence of the *tir* gene of plasmid pOXA-48a.

Cloning experiments and sequencing. The reconstructed *tir* gene was subsequently cloned into the vector pCRBluntII-TOPO and electroporated into rifampin-resistant *E. coli* TOP10, giving rise to *E. coli* TOP10(pTOPO-Tir). As a control, a noncoding sequence of 683 bp was cloned into the vector pCRBluntII-TOPO and electroporated into rifampin-resistant *E. coli* TOP10, giving rise to *E. coli* TOP10(pTOPO-Nc). Sequences of the inserts of these recombinant plasmids were confirmed by sequencing.

Qualitative filter matings. Plasmids pOXA-48a and pNDM-OM from *K. pneumoniae* 11978 and *K. pneumoniae* 601, respectively, were introduced into rifampin-resistant *E. coli* TOP10 by mixing 200 μl of

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)	PCR product size (bp)	Use(s)
preTir-For	GCTAGCTTGGCAATCATTTTCTGTATC	530	Amplification and cloning
TirEnd-Rev	GTTGTACCTCGAACGGAAGACGTTCAGCATGACACCACGG		
TirEnd-For	CCGTGGTGTCATGCTGAACGTCTTCCGTTCGAGGTACAAC	326	Amplification and cloning
preTir-Rev	GCTAGCGGTATGCATTTTCACCTCC		
Tir-GSP1	TCGTCATAAATGGCTCAGCG	327[a]	5′ RACE
Tir-GSP2	GGTCCAGCACTTTACCCAGC	303[a]	
Tir-GSP3	GAAATTCACCGACATACACC	282[a]	
RT-Tir-For	CTGCTCTATGGGCTGGG	273	RT-PCR
RT-Tir-Rev	GCAACGTCAGGATCACRTCG		
RT-TraM-For	GGATCAAACTGACTCGGATC	190	RT-PCR
RT-TraM-Rev	GAAGCCAGTAACCCTGAAG		
gapA-For	TATGACTGGTCCGTCTAAAGACAA	192	RT-PCR
gapA-Rev	GGTTTTCTGAGTAGCGGTAGTAGC		

[a] Distance to the ATG start codon.

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donor cells with 800 μl of recipient cells and filtering 200 μl of the mating mix onto the surface of a 0.45-μm-pore-size filter (Millipore, Molsheim, France). The filter was placed onto the surface of a Trypticase soy plate, which was then incubated at 37°C for approximately 3 h. Following incubation, the bacterial lawn on the surface of the filter was streaked onto selective medium containing ticarcillin and rifampin. Those experiments gave rise to rifampin-resistant *E. coli* TOP10(pOXA-48a) and *E. coli* TOP10(pNDM-OM). Plasmid pOXA-48a was introduced into rifampin-resistant *E. coli* TOP10(pTOPO-Tir) and rifampin-resistant *E. coli* TOP10(pTOPO-Nc) according to the same protocol, using ticarcillin, kanamycin, and rifampin for selection. As a result, *E. coli* TOP10(pOXA-48a, pTOPO-Tir) and *E. coli* TOP10(pOXA-48a, pTOPO-Nc) were obtained.

Quantitative filter matings. Donor and recipient cells were each grown overnight in LB broth supplemented with ticarcillin (50 $\mu g \cdot ml^{-1}$) for plasmid maintenance in donor cells. A 0.25-ml donor culture was mixed with 4.75 ml LB broth and incubated at 37°C for 5 h without shaking. Recipient cultures of *E. coli* JM109 or *E. cloacae* SB grown overnight were diluted 1:50 in LB broth and incubated at 37°C for 5 h with shaking. After incubation, 0.25 ml of the donor culture was gently mixed with 2.5 ml of the recipient culture, and 200 μl of this mating mix was filtered through a 0.45-μm filter (Millipore). Filters were incubated on prewarmed plates at 37°C for 2 h. Mating assays were ended by placement of filters into 4 ml of an ice-cold 0.9% NaCl solution, followed by vigorous agitation for 30 s. The number of transconjugants per donor cell was determined by plating dilutions of the mating mixture onto plates containing antibiotics. Strain donor cells were selected with ticarcillin (50 $\mu g \cdot ml^{-1}$) or temocillin (50 $\mu g \cdot ml^{-1}$) and rifampin (60 $\mu g \cdot ml^{-1}$). Transconjugants were selected with ticarcillin (50 $\mu g.ml^{-1}$) or temocillin (50 $\mu g \cdot ml^{-1}$) and nalidixic acid (20 $\mu g \cdot ml^{-1}$). Transfer frequencies were calculated by dividing the number of transconjugants by the number of donor cells. For the measurement of conjugation frequencies, the standard deviation was calculated from three independent cultures. Statistical analysis was performed by using the Student *t* test; a *P* value of ≤0.05 was considered significant.

