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Worldwide Disseminated *armA* Aminoglycoside Resistance Methylase Gene Is Borne by Composite Transposon Tn*1548*

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The *armA* (*a*minoglycoside *r*esistance *m*ethylase) gene, which confers resistance to 4,6-disubstituted deoxystreptamines and fortimicin, was initially found in *Klebsiella pneumoniae* BM4536 on IncL/M plasmid pIP1204 of ca. 90 kb which also encodes the extended-spectrum β-lactamase CTX-M-3. Thirty-four enterobacteria from various countries that were likely to produce a CTX-M enzyme since they were more resistant to cefotaxime than to ceftazidime were studied. The *armA* gene was detected in 12 clinical isolates of *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli*, *K. pneumoniae*, *Salmonella enterica*, and *Shigella flexneri*, in which it was always associated with $bla_{CTX\text{-}M\text{-}3}$ on an IncL/M plasmid. Conjugation, analysis of DNA sequences, PCR mapping, and plasmid conduction experiments indicated that the *armA* gene was part of composite transposon Tn*1548* together with genes *ant3″9*, *sul1*, and *dfrXII*, which are responsible for resistance to streptomycin-spectinomycin, sulfonamides, and trimethoprim, respectively. The 16.6-kb genetic element was flanked by two copies of IS*6* and migrated by replicative transposition. This observation accounts for the presence of *armA* on self-transferable plasmids of various incompatibility groups and its worldwide dissemination. It thus appears that posttranscriptional modification of 16S rRNA confers high-level resistance to all the clinically available aminoglycosides except streptomycin in gram-negative human and animal pathogens.

Despite the development of new β-lactams and fluoroquinolones, aminoglycosides are still used for the treatment of severe infections due to gram-negative bacteria. Aminoglycosides act by causing translational errors and by inhibiting translocation (5). Their target sites include ribosomal domains in which the accuracy of the codon-anticodon is assessed (19, 26). In particular, they bind to a highly conserved motif of 16S rRNA, which leads to alterations in ribosome function (15, 28). Since their introduction into clinical use, bacterial resistance to aminoglycosides has been reported (21). There are four known mechanisms of resistance to these drugs in bacterial human pathogens: (i) decreased intracellular accumulation of the antibiotic by alteration of outer membrane permeability (9), diminished inner membrane transport (23), or active efflux (12, 17); (ii) enzymatic modification of the drug (21), primarily through N-acetylation, O-nucleotidylation, or O-phosphorylation, which is the most common mechanism; (iii) modification of the target by mutation in ribosomal proteins or in 16S rRNA (18); and (iv) trapping of the drug (13, 14). Microorganisms that produce aminoglycosides have developed an additional pathway to avoid suicide. This self-defense mechanism involves posttranscriptional methylation of rRNA by the use of *S*-adenosylmethionine as a cofactor (1).

The *armA* (*a*minoglycoside *r*esistance *m*ethyltransferase) gene, which confers resistance to 4,6-disubstituted deoxystreptamines (4,6-dds; kanamycin, amikacin, isepamicin, gentamicin, netilmicin, sisomicin, and tobramycin) and fortimicin, was initially found in *Klebsiella pneumoniae* BM4536 on IncL/M plasmid pIP1204 of ca. 90 kb which also encodes the extended-spectrum β-lactamase CTX-M-3 (7). The deduced amino acid sequence of *armA* displays from 37 to 47% similarity to 16S rRNA m^7G-methyltransferases from antibiotic producers (7).

