JOURNAL OF BACTERIOLOGY, Jan. 2001, p. 235–249 Vol. 183, No. 1
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.1.235–249.2001

Characterization of In53, a Class 1 Plasmid- and Composite Transposon-Located Integron of *Escherichia coli* Which Carries an Unusual Array of Gene Cassettes

THIERRY NAAS,[1]* YUZURU MIKAMI,[2] TAMAE IMAI,[2] LAURENT POIREL,[1] AND PATRICE NORDMANN[1]

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique-Hôpitaux de Paris, Faculté de Médecine Paris-Sud, 94275 Le Kremlin-Bicêtre, France,[1] and Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1–8-1 Inotano, Chuo-Ku, Chiba 260-8673, Japan[2]

Received 12 June 2000/Accepted 12 October 2000

Further characterization of the genetic environment of the gene encoding the *Escherichia coli* extended-spectrum β-lactamase, bla_{VEB-1}, revealed the presence of a plasmid-located class 1 integron, In53, which carried eight functional resistance gene cassettes in addition to bla_{VEB-1}. While the *aadB* and the *arr-2* gene cassettes were identical to those previously described, the remaining cassettes were novel: (i) a novel nonenzymatic chloramphenicol resistance gene of the *cmlA* family, (ii) a *qac* allele encoding a member of the small multidrug resistance family of proteins, (iii) a cassette, *aacA1b*/*orfG*, which encodes a novel 6′-*N*-acetyltransferase, and (iv) a fused gene cassette, *oxa10*/*aadA1*, which is made of two cassettes previously described as single cassettes. In addition, *oxa10* and *aadA1* genes were expressed from their own promoter sequence present upstream of the *oxa10* cassette. *arr-2* coded for a protein that shared 54% amino acid identity with the rifampin ADP-ribosylating transferase encoded by the *arr-1* gene from *Mycobacterium smegmatis* DSM43756. While in *M. smegmatis*, the main inactivated compound was 23-ribosyl-rifampin, the inactivated antibiotic recovered from *E. coli* culture was 23-*O*-ADP-ribosyl-rifampin. The integrase gene of In53 was interrupted by an IS*26* insertion sequence, which was also present in the 3′ conserved segment. Thus, In53 is a truncated integron located on a composite transposon, named Tn*2000*, bounded by two IS*26* elements in opposite orientations. Target site duplication at both ends of the transposon indicated that the integron likely was inserted into the plasmid through a transpositional process. This is the first description of an integron located on a composite transposon.

Integrons are genetic elements that consist of an integrase gene with adjacent gene cassettes that commonly contain antibiotic resistance genes. Several classes of integrons have been established based on the structure of the integrase (56). The most commonly encountered integrons are those of class 1. They are characterized by a 5′ conserved segment (5′-CS), which contains the *int* gene, encoding the integrase which catalyzes site-specific recombination (12, 13), and in most cases a 3′ conserved segment (3′-CS), which carries *qacE*Δ*1,* a functional deletion derivative of the *qacE* gene, which specifies resistance to antiseptics and disinfectants, the *sul1* gene, which confers sulfonamide resistance, and an open reading frame (ORF), *orf5*, of unknown function (22, 48, 62). Integrons can integrate gene cassettes, by site-specific recombination, at a recombination site called *attI1* (23, 56). Gene cassettes are individual mobile units bounded by integrase recombination core sites and have conserved features at the 3′ ends of the cassettes with an inverse core site and a 59-base element (21, 61). The consensus core site sequence is GTTRRRY (R is a purine, and Y is a pyrimidine) (61). Integrons have been found in a variety of gram-negative species, including *Pseudomonas aeruginosa* (33, 38, 56). They are often part of transposons or plasmids (33, 56). Integron-located genes other than those conferring antibiotic resistance have been described, such as *qacE,* which encodes an exporter protein mediating resistance to antiseptics and disinfectants (31, 48, 50, 54). Cassettes are always integrated in the same orientation and are cotranscribed from one or two common promoters located in the 5′-CS (14, 32). However, the *qacE* and the *cmlA* gene cassettes carry their own promoter sequences (3, 50, 52, 60).

Rifampin is a valuable antibiotic for treating infections such as tuberculosis, staphylococcal infections, and some infections caused by gram-negative organisms (e.g., *Neisseria meningitidis* and *Acinetobacter* spp.). Its antimicrobial activity is mediated by inhibition of prokaryotic DNA-dependent RNA polymerases, and most rifampin-resistant *Mycobacterium tuberculosis* and *Mycobacterium leprae* strains have an alteration in the β-subunit of this enzyme (25, 47). However, in some resistant organisms, such as *Nocardia, Bacillus,* and *Pseudomonas* spp. and nontuberculosis species of mycobacteria, the resistance to rifampin is not usually due to mutations in the *rpoB* gene (68). Several other resistance mechanisms have been identified, including a rifampin efflux (11) and inactivation of rifampin by decomposition (16), glycosylation (63), phosphorylation (69), and ribosylation (15, 55). The first reported case of rifampin inactivation by ribosylation has been described for *M. smegmatis* DSM43756, a bacterial strain that is naturally resistant to rifampin (15, 55). In this strain, rifampin is modified first to ADP-ribosylated rifampin (RIP-TAs) and then to ribosylated

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre cedex, France. Phone: 33-1-45-21-36-24. Fax: 33-1-45-21-63-40. E-mail: thierry.naas@kb.u-psud.fr.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Strains		
E. coli DH10B	F′ *mcrA* Δ(*mrr-hsdRMS mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL endA1 nupG* Str^r	Life Technologies
E. coli MG-1	Extended-spectrum cephalosporin resistance, Rif^r Kan^r Tob^r Gen^r Amk^r Spt^r Str^r Sul^r Tet^r Cm^r	51
Plasmids[b]		
pPCR-script Amp (SK+)	Amp^r	Stratagene
pPCR-script Cm (SK+)	Cm^r	Stratagene
PBK-CMV	Neo^r Kan^r	Stratagene
pNLT1	Natural plasmid of *E. coli* MG-1 containing bla_{VEB1} ESBL, Rif^r Kan^r Tob^r Gen^r Amk^r Spt^r Str^r Sul^r Cm^r	51
pRLT2	pBKCMV recombinant plasmid containing an 11-kb *Sau*3A fragment with bla_{VEB-1} ESBL, Kan^r Tm^r Amk^r Net^r Cm^r Sul^r	This work
pRLT3	pBKCMv recombinant plasmid containing a 13-kb *Sau*3A fragment with bla_{VEB-1} ESBL, Kan^r Tm^r Amk^r Net^r Cm^r	This work
pRLT4a and b	pPCRscript recombinant plasmid containing a 1.3-kb PCR fragment with *qacI*; a, EtBr^r CTAB^r; b, Etbr^r CTAB^r	This work
pRLT5a and b	pPCRscript recombinant plasmid containing a 1.8-kb PCR fragment with *aacA1*/*orfG*; a, Kan^r Tm^r Amk^r Net^r; b, no resistance observed	This work
pRLT6a and b	pPCRscript recombinant plasmid containing a 2.7-kb PCR fragment with *arr-2*; a, Rif^r; b, no resistance observed	This work
pRLT7a and b	pPCRscript recombinant plasmid containing a 3.8-kb PCR fragment with *cmlA5*; a, Cm^r; b, Cm^r	This work
pRLT8a and b	pPCRscript recombinant plasmid containing a 3.2-kb PCR fragment with *oxa10*/*aadA1*; a, Amp^r Str^r Spt^r; b, Amp^r Str^r Spt^r	This work
pRLT9a and b	pPCRscript recombinant plasmid containing a 1.9-kb PCR fragment with the *oxa10*/*aadA1* fusion cassette; a, Amp^r Str^r Spt^r; b, Amp^r Str^r Spt^r	This work
pRLT10a and b	pPCRscript recombinant plasmid containing a 0.9-kb PCR fragment with the *oxa10* cassette; a, Amp^r; b, no resistance observed	This work

[a] Abbreviations: Kan, kanamycin; Tm, tobramycin; Net, netilmycin; Str, streptomycin; Spt, spectinomycin; Amk, amikacin; Cm, chloramphenicol; Tet, tetracycline; Sul, sulfonamide; Rif, rifampin; ESBL, extended-spectrum β-lactamase.

[b] For plasmids with "a" and "b" designations, "a" indicates recombinant plasmids where the inserted genes are in the same orientation as the *Plac* promoter of pPCRscript and "b" indicates recombinant plasmids where the inserted genes are in the orientation opposite that of the *Plac* promoter of pPCRscript.

rifampin (RIP-Mb) (28, 42). The gene responsible for the rifampin inactivation in *M. smegmatis*, *arr-1*, is chromosomally located. Recently, another chromosomal gene, *arr-2*, which is 54% identical to *arr-1,* was found in *P. aeruginosa*, where it conferred resistance to rifampin (67).

Escherichia coli MG-1, which was previously shown to be resistant to multiple antibiotics, was isolated from a clinical sample from a South Asian patient who had been hospitalized in France (51). Since the gene cassette, bla_{VEB-1}, encoding a novel extended-spectrum β-lactamase of clinical relevance was identified, we characterized the genetic environment of this gene in order to predict its potential for spreading. We report here a novel integron, In53, which is part of a composite transposon that is inserted on a large self-transferable plasmid. Detailed characterization of the nine gene cassettes of In53 is provided, as well as the determination of the physiological effect mediated by one of the integrated genes, *arr-2*.