Primer extension experiments. Total RNA was extracted from the *E. coli* TOP10(pOXA-48a) and *E. coli* TOP10(NDM-OM) strains with an RNeasy Midi kit (Qiagen, Courtaboeuf, France) by using RNAprotect (Qiagen) according to the recommendations of the manufacturer. RNA extracts were previously treated with DNase (Qiagen). The 5′ rapid amplification of cDNA ends (RACE) reactions were performed with 5 μg of total RNA of *E. coli* TOP10(pOXA-48a) and *E. coli* TOP10(pNDM-OM) and a 5′ RACE system kit (version 2.0; Invitrogen, Cergy-Pontoise, France), according to the recommendations of the manufacturer. The design of specific primers (Table 1) was performed as specified by the manufacturer for first-strand cDNA synthesis (primer Tir-GSP1), PCR of dC-tailed cDNA (primer Tir-GSP2), and nested amplification (primer Tir-GSP3) (Table 1). The 5′ RACE PCR products were then cloned into pCRBluntII-TOPO. Analysis of the cloned sequence allowed determination of the transcription initiation site and, subsequently, the promoter sequences.

Expression of the *tir* gene. Real-time reverse transcription-PCR (RT-PCR) was performed to measure mRNA expression levels of the *tir* and the *traM* genes from plasmids pOXA-48a and pNDM-OM. A one-step RT-PCR was performed by using a Rotor-Gene instrument (Qiagen) with the Rotor-Gene SYBR green RT-PCR kit (Qiagen), with 100 ng of total RNA of *E. coli* TOP10(pOXA-48a) and *E. coli* TOP10(pNDM-OM) and 1 μM primer in a total volume of 25 μl (Table 1). Relative transcripts levels

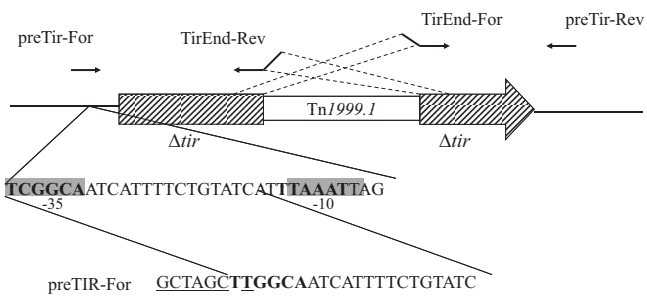

FIG 1 Design of primers for amplification of the *tir* gene. The −35 and −10 sequences of the promoter of the *tir* gene are indicated in boldface type and are shaded in gray. Nucleotides modified or added in primer preTIR-For are underlined. The orientation of the primers is indicated by arrows.

TABLE 2 Transfer frequencies in *E. coli* JM109 and *E. cloacae* SB[a]

Donor strain	Recipient strain	Mean transfer frequency ± SD
E. coli TOP10(pOXA-48a)	*E. coli* JM109	$1.1 \times 10^{-1} \pm 0.02$
E. coli TOP10(pNDM-OM)	*E. coli* JM109	$2.6 \times 10^{-3} \pm 0.016$
E. coli TOP10(pOXA-48a, pTOPO-Nc)	*E. coli* JM109	$1.7 \times 10^{-1} \pm 0.03$
E. coli TOP10(pOXA-48a, pTOPO-TIR)	*E. coli* JM109	$1.6 \times 10^{-3} \pm 0.0005$
E. coli TOP10(pOXA-48a, pTOPO-Nc)	*E. cloacae* SB	$4.9 \times 10^{-2} \pm 0.018$
E. coli TOP10(pOXA-48a, pTOPO-TIR)	*E. cloacae* SB	$1.2 \times 10^{-3} \pm 0.00004$

[a] In each case, three independent experiments were performed, and the means and standard deviations were calculated. Statistical analysis was performed by using the Student *t* test, and a *P* value of ≤0.05 of was considered significant.