The goal of this work was to study the extent and mechanism(s) of dissemination of the *armA* gene among members of the family *Enterobacteriaceae* that produce CTX-M enzymes and that were isolated from several countries. Conjugation experiments, sequence analysis of plasmid pCTX-M-3 from *Citrobacter freundii* (GenBank accession number AF550415), and PCR mapping of pIP1204 (7) indicated that *armA* is borne by a 16.6-kb element flanked by two direct copies of IS*6*. We therefore tested the transposability of this composite element, designated Tn*1548*.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Thirty-four enterobacteria from three hospitals in Paris, France, from two hospitals in Sofia, Bulgaria, and from our laboratory collection that were likely to produce a CTX-M enzyme since they were more resistant to cefotaxime than to ceftazidime were studied (Table 1). *Escherichia coli* BM694 (10), a spontaneous mutant of *E. coli* C1a (20) resistant to nalidixic acid, was used as the recipient for conjugation. The *E. coli* strains and plasmids used in transposition experiments are listed in Table 2. The strains were

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TABLE 1. Properties of the strains studied

Strain (no. of isolates)	Country of origin	Absence or presence of the following[a]:							
		$bla_{CTX-M-2}$	$bla_{CTX-M-3}$	$bla_{CTX-M-9}$	$bla_{CTX-M-14}$	$bla_{CTX-M-15}$	$bla_{CTX-M-28}$	*armA*	IncL/M
C. freundii									
2	Bulgaria	−	+	−	−	−	−	+	+
1	Poland	−	+	−	−	−	−	+	+
E. coli									
1	France	+	−	−	−	−	−	−	−
1	France	−	+	−	−	−	−	+	+
1	Bulgaria	−	+	−	−	−	−	+	+
1	France	−	−	+	−	−	−	−	−
2	France	−	−	−	+	−	−	−	−
8	Bulgaria	−	−	−	−	+	−	−	−
1	India	−	−	−	−	+	−	−	−
4	France	−	−	−	−	−	+	−	−
K. pneumoniae									
2	Bulgaria	−	+	−	−	−	−	+	+
2	France	−	+	−	−	−	−	+	+
1	Turkey	−	−	−	−	+	−	−	−
K. oxytoca (1)	Bulgaria	−	−	−	−	+	−	−	−
E. cloacae (1)	France	−	+	−	−	−	−	+	+
E. aerogenes (1)	Bulgaria	−	−	−	−	+	−	−	−
P. mirabilis (1)	France	−	+	−	−	−	−	−	−
S. enterica (1)	Bulgaria	−	+	−	−	−	−	+	+
S. flexneri (1)	Bulgaria	−	+	−	−	−	−	+	+
S. marcescens (1)	France	−	+	−	−	−	−	−	+

[a] − and +, absence and presence of the gene, respectively.

grown in brain heart infusion broth or on agar (Difco Laboratories, Detroit, Mich.) at 37°C.

DNA preparation and transfer. Isolation of total DNA and small- and large-scale preparation of plasmid DNA were performed as described previously (20). Restriction with endonucleases was performed according to the supplier's recommendations. Amplification of DNA was performed in a 2400 thermal cycler (Perkin-Elmer Cetus, Notwalk, Conn.) with *Taq* (Qbiogene, Inc., Carlsbad, Calif.) or *Pfu* (Stratagene, La Jolla, Calif.) DNA polymerase, as recommended by the manufacturers. PCR elongation times and temperatures were adjusted according to the expected size of the PCR product and according to the nucleotide sequence of the primers, respectively. Detection and identification of the genes in pIP1204 were done by PCR with primers described previously (7). Primers IncF and IncR were designed from the sequence of the replicon of pMU407.1 (GenBank accession number U27435), a naturally occurring conjugative plasmid of the IncL/M incompatibility group (Table 3). These primers allowed amplification of a 480-bp portion of the vegetative replication origin of pIP1204, which was 92% identical to that of pMU407.1. The target sites of Tn*1548* were determined by sequencing of pBR322::Tn*1548* junctions by using the primers described in Table 3. The amplification products were purified by using the QIAquick PCR purification kit (QIAGEN, Inc., Chatsworth, Calif.).