MATERIALS AND METHODS

Enzymes and chemicals. T4 DNA ligase and restriction endonucleases were used according to the manufacturer's recommendations (Amersham Pharmacia Biotech, Orsay, France). Cetyltrimethylammonium bromide (CTAB), ethidium bromide (EtBr), chloramphenicol, rifampin, and kanamycin were from Sigma (Sigma, St. Quentin Fallavier, France). *Taq* DNA polymerase was from Perkin Elmer (Perkin Elmer, Les Ullis, France). Antibiotic disks were used for routine antibiograms (Sanofi-Diagnostics Pasteur, Marnes-la-Coquette, France). The antimicrobial agents and their sources have been described elsewhere (51).

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in the study are listed in Table 1. Cells were grown at 37°C under aerobic conditions in Trypticase soy (TS) broth (Gibco-BRL-Life Technologies, Eragny, France) containing the appropriate antibiotic or on Mueller-Hinton (MH) agar plates (Sanofi-Diagnostics Pasteur). Antibiotic concentrations for selection were as follows: ampicillin, 100 μg/ml; chloramphenicol, 15 μg/ml, kanamycin, 50 μg/ml; and rifampin, 200 μg/ml. Antibiotic susceptibility was determined by disk diffusion on MH agar (51). The method of Steers et al. (59) was used to determine the MICs. For each strain, 10^4 CFU per spot were delivered onto MH plates containing antibiotics in twofold dilutions. The MIC was determined as the lowest concentration of an antibiotic at which no visible growth was observed after 20 h of incubation at 37°C. Induction of chloramphenicol resistance by growth in the presence of 1 μg of chloramphenicol per ml was performed as described previously (17).

DNA techniques. Electrotransformation of *E. coli* DH10B was performed as described previously (43). Whole-cell DNA preparation, small- and large-scale plasmid DNA preparations, and agarose electrophoresis were also done as described previously (43, 58).

Standard PCR experiments were performed as described previously (29, 58). For each PCR amplification experiment, 500 ng of total DNA of *E. coli* MG-1 was used in a standard PCR mixture of 100 μl with the following amplification program: 10 min, 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C; and a final extension of 10 min at 72°C. The sequences of the PCR primers are available upon request. PCRs were performed in a DNA thermal cycler 9600 (Perkin-Elmer).

Cloning, DNA sequencing, and sequence analysis. *Sau*3AI fragments of *E. coli* MG-1 were size selected and cloned into the pBKCMV vector by selecting *E. coli* DH10B transformants on TS plates supplemented with 100 μg of ampicillin (51). Sequencing of the inserts on both strands was performed using laboratory-designed primers on an Applied Biosystem sequencer (ABI 377; PE-Biosystem, Les Ullis, France). Nucleotide and amino acid sequences were analyzed by using the software available online over the Internet at Pedro's Biomolecular Research

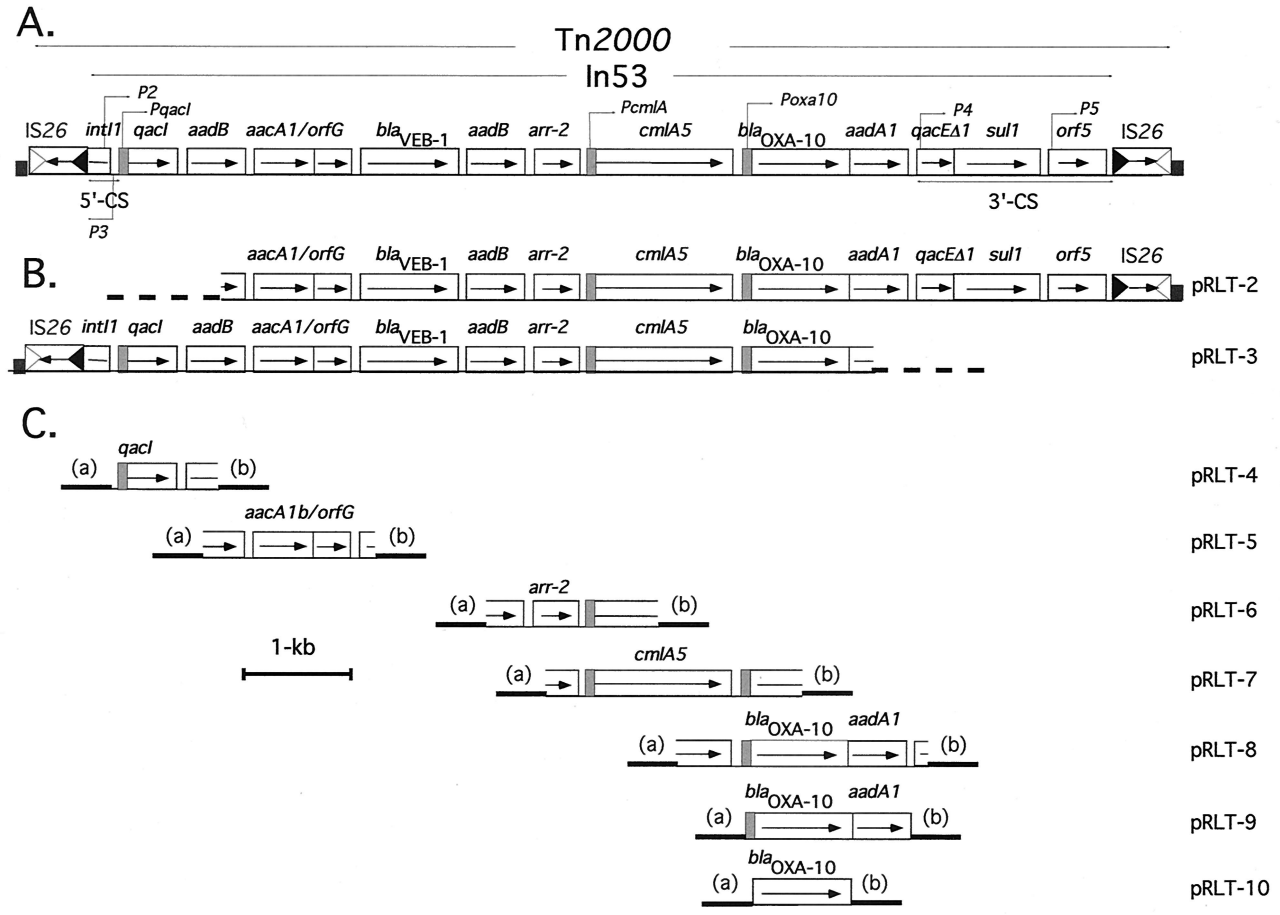

FIG. 1. (A) Schematic representation of Tn*2000* that contained In53 from *E. coli* MG-1. The 5′ and the 3′-CSs are indicated by a double arrow. ORFs or genes are shown as boxes with an arrow indicating the orientation of the coding sequence and with the gene name above the corresponding box. The different promoter sequences, P_2, P_3, P_4, P_5, P_{qacI}, P_{cmlA}, and P_{oxa10} are represented with a gray box and with an arrow. The hatched boxes at each end of the transposon represent the target site duplication. IS*26*-related inverted right and left repeats are shown by empty and filled triangles, respectively. The coding orientation of the IS*26* transposase gene is represented by an arrow. The Tn*2000* and the In53 structures are indicated between two divergent arrows. (B) Schematic representation of pRLT-2 and pRLT-3, two plasmids carrying large DNA inserts from *E. coli* MG-1 cloned into pBKCMV. The thick dotted line represents the pBKCMV vector sequence. (C) Schematic representation of pRLT-4 to -10, which correspond to plasmids constructed from various PCR products cloned into pPCRscript vectors (either Amp^r or Cm^r). Each construct exists in both orientations (a and b) with respect to P_{lac}, the plasmid-located promoter.

Tools website (http://www.fmi.ch/biology/research_tools.html) and the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment of deduced peptide sequences was carried out over the Internet at the University of Cambridge website using ClustalW (http://www.ebi.ac.uk/clustalW/). Promoter prediction by neural network was carried out over the Internet at http://www.fruitfly.org/seq_tools/promoter.html.

Parts of the identified class 1 integron were PCR amplified, and the DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France) and subsequently cloned in both orientations into the pPCRscript vector (Amp^r or Cm^r) (Stratagene, Amsterdam, The Netherlands). The inserts of these resulting recombinant plasmids were resequenced.

Biochemical characterization of ARR-2 and structural analysis of the inactivated product. *E. coli* DH10B harboring recombinant plasmid pRLT-6a was grown in 200 ml of TS broth with 100 μg of ampicillin per ml on a Brunswick rotary shaker for 18 h at 37°C. Cells were collected by centrifugation and resuspended in 5 ml of extraction buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5 mM dithiothreitol). The cell suspension was sonicated three times on ice for 30 s at 40 W (Bioruptor; Cosmo Bio, Tokyo, Japan). To 4 ml of this cell homogenate, 4 ml of reaction mixture containing 60 mg of rifampin and 100 mg of NADH was added. After incubation at 37°C for 3 h, the reaction mixture was centrifuged and the supernatant was freeze-dried. Dried samples were extracted with 8 ml of methanol, and the extract was chromatographed on an LH-20 Sephadex column (28 by 290 mm; Amersham Pharmacia Biotech) with methanol.

The molecular weight and molecular formula of RIP-TAs were determined by positive- and negative-ion fast atom bombardment mass spectrometry (FAB-MS) and high-resolution FAB-MS (HRFAB-MS) on a JEOL JMS-HX110 instrument (JEOL, Tokyo, Japan) (42). The inactivated antibiotic compounds were analyzed by reverse-phase thin-layer chromatography (KC18F; J. T. Baker, Inc., Tokyo, Japan) with a development solvent system of 0.2 M sodium chloride–dimethyl sulfoxide–acetonitrile (4:1.5:4). Analysis of the inactivated compounds was also done by reverse-phase high-pressure liquid chromatography on a LiChrosphere 100 RP-18(e) column (Cica-Merck, Tokyo, Japan; column dimensions, 4.6 by 150 mm; eluent, 38% acetonitrile [CH_3CN] with 0.05% trifluoroacetic acid) at a flow rate of 1 ml per min, with a detection system set at UV 270 nm (28, 42).