FIG 2 Promoter structures for the *tir* gene in plasmids pOXA-48a (a) and pNDM-OM (b). The −35 and −10 sequences of the promoter are indicated in boldface type and are shaded in gray. The +1 transcription start sites are indicated in boldface type.

were calculated by using the $2^{-\Delta\Delta CT}$ method (25). Levels of *gap* gene (encoding D-glyceraldehyde-3-phosphate dehydrogenase) transcription were used as internal controls to normalize the data. At least three independent RNA samples isolated from three separate cultures were used to determine average transcript levels of each strain.

RESULTS AND DISCUSSION

Comparison of the transfer frequencies of plasmids pOXA-48a and pNDM-OM. Transfer frequencies of two IncL/M plasmids, pOXA-48a, carrying the bla_{OXA-48} gene, and pNDM-OM, carrying the bla_{NDM-1} gene, were compared. Results of these experiments are shown in Table 2. The transfer efficiency of pOXA-48a was about 40-fold higher than that of pNDM-OM. Noteworthy, those two plasmids exhibit different sizes (61,881 bp for pOXA-48a and 87,185 bp for pNDM-OM) and display significant heterogeneity in several genes of the *tra* locus (*traX*, *traY*, and *excA*) that might be involved in transfer efficiency differences. Nevertheless, we hypothesized that the differences observed in terms of transfer frequency might also be related to the disruption of the *tir* gene resulting from the insertion of transposon Tn*1999*, leading to a lack of production of the inhibition protein for pOXA-48a transfer.

Disruption of the *tir* gene and inhibition of the conjugative transfer of plasmid pOXA-48a. The transfer frequencies of pOXA-48a in the presence or absence of the Tir protein were therefore evaluated by performing mating-out assays with *E. coli* TOP10(pOXA-48a, pTOPO-Tir) or *E. coli* TOP10(pOXA-48a, pTOPO-Nc) as the donor, respectively, and using *E. coli* JM109 or *E. cloacae* SB as the recipient (Table 2). Each of these donor strains harbored two plasmids, both having the natural plasmid pOXA-48a together with a recombinant plasmid, either pTOPO-Tir, encoding the transfer inhibition protein TIR, or pTOPO-Nc, possessing a noncoding DNA sequence, respectively. Interestingly, *trans*-complementation with the TIR protein resulted in approximately 100- and 50-fold decreases of the efficiency of pOXA-48a transfer in *E. coli* and *E. cloacae*, respectively (Table 2). These results confirm the role of TIR in the inhibition of plasmid transfer.

Characterization of *tir* gene promoter sequences in pOXA-48a and pNDM-OM. Using the 5′ RACE technique, the +1 transcription initiation site and, subsequently, the promoter sequences of the *tir* gene were mapped for both pOXA-48a and pNDM-OM, respectively. In pOXA-48a, the +1 transcription site was located 53 bp upstream of the start codon of the *tir* gene. The promoter of the *tir* gene was made of a −35 box (TCGGCA) and a −10 box (TTAAAT) separated by 17 bp (Fig. 2). The promoter of the *tir* gene was made of a −35 box (TCGGTA) separated by 17 bp from the −10 box (TTAAAT) in pNDM-OM (Fig. 2). In comparison with the −35 box promoter sequences of *E. coli* TOP10 (pNDM-OM) (TCGGTA), the −35 box promoter sequences of *E. coli* TOP10(pOXA-48a) (TCGGCA) displayed a single mutation, leading to a −35 box closer to the consensus sequence (24), suggesting a putative stronger promoter and, consequently, stronger expression of the *tir* gene in *E. coli* TOP10(pOXA-48a).