Mating experiments. Conjugation from clinical isolates to *E. coli* BM694 was performed with selection on nalidixic acid (40 μg/ml) and amikacin (20 μg/ml) or

TABLE 2. Strains and plasmids used in transposition experiments

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH1	F^- *supE44 recA1 endA1 gyrA96* (Nal^r) *thi-1 hsdR17* (r_K^- m_K^+) *relA1*	20
HB101	F^- *hsd20*(r_B^- m_B^-) *proA2 lacY1 recA13 rpsL20* (Str^r) *ara-14 galK2 xyl-5 mtl-1 supE44* λ^-	2
K802N	*hsdR hsdM*$^+$ *gal met sup rpoB nalA* (Nal^r)	25
Plasmids		
pOX38-Neo^r	F derivative Tra^+ Neo^r; 58.2 kb	3
pSU18-MluI	Tra^- Mob^- Cm^r; 2.3 kb	This study
pAT783	pSU18-MluIΩ22 kb MluI-AscI from pIP1204 Tra^- Mob^- Cm^r Sm^r Sul^r Tp^r 4,6-dds^r	This study
pBR322	Tra^- Mob^- Ap^r Tc^r; 4.4 kb	22
pIP1204	IncL/M Tra^+ Ap^r Cef^r Sm^r Su^r Tp^r 4,6-dds^r; ca. 90 kb	7

[a] Tra^+, self-transferable; Tra^-, non-self-transferable, Mob^-, nonmobilizable; Ap^r, ampicillin resistance; Cef^r, cefotaxime resistance; Cm^r, chloramphenicol resistance; Nal^r, nalidixic acid resistance; Neo^r, neomycin resistance; Sm^r, streptomycin-spectinomycin resistance; Str^r, streptomycin resistance; Su^r, sulfonamide resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance; 4,6-dds^r, 4,6-disubstituted deoxystreptamine resistance.

TABLE 3. Primers used for amplification and sequencing

Locus	Primer name	Sequence (5′→3′)	Position[a]	Source or reference
incL/M	IncF	CCCAAAGATGCAAAAAGAATG	270 to 290	This study
	IncR	AAAGACCTTCTACTGACTCAA	749 to 729	
bla_{TEM-1}	A216	ATAAAATTCTTGAAGACGAAA	−5 to 20	11
	A217	GACAGTTACCAATGCTTAATCA	1074 to 1053	
*tnp*IS*6*	IS6rev	ACAGAATGATGTCACGCTG	109 to 91	This study
	IS6for	TGCACTACGCAAAGGCCAG	681 to 699	

[a] The coordinates refer to the replicon of pMU407.1 (GenBank accession number U27345), the Sutcliffe numbering of the bla_{TEM} genes (22), and to the first base of IS*6* (16).

ampicillin (100 μg/ml). The transposition and cointegrate-forming properties of Tn*1548* were studied in a mating assay, as described previously (6, 24). In this system, the mobility of a transposable element carried by a nontransferable and nonmobilizable plasmid (pAT783) to a self-transferable plasmid (pOX38-Neo^r) was revealed in a standard mating assay between the *recA* strains *E. coli* HB101, used as the donor, and *E. coli* DH1, used as the recipient. In every mating, randomly selected transconjugants were purified and tested for antibiotic resistance to eliminate donor mutants and, when necessary, to discriminate between simple transposition or cointegrate formation.

Susceptibility testing. Antibiotic susceptibility was determined by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France), according to Comité de l'Antibiogramme de la Société Française de Microbiologie standards (4).

DNA sequence determination and analysis. Both strands of the DNA were sequenced by using synthetic primers and a CEQ 2000 DNA analysis system automatic sequencer (Beckman Coulter, Fullerton, Calif.). The nucleotide and deduced amino acid sequences were analyzed with the GCG sequence analysis software package (version 10.1; Genetics Computer Group, Madison, Wis.). BLAST program searches were performed by using the National Center for Biotechnology Information website (http://www.ncbi.nim.nlh.gov/).