Nucleotide sequence accession number. The nucleotide sequence of the entire In53 integron along with flanking sequence has been deposited in the GenBank database under the accession no. AF205943.

RESULTS AND DISCUSSION

Antibiotic resistance of *E. coli* MG-1 is of plasmidic origin. In addition to having a previously described extended-spectrum resistance to β-lactams, *E. coli* MG-1 was resistant to tetracycline, netilmicin, tobramycin, amikacin, gentamicin, CTAB, EtBr, sulfonamide, streptomycin, spectinomycin, rifampin, and

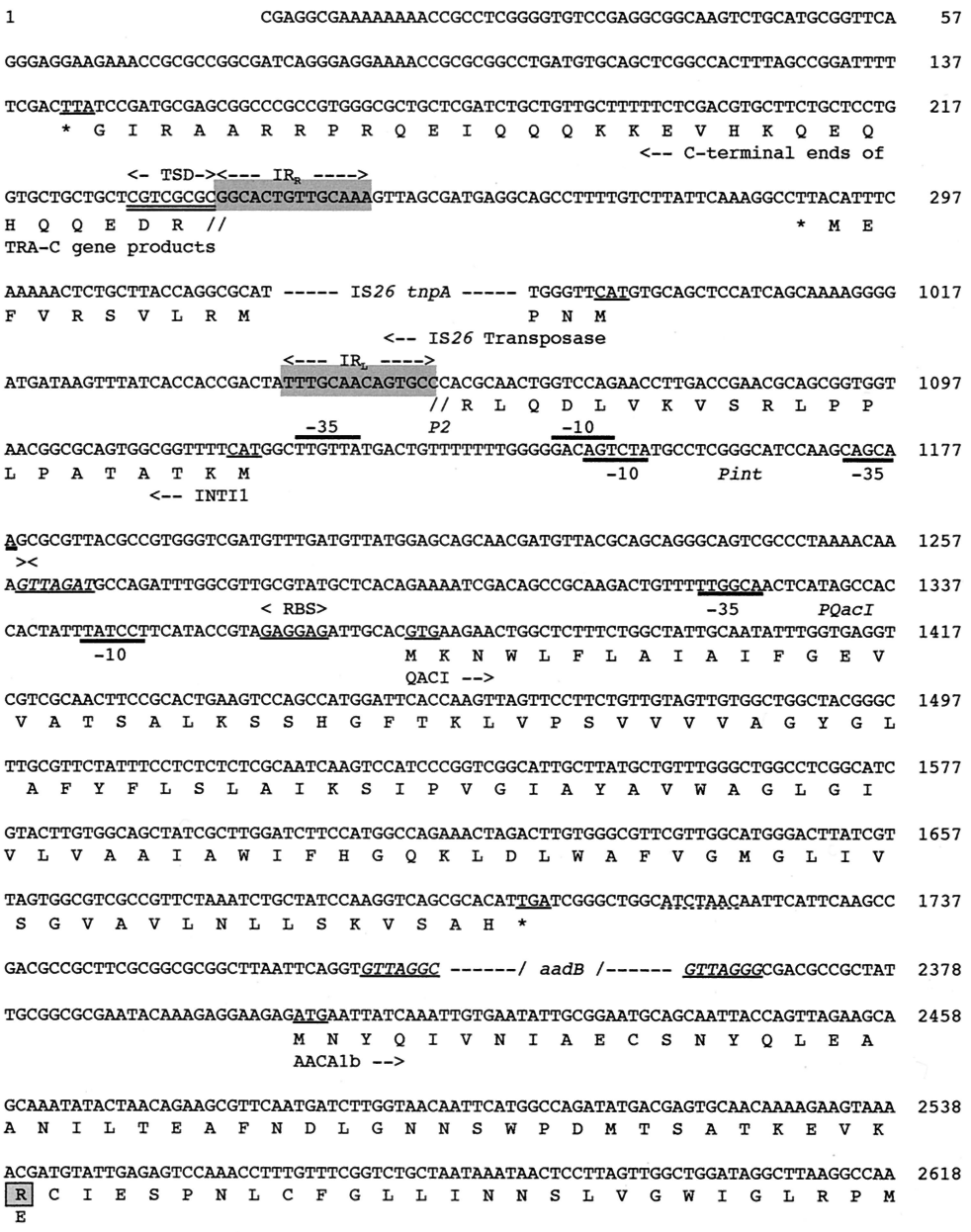

FIG. 2. Nucleotide sequence of a 12,374-bp fragment of In53 surrounded by the two insertion sequences, IS*26*, part of *intI1*, *attI1*, and some flanking sequences. The deduced amino acid sequence is designated in single-letter code below the nucleotide sequence. Slashes flanked by dashes indicate break points within the cassette sequences, while dashes alone represent sequence breakpoints within coding sequences. Facing arrows indicate gene cassette boundaries. The start and stop codons of the various genes of interest are underlined. Gene names followed by arrows indicating their translational orientation are below their initiation codons. Right and left IS-related inverted repeats (IR_R and IR_L) are shown by gray boxes and by two divergent arrows with appropriate labels. IS-related target site duplications (TSD) are double underlined. The various *oxa10*, *aadA1*, and *oxa10*/*aadA1* fusion cassette core and inverse core sites are indicated with boxes and dashed boxes, respectively. C, core site; IC, inverse core site; RBS, ribosome-binding site. The computer-predicted promoter $P_{oxa10(1)}$, $P_{oxa10(2)}$, P_2, and P_{int} sequences are represented, with $P_{oxa10(1)}$ and P_2 indicated by thick overlining and $P_{oxa10(2)}$ and P_{int} indicated by thick underlining. The two divergent arrows above underlined sequences represent a symmetry element generally encountered in 59-base elements. Within the amino acid sequence of AACA1b, the modified positions with respect to AACA1 are boxed, and the amino acid found in AACA1 (64) is indicated below these positions.

chloramphenicol. Except for tetracycline, these resistance markers were transferred from *E. coli* MG-1 to *E. coli* JM109 at a frequency of 10^{-8}, similar to that of the extended-spectrum β-lactamase gene bla_{VEB-1} (data not shown). The transconjugants were resistant to netilmicin, tobramycin, amikacin, gentamicin, CTAB, EtBr, streptomycin, spectinomycin, rifampin, and sulfonamides. They were also resistant, albeit at a lower level, to chloramphenicol (Table 2). DNA analysis of the transconjugants revealed the presence of a large plasmid with an estimated size of 160 kb that was able to confer the extended-spectrum resistance profile along with the other resistance markers. This plasmid was previously named pNLT-1 (51). The

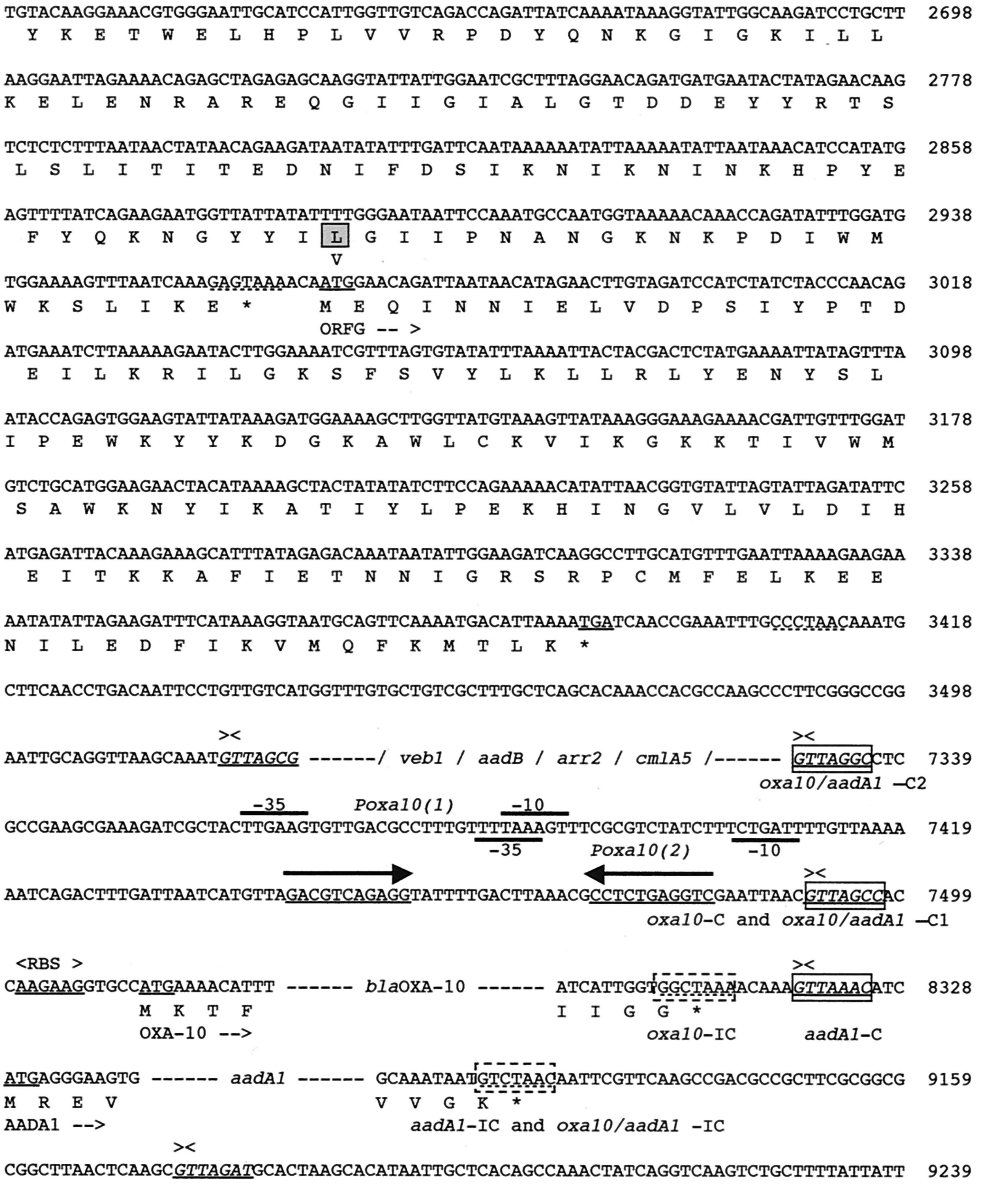

FIG. 2—*Continued*.

resistance determinant of tetracycline was harbored on a second plasmid along with a bla_{TEM-1} resistance gene, encoding a narrow-spectrum penicillinase.