Expression of *tir* genes in pOXA-48a and pNDM-OM. Quantitative RT-PCR was performed to measure the expression of the *tir* gene in *E. coli* TOP10(pOXA-48a) and in *E. coli* TOP10(pNDM-OM). The *tir* gene transcript levels were compared to those of the *gap* gene, which was taken as a chromosomal reference for gene expression, and to those of the *traM* gene, taken as a plasmid reference for gene expression for pOXA-48a and pNDM-OM. The *traM* genes of these two plasmids were very similar (only a single amino acid substitution in the corresponding

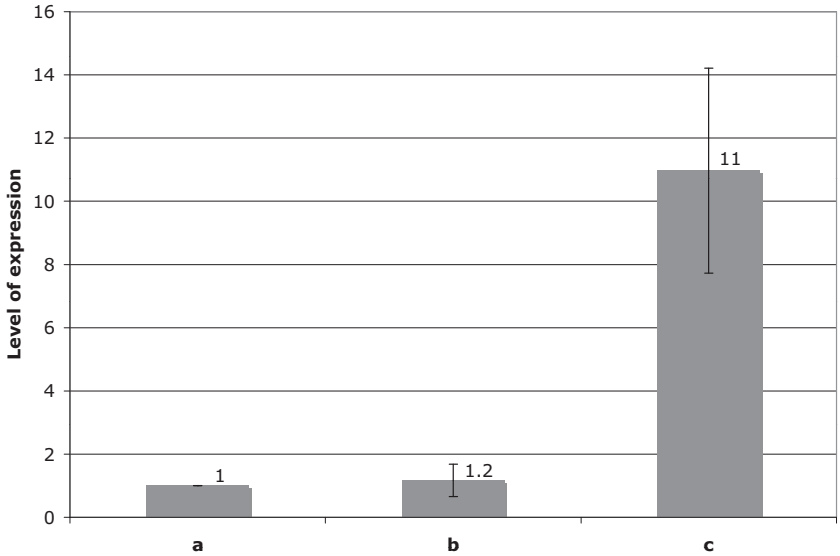

FIG 3 Mean relative expression ratio of the *traM* (b) and the *tir* genes (c) for *E. coli* TOP10(pOXA-48a) compared to *E. coli* TOP10(pNDM-OM). The expression level of the *gap* gene (a) in *E. coli* TOP10 was used as a reference with a value of 1.

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protein), whereas the promoter sequences of these genes were strictly identical. As expected, the transcription levels of the *traM* genes of pOXA-48a and pNDM-OM, sharing identical promoter sequences, were almost identical (Fig. 3). Transcriptional profile analysis indicated that the *tir* genes were significantly expressed in both pOXA-48a and pNDM-OM. Furthermore, an 11-fold-increased expression level of the *tir* gene was observed in *E. coli* TOP10(pOXA-48a) compared to *E. coli* TOP10(pNDM-OM), in accordance with a stronger −35 promoter sequence of the *tir* gene in pOXA-48a (Fig. 3). However, the TIR protein was not functional when using pOXA-48a, with its corresponding gene being disrupted by Tn*1999*. Analyzing the sequence of another IncL/M-type plasmid, namely, pEL60 from *Erwinia amylovora*, considered to possess a typical IncL/M backbone (neither resistance gene nor insertion sequence), the −35 promoter sequence of the *tir* gene was found to be distantly related to that consensus, ACGGTA (1). Therefore, it appears that the expression of the *tir* gene was maintained at a low level in order to avoid the inhibition of plasmid transfer and therefore to increase the transfer frequency. In the case of pOXA-48a, the promoter of the *tir* gene was stronger; however, the gene was truncated and could not encode a functional protein. Altogether, these elements may constitute a negative regulation system contributing to the dissemination of IncL/M plasmids, which are known to be efficient vectors for resistance genes.

The current spread of the $bla_{\text{OXA-48}}$ gene is largely the consequence of the dissemination of a single epidemic plasmid. In this study, we showed that the inactivation of the *tir* gene, encoding a transfer inhibition protein, by the insertion of Tn*1999* may contribute to the efficient transfer of plasmid pOXA-48a among various genetic backgrounds. However, other genes of the operon transfer, namely, *traX*, *traY*, and *excA*, are very specific to the pOXA-48a plasmid, and further experiments will be necessary to evaluate their role in the transfer of pOXA-48a.

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