RESULTS

Distribution of the *armA* gene in enterobacteria. Among the 34 clinical isolates of enterobacteria, collected in several countries, likely to produce a CTX-M enzyme since they were more resistant to cefotaxime than to ceftazidime (Table 1), the $bla_{CTX-M-3}$ gene was identified in 14 strains, the $bla_{CTX-M-2}$ gene was identified in 1 strain, the $bla_{CTX-M-9}$ gene was identified in 1 strain, the $bla_{CTX-M-14}$ gene was identified in 2 strains, the $bla_{CTX-M-15}$ gene was identified in 12 strains, and the $bla_{CTX-M-28}$ gene was identified in 4 strains. The *armA* gene was detected in 12 isolates, including *C. freundii* (3 of 3 strains), *Enterobacter cloacae* (1 of 1), *E. coli* (2 of 19), *K. pneumoniae* (4 of 5), *Salmonella enterica* serotype Enteritidis (1 of 1), and *Shigella flexneri* (1 of 1). The strains harboring the *armA* and the $bla_{CTX-M-3}$ genes, except for the two *Citrobacter freundii* strains from Bulgaria, were isolated in different hospitals. The $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, and $bla_{CTX-M-28}$ genes were not carried by IncL/M plasmids.

In two strains harboring $bla_{CTX-M-3}$, the *armA* gene could not be detected by PCR. In a *Proteus mirabilis* strain, no plasmid belonging to incompatibility group IncL/M was found. In a *Serratia marcescens* strain, the IncL/M origin of vegetative replication and the bla_{TEM-1} and $bla_{CTX-M-3}$ genes were detected by PCR and sequencing.

Conjugation experiments. Transfer of high-level aminoglycoside resistance from the 12 *armA*-containing *E. coli*, *K. pneumoniae*, *C. freundii*, *S. enterica*, and *S. flexneri* strains to *E. coli* BM694 was obtained with selection on nalidixic acid and amikacin. By disk diffusion, PCR with specific primers, and sequence analysis, the *E. coli* transconjugants were found to express resistance to 4,6-disubstituted deoxystreptamines because of *armA*, to β-lactams because of acquisition of bla_{TEM-1} and $bla_{CTX-M-3}$, to certain aminoglycosides because of *aac3*, to streptomycin-spectinomycin because of *ant3″9*, to sulfonamides because of *sul1*, and to trimethoprim because of *dfrXII*. All the resistance genes were always carried by an IncL/M plasmid of ca. 80 to 90 kb.

When the transfer from the same strains to *E. coli* BM694 was selected on nalidixic acid and ampicillin, for 11 of the 12 donors transfer of *armA*, bla_{TEM-1}, and $bla_{CTX-M-3}$ was associated with that of the *aac3*, *ant3″9*, *sul1*, and *dfrXII* genes, as tested by PCR with specific primers. The transconjugant from *S. enterica* serotype Enteritidis was resistant to β-lactams after the acquisition of bla_{TEM-1} and $bla_{CTX-M-3}$ and was resistant to certain aminoglycosides, such as gentamicin, kanamycin, netilmicin, and tobramycin, by the presence of the *aac3* gene; but it remained susceptible to amikacin, fortimicin, streptomycin-spectinomycin, sulfonamides, and trimethoprim, probably due to the loss of the *armA*, *ant3″9*, *sul1*, and *dfrXII* genes during conjugation.

These data, together with those drawn from sequence analysis of plasmid pCTX-M-3 from *C. freundii* (GenBank accession number AF550415) and PCR mapping of pIP1204 (7) and of the plasmids from the *S. enterica* serotype Enteritidis donor and from the corresponding transconjugant (data not shown), indicated that the *ant3″9*, *sul1*, *dfrXII*, and *armA* genes were part of a 16.6-kb element flanked by two direct copies of IS*6*. This composite element was designated Tn*1548*.