The bla_{VEB-1} gene cassette of pNLT-1 is inserted into a class 1 integron. In order to determine the genetic environment of bla_{VEB-1}, whole-cell DNA from *E. coli* MG-1 was partially digested with *Sau*3AI and cloned into pBKCMV. Only recombinant clones with large inserts were selected upon a first screening. Two of the largest recombinant plasmids conferring ampicillin resistance, pRLT-2 and pRLT-3 (Fig. 1A and B), containing, respectively, an 11-kb and a 13-kb insert, were retained for further study. The sequences of the inserts present in pRLT-2 and pRLT-3 were determined, and their genetic organization is shown in Fig. 1A and B. Besides the classical ORFs encountered in class 1 integrons, 10 additional ORFs were present. At the 5′ and 3′ ends of the insert, the sequences were identical to parts of the 5′- and 3′-CSs of class 1 integrons (4).

The 5′-CS was truncated, and only the first 200 bp of the class 1 integrase gene was present, resulting in a nonfunctional integrase (4). Therefore, the cassettes are likely unable to move by means of this integrase. While the cassette promoter P_2 located in the integrase gene was still present, the promoter P_1 was deleted. The expression of the inserted genes was likely driven by this P_2 promoter (−35, TTGTTA, and −10, CAC AGT), which was in its strong promoter configuration; this configuration arose by means of a three-guanosine insertion between the −35 and −10 boxes, bringing its spacing to an optimal 17 bp (14, 32) (Fig. 2). However, this −10 box differs from the known −10 boxes of P_2 promoters by a T-to-C replacement at the first position (14, 32). This position is the most conserved position in the −10 boxes of prokaryotic pro-

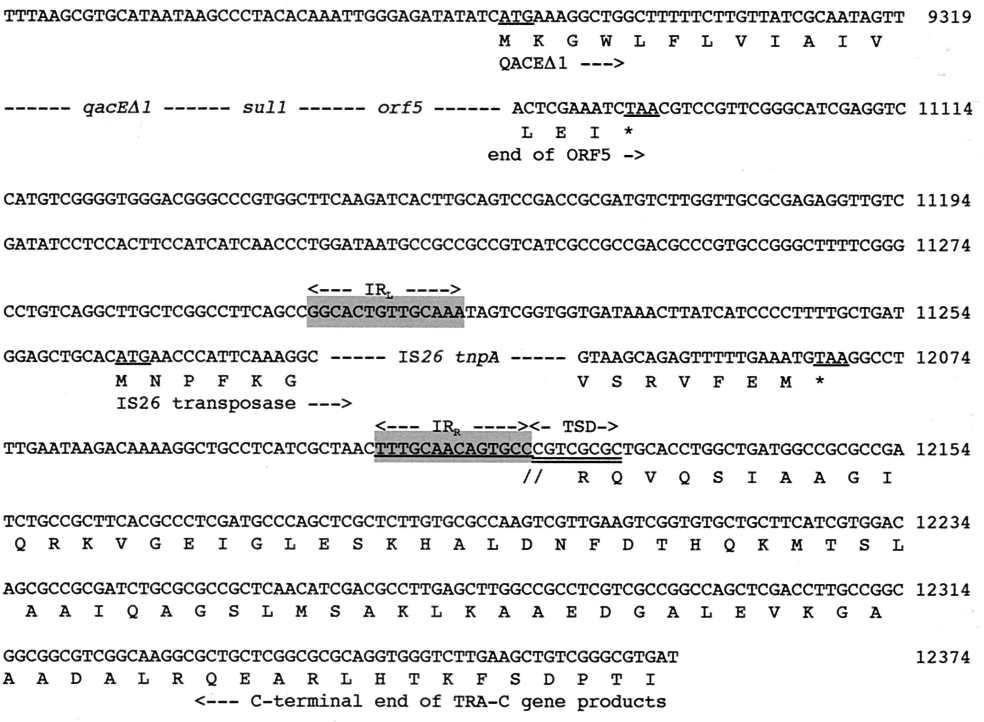

FIG. 2—*Continued*.

moter sequences (14, 32). Sequence analysis of the DNA sequence further upstream of the truncated integrase gene revealed the presence of an insertion sequence, IS*26* (41) (Fig. 1 and 2). Although a −35 sequence in the inverted repeat of IS*26* can form a promoter when juxtaposed to a −10 sequence (37, 43), this is not the case in In53. Therefore, it is likely that the cassettes are primarily expressed from the P_2 promoter.

The 3′-CS consisted of *qacEΔ1* fused to the *sul1* gene and of an entire *orf5* typical of *sul1*-associated integrons (22). The 3′-CS contained the same sequence as that found in the 3′-CS of In5 (22) except that the 3′-CS of In53 is 2,126 bp long (versus 2,386 bp in In5, which is the longest known 3′-CS [22]) and merged directly into an IS*26* insertion sequence. The *sul1* gene present in the 3′-CS of In53 is functional since *E. coli* DH10B(pRLT-2) exhibited decreased susceptibility to sulfonamides (Table 2).

These data indicate that the insert in plasmid pNLT-1 contains a truncated class 1 integron, designated In53, with an unusually large variable region and a sequence encoding an integrase that is not functional. PCR using integrase-specific primers failed to detect any intact type 1, type 2, or even type 3 integrase gene present in *E. coli* MG-1 (data not shown), suggesting that In53 is an inactive integron with respect to cassette movement, at least in *E. coli* MG-1.

The gene cassettes of In53. The 5′- and 3′-CS flanked 10 ORFs. Gene cassettes are individual mobile units bounded by integrase recombination core sites and have conserved features at the 3′ ends of the cassettes with an inverse core site followed by a 59-base element (21, 61). The 59-base element is an imperfect inverted repeat sequence which acts as a recombination site. The recombination crossover occurs after the first guanine of the conserved GTTRRRY (R = purine, Y = pyrimidine) core site, located at the end of the 59-base element (21, 60). At the 3′ end of most ORFs, structures homologous to the 59-base element were present, suggesting that most ORFs were part of gene cassettes (13, 60). Two ORFs lacked a 59-base element sequence, suggesting that they may belong to fused gene cassettes.

(i) The *aadB* cassettes. In53 contained two *aadB* cassette versions that differ only by one base pair, which remains silent

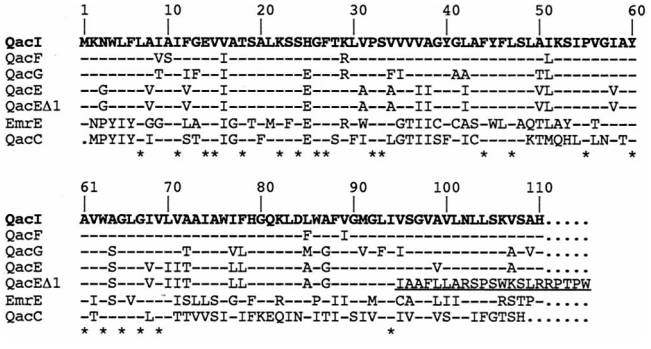

FIG. 3. Comparison of the deduced amino acid sequence of the *qacI* product (QacI) with that of other proteins of the small multidrug resistance family (49). QacF is encoded by a gene cassette from In40 of *Enterobacter aerogenes* BM2688 (50); QacG is encoded by the gene cassette from In31 of *P. aeruginosa* 101/1477 (31); QacE is encoded by the gene cassette from In16 (48); QacEΔ1 is a QacE derivative encoded by the truncated *qacEΔ1* allele found in the 3′-CS of several *sulI*-associated integrons (48, 62). EmrE is an ethidium efflux protein from *E. coli* (53); QacC is a protein from *Staphylococcus aureus* (34). Identical residues are indicated by dashes, and conserved residues are shown by asterisks. The underlined residues from QacEΔ1 indicate the deleted portion of the protein compared to QacE.