Transposition of Tn*1548*. Transposition of Tn*1548*, which conferred resistance to 4,6-disubstituted deoxystreptamines, fortimicin, streptomycin-spectinomycin, sulfonamides, and trimethoprim, was studied by plasmid conduction in *E. coli* by using pOX38-Neo^r. Plasmid pOX38 is a conjugative F derivative which does not carry any known insertion sequence except a small region of IS*3*. Plasmid pOX38-Neo^r was constructed by cloning the *aph3* gene, which confers neomycin resistance, into pOX38 (3). A ca. 22-kb MluI-AscI fragment from pIP1204 (7) encompassing the entire Tn*1548* putative transposon was cloned into pSU18-MluI, generating plasmid pAT783 (Tra$^-$ Mob$^-$ Cm^r 4,6-dds^r Sm^r Sul^r Tp^r; 24.3 kb), which was introduced by transformation into *E. coli* HB101 (*recA* Str^r) harboring pOX38-Neo^r (Tra$^+$ Neo^r) (Table 2). Transfer by mobilization of amikacin resistance (Ak^r) from the resulting strain, HB101(pOX38-Neo^r pAT783), into *E. coli* DH1 (*recA*

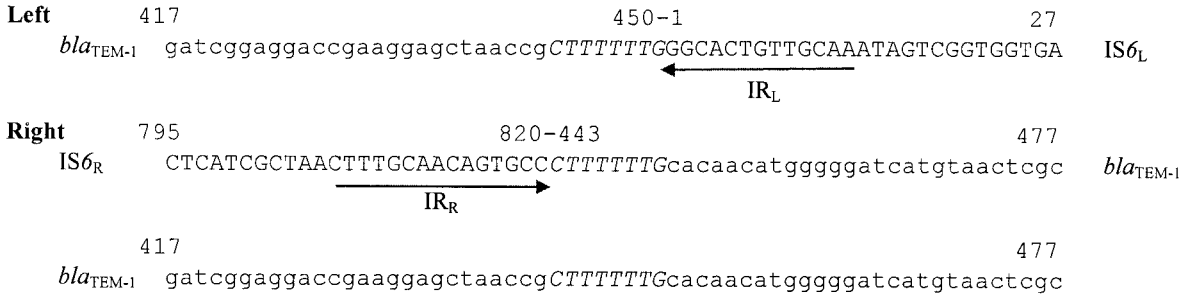

FIG. 1. Sequences of the left and right pBR322::Tn*1548* junctions and the bla_{TEM-1} target gene. The sequences of bla_{TEM-1} and IS*6* are indicated in lower- and uppercase letters, respectively. Horizontal arrows delineate the 14-bp inverted repeats (IR) of IS*6*, and the 8-bp duplicated motif at the insertion site is in italics. The coordinates refer to the first bases of bla_{TEM-1} and IS*6* (16).

Nal^r) was obtained at a frequency of ca. 10^{-4} per donor cell. A DH1 transconjugant was selected, and in mating experiments into HB101, it cotransferred the antibiotic resistance conferred by pAT783 and pOX38-Neo^r, suggesting the formation of a pOX38-Neo^r::pAT783 cointegrate.

The pOX38-Neo^r::pAT783 cointegrate was transferred by conjugation from HB101 into *rec*$^+$ *E. coli* K802N, and transconjugants were selected on plates containing amikacin plus nalidixic acid. The HB101(pOX38-Neo^r::pAT783) donors and the K802N(pOX38-Neo^r::pAT783) transconjugants were grown for approximately 100 generations in the absence of antibiotic and plated onto brain heart infusion agar, and the colonies were tested for their antibiotic susceptibilities by disk diffusion. All HB101 donors tested (200 colonies) remained resistant to amikacin, streptomycin-spectinomycin, sulfonamides, trimethoprim, neomycin, and chloramphenicol. By contrast, in the K802N recipient, the loss of resistance (i) to amikacin, streptomycin-spectinomycin, sulfonamides, and trimethoprim secondary to excision of Tn*1548*, (ii) to neomycin due to segregation of pOX38-Neo^r, and (iii) to chloramphenicol following the loss of pSU18-MluI was observed (in 2 of 200 colonies tested), indicating resolution of the pOX38-Neo^r::pAT783 cointegrate in a recombination-proficient host. The cointegrates generated by IS*6* were stable in a *recA* genetic background, since this element does not encode a site-specific resolvase (16, 24). However, in a *rec*-positive bacterium, homologous recombination can occur between two directly oriented copies of IS*6* that are generated during replicative transposition (24).