TABLE 2. In vitro susceptibility to various antimicrobial agents of *E. coli* MG-1, *E. coli* DH10B, *E. coli* DH10B harboring natural plasmid pNLT1 and of *E. coli* DH10B harboring recombinant plasmids that display some of the resistance markers of In53

Antibiotic	MIC (μg/ml) for[a]:																		
	E. coli MG-1[b]	*E. coli* DH10B																	
		pNLT1[c]	pRLT2[d]	pRLT3[d]	pRLT4[e]		pRLT5[e]		pRLT6[e]		pRLT7[f]		pRLT8[e]		pRLT9[e]		pRLT10[e]		*E. coli* DH10B
					a	b	a	b	a	b	a	b	a	b	a	b	a	b	
Imipenem	0.25	0.25	0.25	0.25	—	—	—	—	—	—	—	—	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Ceftazidime	256	256	>256	>256	—	—	—	—	—	—	—	—	4	4	4	4	4	0.25	0.25
Amoxicillin	>512	>512	>512	>512	—	—	—	—	—	—	—[g]	—[g]	>512	>512	>512	>512	>512	2	2
Cephalothin	>256	128	128	128	—	—	—	—	—	—	—	—	256	256	256	256	256	2	2
Streptomycin	>400	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]	—[h]
Spectinomycin	>400	>400	>400	>400	—	—	—	—	—	—	—	—	>400	>400	>400	>400	—	—	4
Kanamycin	>256	>256	—[g]	—[g]	—	—	>128	—	—	—	—	—	—	—	—	—	—	—	<0.5
Gentamicin	32	16	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	<0.5
Tobramycin	128	64	—	—	—	—	>128	—	—	—	—	—	—	—	—	—	—	—	<0.5
Amikacin	64	32	—	—	—	—	<128	—	—	—	—	—	—	—	—	—	—	—	1
Netilmicin	32	32	—	—	—	—	>128	—	—	—	—	—	—	—	—	—	—	—	<0.5
Chloramphenicol	16	16	32	32	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	32	16	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	2
Tetracycline	>32	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
CTAB	300	300	300	300	400	400	—	—	—	—	—	—	—	—	—	—	—	—	100
EtBr	200	200	200	200	250	250	—	—	—	—	—	—	—	—	—	—	—	—	75
Rifampin	>256	>256	>256	>256	—	—	—	—	>256	8	—	—	—	—	—	—	—	—	8
Sulfonamide	>1,000	1,000	>1,000	>1,000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15

[a] For "a" and "b" plasmids, the gene is inserted in the same orientation as the P*lac* promoter and in the opposite orientation, respectively. —, not assayed.
[b] *E. coli* MG-1 produces VEB-1 β-lactamase along with a TEM-1 penicillinase.
[c] Natural plasmid containing bla_{VEB-1}.
[d] Recombinant plasmid backbone is pBKCMV (Km^r).
[e] Recombinant plasmid backbone is pPCRscript-Cam (Cm^r).
[f] Recombinant plasmid backbone is pPCRscript-Amp (Amp^r).
[g] Resistance carried on the cloning vector.
[h] Resistance carried by the host strain.

in terms of amino acid sequence (9) (Fig. 1 and 2). These cassettes are widespread among gram-negative bacteria and confer resistance to kanamycin, tobramycin, and gentamicin (38, 44, 56). The presence of two almost identical cassettes in integrons has been reported for other gene cassettes, such as *oxa2* (56, 66). It is interesting that in all the bla_{VEB-1}-containing integrons described to date (44, 51, 67), an *aadB* gene cassette is found 3′ to bla_{VEB-1} (38).

(ii) The *qacI* cassette. The first cassette contained an ORF of 345 nucleotides (Fig. 1A and 2). A putative initiation GTG codon was preceded at 7 bp by a ribosome-binding site-like sequence (Fig. 2). The coding sequence, designated *qacI,* which may direct the synthesis of a 110-amino-acid protein, had 90% identity with *qacF* from *Enterobacter aerogenes* (50) and 67.8% identity with the sequence of the *qacE* gene (48). The *qacE* gene specifies an exporter protein that mediates resistance to intercalating dyes and quaternary ammonium compounds and that has been found in the class 1 integron of transposon Tn*402*, later designated Tn*5090* (54). The *qacI* and *qacE* cassettes diverge at their extremities and particularly at their 3′ ends; a 60-bp sequence was present downstream from *qacI,* whereas a 141-base element has been associated with *qacE* (54). The *qacI* 59-base element was identical to that of *aadA6*, which encodes an adenylyltransferase (45). A similar 59-base element was found at the 3′ end of *qacF* (50). The members of the family of 59-base elements are long imperfect inverted repeats that vary in length (from 60 bp up to 141 bp) but retain similarity to the consensus at their termini and are active in integrase-mediated site-specific recombination (13, 61). Hypotheses for the mechanisms of cassette movements have been proposed (56), but the question of whether the genes and the 59-base element have independent origins remains to be elucidated. Closely related genes associated with closely related 59-base elements have been described, e.g., *catB3* and *catB5* cassettes (8). In contrast, *aadA6* and *qacI* gene cassettes represent an interesting example of 59-base elements associated with genes encoding different functions. A similar observation was made with the 90% sequence identity between the *Vibrio cholerae* repeated sequences and the 59-base element associated with *blaP3,* an integron-associated gene encoding a β-lactamase (39). These data may suggest that cassettes can exchange 59-base elements via integrase-mediated recombination at the internal boundaries of the two 59-base elements instead of the normal position at the outer boundaries of the 59-base element (56).

The long leader sequence in the *qacE* cassette has been shown to contain promoter sequences (32). These promoter sequences were also putatively identified in the long leader of the *qacI* cassette (Fig. 1A and C and 2). Computer-assisted promoter prediction programs by neural network identified these sequences as highly likely active promoter sequences (threshold, 0.98). The deduced protein, QacI, shares 90% amino acid identity with QacF, 75% identity with QacE (48), 37.6% with QacC (an antiseptic resistance protein from *Staphylococcus aureus*) (34), and 70.1% with EmrE (an *E. coli* protein mediating resistance to EtBr) (53) (Fig. 3). These proteins form a family of small multidrug export proteins that use proton motive force to energize transport and mediate resistance to antiseptics and disinfectants (48). In order to study the phenotype conferred by *qacI,* a 1.3-kb PCR fragment from pRLT-2 was cloned into pPCRscript, generating pRLT-4a and pRLT-4b, depending on the orientation of the insert with respect to the vector promoter sequence. The MICs of CTAB and EtBr for *E. coli* DH10B(pRLT-4a) and *E. coli* DH10B(pRLT-4b) were 100 and 400 μg/ml, respectively, indicating that QacI confers resistance to quaternary compounds. The fact that the MICs of the quaternary ammonium compounds and EtBr were the same for *E. coli* DH10B(pRLT-4a) and *E. coli* DH10B(pRLT-4b) is consistent with the hypothesis that there is a promoter sequence in the long leader sequence of *qacI*.

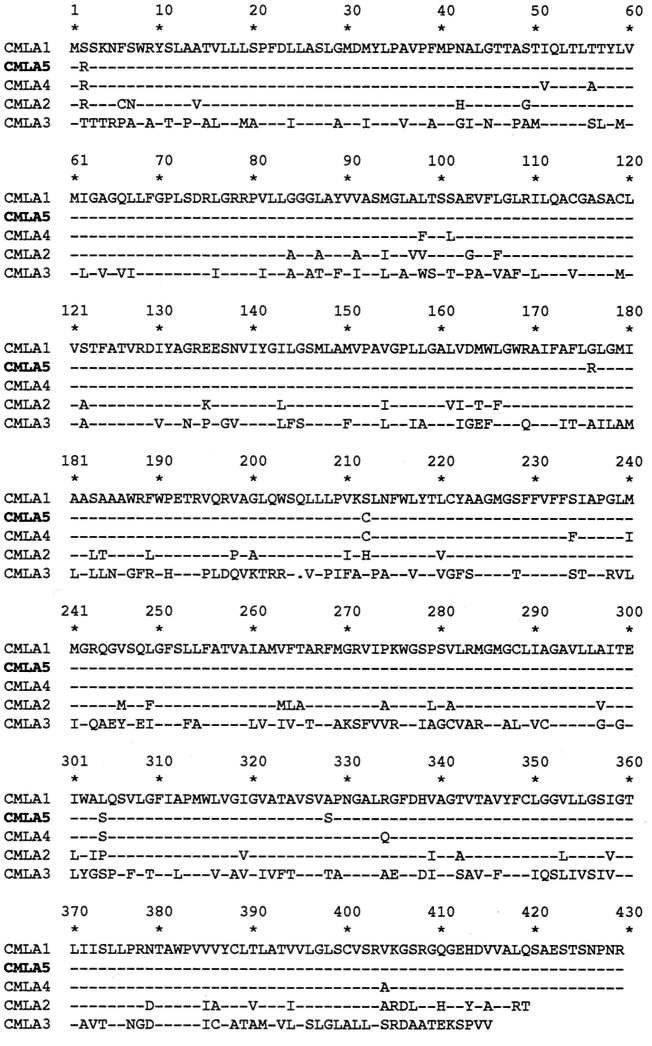

FIG. 4. Comparison of the deduced amino acid sequences of the *cmlA5* product (CMLA5) with those of other proteins of the CMLA family (49). Dashes represent conserved amino acids, and one gap introduced for the alignment is indicated by a dot. While CMLA1 is encoded by a gene cassette from In4 (3), CMLA2 is encoded by a gene cassette from In40 (50), CMLA4 is encoded by a gene cassette from In52 (51), and the genes for CMLA3, CMLA3a and -3b (2, 6, 7) are chromosomal and not present on gene cassettes. CMLA3a and -b differ from CMLA3 by one single-amino-acid substitution each (L308F and V274A, respectively); only the sequence of CMLA3 is displayed.

(iii) The *cmlA5* cassette. The seventh cassette, spanning 1,548 nucleotides (Fig. 1A and 2), contained an ORF of 1,230 nucleotides starting at a putative GTG initiation codon at

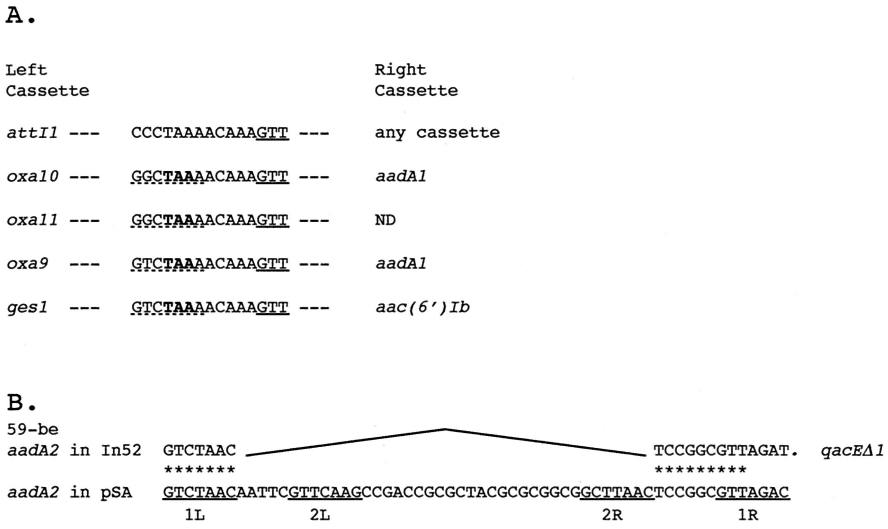

FIG. 5. Various structures of fused gene cassettes. (A) Intergenic regions of fused cassettes in comparison with the *attI1* sequence. Single underlining shows the recombination cleavage site (GTT). Dashed underlining shows the remainder of the inverse core site of the preceding cassette, and boldface shows the stop codon of the preceding gene. ND, not determined. (B) Structure of the sequence of a deleted 59-base element (59-be) found in In52 (52), which is likely to be the result of an integrase-mediated reaction between 1L and 2R regions. 1L, 2L, 2R, and 1R correspond to conserved regions within the 59-base elements (61) and are underlined. The origins of the displayed sequences are as follows: *attI*, references 56 and 61; *oxa10*/*aadA1*, present work; *oxa11*, references 20 and 46; *oxa9*/*aadA1*, reference 66; *aadA2*/*qacEΔ1*, reference 52; *aadA2* in pSA, reference 5; and *ges1*/*aac(6′)Ib*, reference 52.

position 5988 that was preceded at 8 bp by a ribosome-binding site-like sequence (AAGGAG) (data not shown). This coding sequence, designated *cmlA5*, shared 97% identity with the *cmlA1* gene of the class 1 integron In4 in Tn*1696*, which confers a nonenzymatic chloramphenicol resistance (3, 60). The *cmlA5* 59-base element was 70 bp in length, and the sequence differs at two positions from that of the 59-base element of *cmlA1* (data not shown). Eight DNA mismatches lead to five amino acid changes (Fig. 4). A 2.2-kb PCR fragment obtained with primers hybridizing within flanking sequence of *cmlA5* (Table 1 and Fig. 1), thus containing the entire *cmlA5* cassette, was cloned into the pPCRScript/Amp vector in both orientations. The resulting plasmids, pRLT7-a and pRLT7-b (Fig. 1C), conferred chloramphenicol resistance on *E. coli* DH10B at similar levels (MICs of 32 and 16 μg/ml, respectively) (Table 2). The deduced protein of 409 amino acids, CMLA5, shared 97% identity with CMLAl and 54% identity with the polypeptide predicted from the *Salmonella enterica* serotype Typhimurium gene, which confers resistance to florfenicol and chloramphenicol (2, 6, 7) (Fig. 4). CMLAl is an efflux protein of the major facilitator family and confers resistance by chloramphenicol efflux (3, 60).

While most gene cassettes are usually inserted in the same orientation and are under the control of the common promoters P_1 and P_2 located in the 5′-CS (14), a few cassettes described to date, including *cmlA* variants, *qacE*, and *qacEΔ1*, contain a promoter-like sequence (3, 19, 31, 50, 52, 60). Analysis of the region upstream from *cmlA5* showed a putative promoter consisting of −35 (TCGCGG) and −10 (TACGAT) motifs separated by 17 nucleotides. The −10 and −35 motifs were identical to those proposed for *cmlA1*, which indicates that the *cmlA5* gene may also be expressed from its own promoter. The region upstream from *cmlA5* contains a small ORF that may encode a nine-amino-acid peptide that is closely related to the leader peptides of *cat* genes and that differs from that of *cmlA1* by having a Lys instead of an Asn at position 6 (data not shown). This region also contains inverted repeats capable of forming alternate stem-loop structures (18, 60). These features are similar to those found upstream from the inducible *cat* and *erm* genes, which are regulated by transcriptional attenuation (18, 35). The *cmlA5* gene in plasmid pRLT-7a and -7b conferred low-level chloramphenicol resistance on *E. coli* DH10B (Fig. 1C and Table 2). Inducibility of expression of CMLA1 from plasmid R26 in *E. coli* K-12 by subinhibitory concentrations of chloramphenicol has been demonstrated previously (17). The similarities in structure and in sequence of the two cassettes suggest that the regulation of *cmlA5* might be similar to that of *cmlA1*. Indeed, when plasmid pRLT-7a or -7b was expressed in *E. coli* DH10B, a twofold increase in the MIC of chloramphenicol was observed upon induction.

(iv) The *aacA1*/*orfG* fusion cassette. The *aacA1* gene did not appear to possess an associated 59-base element but instead was followed 4 bp downstream by an additional ORF, named *orfG* (GenBank accession number AF047479) (A. Gravel, R. Parent, and P. H. Roy, unpublished data). Although most of the gene cassettes thus far discovered carry antibiotic resistance genes, there are a few other examples of cassettes that carry genes that are not involved in antimicrobial resistance or whose function remains unknown (56). The ORFG product falls in the latter category. ORFG is 142 codons long and is followed by a 59-base element of 107 bp. It is interesting that neither *aacA1b* nor ORFG has been found as a single gene cassette (64) (GenBank accession number AF047479) (Gravel et al., unpublished). The *aacA1* and *orfG* genes have very

A

```
          1         10         20         30         40         50         60
          |         |          |          |          |          |          |
ARR-1   MVANPP...KPFEVHESGAYLHGTKADLKVGDRLVPGRESNFEAGRIMKHVYITQTLDAA
ARR-2   --KDWIPISHDNYKQVQ-PFY-----N-AI--L-TT-FI-H--D---L--I-FSALMEP-
ARR-3             MD--LDE-PFF-----D-R---H-TA-FP--YRPEIL-N-I-F-ALR-G-
CONS                      G   HGTKA L  GD L  G  S          H Y       A
        61        70         80         90        100        110        120
          |         |          |          |          |          |          |
ARR-1   VWGAELAVG....EGRGRIYIVEPEGEIEDDPNVTDKKLPGNPTRSYRTREPVRIVGELT
ARR-2   -------MSLSGL----Y------T-PF-----L-N--F-----Q----C--L----VVE
ARR-3   GLA----A-....D-EP-V-A---T--F-N--------F---------ST--L-V---VV
CONS       AELA        G    Y VEP G  E DPN T KK PGNPT SYR  EP R VG

        121       130        140        150
          |         |          |          |
ARR-1   DWEGHSPEQIAAMREGLEDLRRKGLAVIYD
ARR-2   -----PV-L-RG-LDS----K-R--H--E-
ARR-3   --TRQT--ALRTW-ER-DAM-LE-R-E-IN
CONS    DW     E        L      G   I
```

B

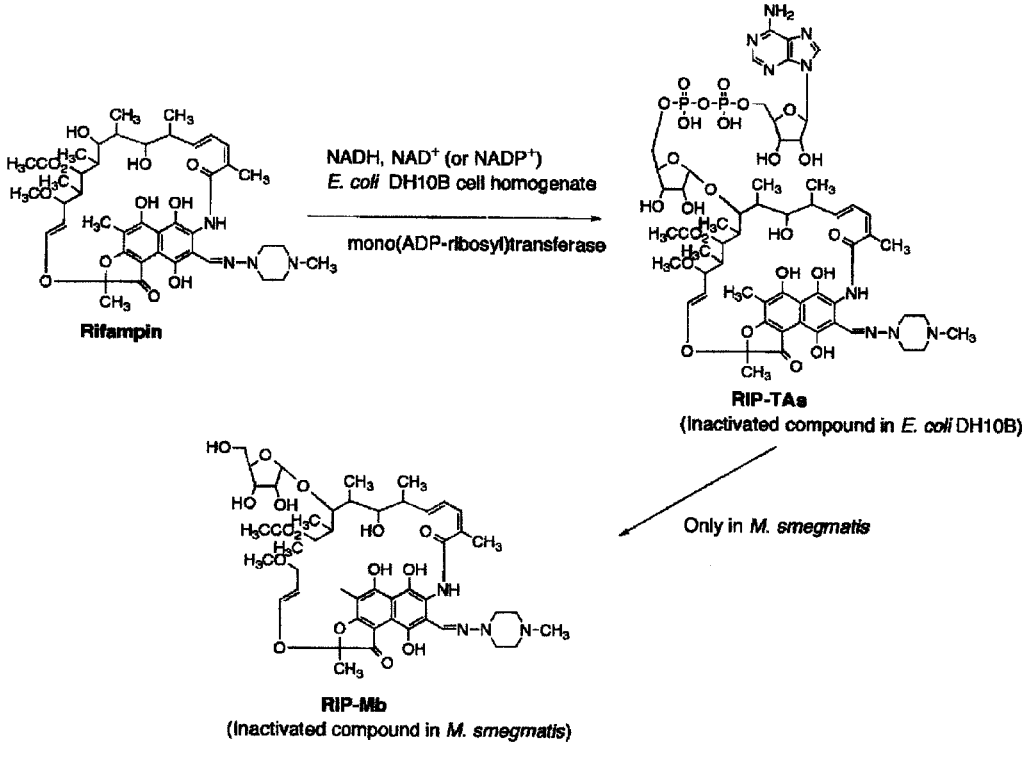

FIG. 6. (A) Amino acid alignment of the three known ARR proteins: ARR-1 from *M. smegmatis* (15), ARR-2 from *E. coli* MG-1 and *P. aeruginosa* pTh2 (reference 67 and the present work), and ARR-3 from *Streptomyces* (57). Dashes represent identical amino acids. A consensus (CONS) sequence is derived from the alignment. (B) Schematic representation of the inactivation pathway of rifampin by ARR-2 and ARR-1 (28).