Target sites of Tn*1548*. Plasmid pBR322 (Tra$^-$ Mob$^-$ Ap^r Tc^r) was introduced by transformation into HB101 (pOX38-Neo^r::Tn*1548*). Transfer of resistance to amikacin and to ampicillin or tetracycline from HB101(pOX38-Neo^r::Tn*1548* pBR322) to *E. coli* K802N was obtained at a frequency of ca. 10^{-5} per donor cell. The transconjugants were tested for their antibiotic susceptibilities by disk diffusion. One transconjugant susceptible to ampicillin, in which integration of pOX38-Neo^r::Tn*1548* occurred in or in the regulatory region of the β-lactamase gene, was selected for sequencing of the junctions between pBR322 and Tn*1548*. Primer pairs consisting of primers A216 and IS6rev and primers IS6for and A217 (Table 3) were used to amplify and sequence the junction fragments. The ends of the insert in the bla_{TEM-1} gene of pBR322 corresponded to the terminal inverted repeats of Tn*1548*. Eight base pairs (CTTTTTTG) of bla_{TEM-1} duplicated in direct orientation were found at the borders of the transposon (Fig. 1).

DISCUSSION

We have studied the extent and the mechanism(s) of dissemination of the *armA* gene among enterobacteria from several countries. The *armA* gene is of clinical importance since it confers high-level resistance to all the clinically available aminoglycosides except streptomycin. The results indicate that *armA* was disseminated by a broad-host-range IncL/M conjugative plasmid. It was linked to bla_{TEM-1} and $bla_{CTX-M-3}$, which confer resistance to all β-lactams with the exception of carbapenems, and also to the *aac3*, *ant3″9*, *sul1*, and *dfrXII* genes. Aminoglycoside resistance due to *armA* has recently been reported in human clinical isolates of enterobacteria and *Acinetobacter baumannii*. Transfer of high-level aminoglycoside resistance to *E. coli* was obtained from 15 of 28 *armA*-containing *E. coli* and *K. pneumoniae* isolates from Taiwan, and cotransfer with $bla_{CTX-M-3}$ and bla_{TEM-1} was also observed (27). In addition, the *armA* gene has been shown to be transferable by conjugation from *E. coli* and *S. marcescens* clinical isolates from Japan to *E. coli* but not from *A. baumannii* to *E. coli* or *Acinetobacter calcoaceticus* (J. Wachino, K. Yamane, H. Kurokawa, S. Suzuki, N. Shibata, and Y. Arakawa, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-1889, 2004). However, in both studies the incompatibility group of the carrier plasmid was not determined, and in the latter work, cotransfer of another resistance gene(s) was not screened for. More recently, the presence of *armA* on a self-transferable IncN plasmid in an *E. coli* pig isolate from Spain has been reported (8).

We have demonstrated that *armA*, together with the *ant3″9*, *sul1*, and *dfrXII* genes, was part of functional transposon Tn*1548* in plasmid pIP1204. This 16.6-kb transposon is a typical composite element flanked by two copies of IS*6* in direct orientation. Tn*1548* transposed consecutively in *E. coli* by replicative transposition, which led to the formation of cointegrates and which generated a target duplication of 8 bp. The functionality of Tn*1548* under natural conditions was confirmed by its presence on plasmids of different incompatibility groups (7, 8).

Taken together, these data support the notion that the spread of *armA* results from both conjugation and transposi-

tion. These combinatorial genetics account for the documented worldwide dissemination of aminoglycoside resistance by 16S rRNA methylation in enterobacteria of human or animal origin and in *A. baumannii* (7, 8, 27; Wachino et al., 44th ICAAC).

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