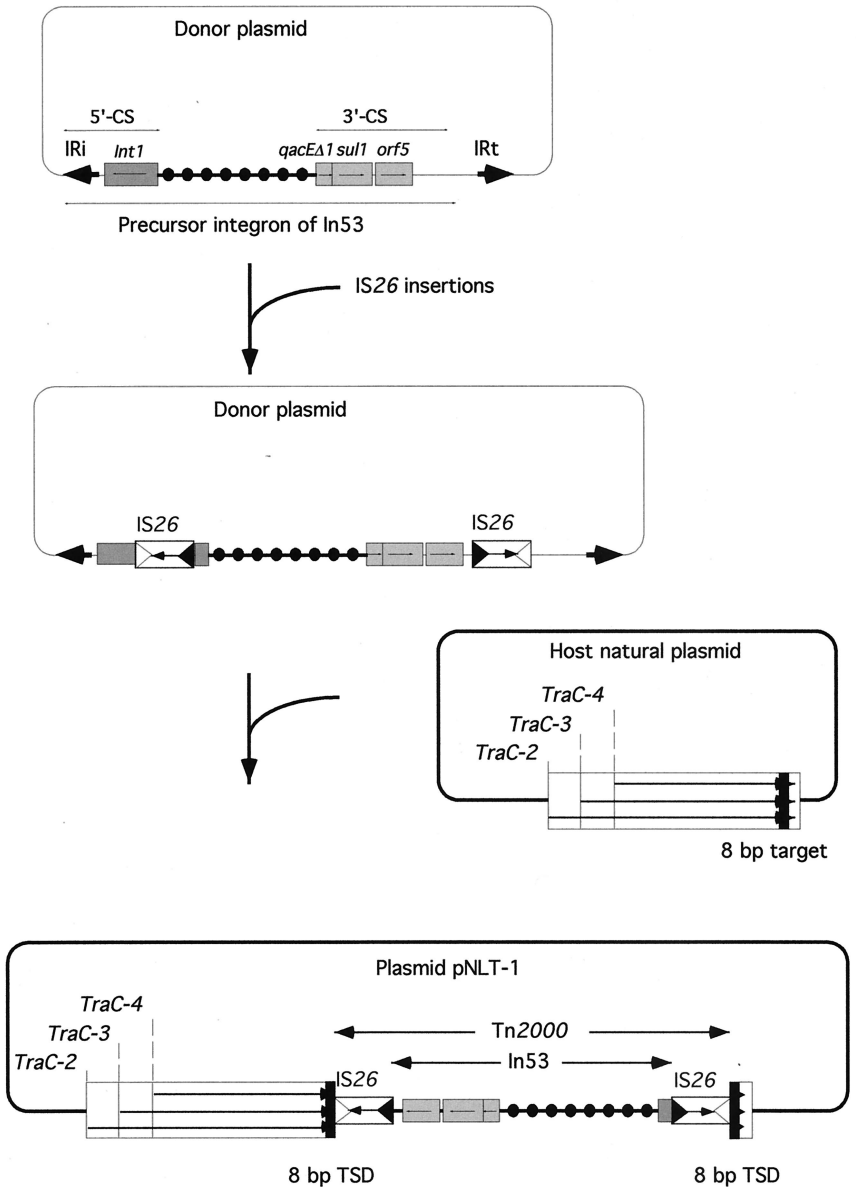

FIG. 7. Schematic representation of the genesis of Tn*2000*, which contains In53 from *E. coli* MG-1. ORFs and genes are shown as boxes with an arrow indicating the orientation of the coding sequence and with the gene name above the corresponding box. IS*26*-related inverted right and left repeats are shown by empty and filled triangles, respectively. The coding orientation of the IS*26* transposase is represented by an arrow. The Tn*2000* and the In53 structures are indicated between two divergent arrows. IS*26*-related target site duplications (TSD) are displayed as filled squares at each end of Tn*2000*. IRi and IRt, integron-specific inverted repeats found at the ends of class 1 integrons (31, 56). The mechanism involved in Tn*2000* transposition could be inverse transposition as was described for Tn*10* (30).

similar G+C contents and codon usage, and thus they are likely to form a cassette as a single unit.

A 1.8-kb PCR fragment obtained with primers hybridizing within flanking sequences of the *aacA1*/*orfG* cassette (Table 1 and Fig. 1C) was cloned into the pPCRscript/Amp vector in both orientations. Only plasmid pRLT5-a, where the *aacA1*/*orfG* cassettes were colinear with the vector promoter (Fig. 1C), conferred kanamycin, tobramycin, amikacin, and netilmicin resistance on *E. coli* DH10B (MIC of >128 μg/ml). The deduced protein of 183 amino acids shared 98% identity (two amino acid changes) (Fig. 2) with AACA1 found in the *Citrobacter diversus* R plasmid and in In21 (GenBank accession number AF047479) (64; Gravel et al., unpublished) and thus was named AACA1b.

(v) Promoter/*oxa10*/*aadA1* fusion cassette. In In53, the *oxa10* gene cassette did not appear to possess an associated 59-base element but instead was fused to an *aadA1* cassette. The *oxa10* coding sequence has so far been described only as a single gene cassette, while *aadA1* has been found only once as a fused cassette in Tn*1331* (26, 45, 56, 66). Loss of a 59-base element may have occurred in a variety of ways, e.g. slippage during DNA replication caused by stem-loop structure in a single-stranded 59-base element DNA template (56). For *aadA2* in In52 the mechanism by which the fusion occurred may be

proposed as an internal deletion at the two conserved regions 1L and 2R of the *aadA2* 59-base element (Fig. 5B) (61). Several other fused cassettes where one 59-base element has likely been deleted have been documented. This is the case for *aadA1*/*oxa9* in Tn*1331* (66) and for bla_{GES-1}/*aac(6′)Ib* in In52 (52), where the sequence found between the two genes is identical to part of *attI* (Fig. 5A). In the *oxa10*/*aadA1* fusion, the typical nine nucleotides that are closely related to *attI* were also found, indicating that the deletion may have arisen from an identical 59-base element and thus indicate a common process of 59-base-element deletion (Fig. 5A).

Two putative recombination core sites were found 5′ to the bla_{OXA-10} gene (Fig. 2). The most likely recombination core site for the fusion cassette corresponds to the closest site 5′ to the translational start site, *oxa10*/*aadA1*-C1 (GTTAGCC), which corresponds to the core site normally encountered in *oxa10* gene cassettes. As expected for fusion cassettes, the core site *oxa10*/*aadA1*-C1 has one mismatch with the inverse core site of *aadA1*. The second possible recombination core site, *oxa10*/*aadA1*-C2 (GTTAGGC), which is located immediately after the *cmlA5* 59-base element, also has one mismatch with the inverse core site of *aadA1*. It seems that the *cmlA* and the *oxa10*/*aadA1* cassettes are separated by 161 bp of unrelated sequence. A BLAST search against the GenBank database did not reveal homology with any known sequence, indicating that these 161 bp do not belong to either of the two cassettes. Computer-assisted promoter prediction programs by a neural network identified within these 161 bp revealed two putative promoter sequences, $P_{oxa10(1)}$ and $P_{oxa10(2)}$ (threshold, 0.99) (Fig. 2). How this sequence got inserted in front of bla_{OXA-10} is unknown. It could be hypothesized that it is the remnant of a deleted cassette that was inserted between the *cmlA5* and *oxa10* cassettes. This hypothesis is strengthened by the presence of a symmetry element, GACNTCAGAGG (whose complement is CCTCTGANGTC), that probably represents the center of a truncated 59-base element for the preceding sequence containing the promoters (Fig. 2). The promoter, whose sequence has not been seen in an integron before, may represent a new cassette with its structural gene and inverted core site deleted. Whether this truncated cassette may act as a mobile promoter cassette is unknown.

In In53, the *oxa10* gene cassette was located downstream from the *cmlA5* cassette and could be transcribed from the *cmlA5* promoter. However, Ploy et al. have shown that the genes located downstream of *cmlA2* are silent because of transcriptional silencing due to the *cmlA2* terminator (50). In the case of *cmlA5*, the same phenomenon may be true, although *E. coli* MG-1 expressed OXA-10, according to isoelectric focusing results showing a pI value of 6.1, which is consistent with OXA-10 expression (data not shown). These results may indicate that the fused *oxa10*/*aadA1* cassette may harbor an efficient *E. coli* promoter or that the *cmlA5* silencer is not effective in *E. coli* MG-1. In order to test these hypotheses, three distinct PCR products were cloned in both orientations into pPCRscript. Plasmids pRLT-8a and pRLT-8b contained the 3′ half of the coding sequence of *cmlA5* (including the transcriptional silencer) and the entire *oxa10*/*aadA1* fusion cassette, pRLT-9a and -9b contained the entire *oxa10*/*aadA1* fusion cassette including the preceding 161 bp, and pRLT-10a and -10b contained only the *oxa10* cassette without the preceding 161 bp (Fig. 1C). The recombinant plasmids were tested for β-lactamase activity and for spectinomycin resistance. No significant difference in expression between the two orientations was observed for plasmid pRLT-8 and pRLT-9. pRLT-10a conferred ampicillin, ticarcillin, and cephalothin resistance on *E. coli* DH10B as expected for OXA-10 expression, while pRLT-10b failed to express OXA-10 since the gene is in antisense orientation with respect to the vector promoter. These results were consistent with the hypothesis that an active *E. coli* promoter is present in front of bla_{OXA-10} (Fig. 1C and Table 2). Therefore, it is very likely that the fused gene cassette *oxa10*/*aadA1* harbored its own promoter, bringing the number of cassettes with long leader sequences harboring a promoter sequence to three.

(vi) The *arr-2* cassette. The DNA sequence around the rifampin resistance gene revealed characteristic gene cassette features. This gene was identical to the previously described *arr-2* gene from *P. aeruginosa* (67), which implies interspecies transfer of that gene. This gene putatively coded for a 150-amino-acid protein that conferred rifampin resistance in *P. aeruginosa* and in *E. coli* (67). The closest homologues in the GenBank database were the ADP-ribosylating transferase encoded by the *arr* gene in *M. smegmatis* (55) and an unpublished sequence, *arr-3* from *Streptomyces coelicolor* (57). ARR-2 showed 54% identity, with only two gaps in the alignment, with ARR-1 and 59% identity with ARR-3 from *S. coelicolor* (Fig. 6A). The mechanism of resistance of *arr-1* is inactivation of rifampin by ribosylation (15, 28, 42). The MICs of rifampin increased to >256 μg/ml for *E. coli* DH10B with pRLT-2 or pRLT-6. The finding that *arr-2* conferred rifampin resistance, in addition to its homology with *arr-1*, strongly suggests that the ARR-2 is an ADP-ribosylating transferase.

Production and extraction of rifampin inactivation product by *E. coli* DH10B(pRLT-4). Although *arr-2* had been previously identified, the physiological role of ARR-2 had not been investigated. When rifampin was added to the reaction mixture including a cell homogenate of *E. coli* DH10B harboring the recombinant plasmid pRLT-6 and NADH, the antibiotic was found to be inactivated within 2 h. To purify and to identify the inactivated compound, the reaction mixture was first extracted with ethyl acetate. No colored compounds were observed in the ethyl acetate phase, but the presence of colored rifampin-related compounds was suggested in the aqueous phase. After the pH of the fraction was changed to 7.0, the colored fraction was freeze-dried and extracted with methanol and the solvent extract was concentrated under vacuum. LH-20 Sephadex chromatography of the concentrated colored compound allowed us to obtain purified rifampin-related products. From 60 mg of rifampin, 43 mg of purified inactivated compound (designated RIP-TAs) was obtained. The reverse-phase thin-layer chromatography profile showed that the inactivated compound was identical to RIP-TAs, showing a retention front value of 0.8 (data not shown). The identity of the colored compound with RIP-TAs was also confirmed by reverse-phase high-pressure liquid chromatography, which showed the same retention time (7.3 min) as RIP-TAs. Positive- and negative-ion FAB-MS data for the purified inactivated product indicated the molecular mass to be 1,363 kDa, and based on the HRFAB-MS data, the molecular formula was determined to be $C_{58}H_{79}N_9O_{25}P_2$. Taken together, these data suggested that the *arr-2* gene en-

codes a mono(ADP-ribosyl)transferase that produces an inactivation product of RIP-TAs [23-(*O*-ADP-ribosyl)rifampin]. Although the main inactivated compound is 23-ribosyl-rifampin (RIP-Mb) in *M. smegmatis* DSM43756, interestingly, *E. coli* DH10B produces only RIP-TAs as the inactivated product. No other inactivation product, such as RIP-Mb, was observed. Therefore, these data suggested that *E. coli* DH10B has no enzyme which can remove the AMP and phosphate from RIP-TAs, as shown in Fig. 6B, to generate RIP-Mb (28, 42). In *M. smegmatis* the RIP-TAs is converted to ribosylated rifampin (RIP-Mb) by the action of an ADP-ribose phosphohydrolase (55). It seems that such an enzyme is absent from *E. coli* or simply is unable to perform the reaction on RIP-TAs, since the only inactivated product isolated from *E. coli* was RIP-TAs.

The mono(ADP-ribosyl) transferase (36) transfers the ADP-ribose moiety of NADH to acceptor molecules, usually proteins (24). Many bacterial mono(ADP-ribosyl)transferases are toxins, such as those of *Corynebacterium diphtheriae*, *V. cholerae*, *Bordetella pertussis*, and *Clostridium botulinum* (40). Endogenous mono-ADP-ribosylation has been demonstrated in several bacteria, including *P. aeruginosa* (24), but little is known about the physiological role of this modification process (24). In these cases, the acceptors were proteins. However, in the present study the acceptor was a low-molecular-weight antibiotic, rifampin, and to our knowledge, this is the second example of ADP-ribosylation as a mechanism of antibiotic inactivation and the first that is integron and plasmid located. Moreover, the ADP-ribosyl moiety is joined to an oxygen atom, in contrast to the examples cited above, where it is joined to a nitrogen atom (Fig. 6B).

In53 is contained on a composite transposon, Tn*2000*. On either side of In53, an IS*26* element was found and in opposite orientations. The DNA sequence immediately next to these insertion sequences was identical to those of the *traC* genes of *E. coli* plasmid R751, which code for the conjugation proteins TRA-C-2, -3, and -4 (65). On both sides of the IS*26* elements not facing the integron, a target site duplication of 8 bp (characteristic of IS*26* transposition) was found, suggesting that the two IS*26* elements may form a composite transposon along with the resistance genes. This transposon, named Tn*2000,* may be responsible for the integron movement and its insertion into plasmid pNLT-1. IS*26* belongs to the IS*6* family of insertion sequences. This family is characterized by the fact that it gives rise exclusively to replicon fusions (cointegrates) in which the donor and target replicons are separated by two directly repeated IS copies (37, 41). Two IS*26* in direct repeat were found at both ends of the kanamycin resistance transposon Tn*2680* (27). They were able to mediate cointegration in *E. coli* K-12 that contains no IS*26* in its chromosome (27). Upon cointegration, mediated by either of the two IS*26* elements, the IS element is duplicated in a direct repeat. However, this cannot be the case for Tn*2000*, where the two elements are in opposite orientations. Some composite transposons, such as Tn*10*, Tn*5*, and Tn*9*, are made of insertion sequences that are in opposite orientations with the 3′ ends of the IS elements facing outwards. For Tn*2000,* the 3′ ends of the elements are facing the integron sequence. This kind of structure may result by a so-called "inside-out," or inverse, transposition as observed for Tn*10* (30). In this kind of transposition, the 5′ ends of the elements are recognized, at a much lower frequency, rather than the 3′ ends (30). Figure 7 outlines a possible mechanism that led to the genesis of Tn*2000*. Whether Tn*2000* is still active in transposition remains to be determined.

Conclusion. This work describes a novel integron, In53, which, instead of residing on a defective Tn*402*-based transposon, acquired mobility by the insertion of IS*26* elements into the 5′ and 3′-CSs. In53 is a peculiar class 1 integron lacking a functional integrase. It is the largest class 1 integron, containing nine different antibiotic resistance genes of different classes including those for β-lactams, aminoglycosides, phenicol, rifampin, and sulfonamides and antiseptic resistance genes. Use of each class of antibiotic or/and antiseptic may result in the selection in vivo of such integron-containing enterobacterial strains. Additionally, its plasmid and transposon locations may provide an easy means of dissemination, as already exemplified by the isolation of other enterobacterial strains, such as *Klebsiella pneumoniae* MG-2 (51) and *Proteus mirabilis* Lil-1 (T. Naas, unpublished data), that carried the same resistance gene.

The sequences of the different cassettes revealed information on the origins of some of them. *cmlA5* is closely related to the *cmlA1* gene cassette, and they probably derive from a common ancestor. In contrast, the *qacE* and *qacI* genes, which are also closely related, are part of cassettes that contain distinct 59-base elements. This observation implies the independent genesis of two cassettes by acquisition of 59-base elements following an unexplained mechanism (56). Furthermore, our results indicate that promoter-containing gene cassettes may arise from existing cassettes that normally lack any promoter sequence.

Our results on *arr-2,* along with those on *arr-1* from *M. smegmatis,* raise the questions of how these two genes have evolved and if they have been transferred between *Mycobacterium* spp., *E. coli*, and *Pseudomonas* spp. These bacterial species are found in soil, where genetic exchange may have occurred. In this respect, *E. coli* and *P. aeruginosa* strains that both contained *arr-2*/$bla_{\text{VEB-1}}$/*oxa10* gene cassettes have been isolated from clinical specimens from patients with the same geographical origin, Vietnam and Thailand, respectively (44, 51, 67). The identification of *arr-2* on a plasmid and on different integrons in several gram-negative species of medical interest is of concern. While in *P. aeruginosa* pTH2 (67) the gene was chromosomal, we identified a plasmid- and transposon-borne gene in *E. coli*. Rifampin is currently used for treating infections such as meningitis due to gram-negative nosocomial pathogens and thus may favor selection of rifampin resistance genes.

The identification of $bla_{\text{OXA-10}}$ in In53 is consistent with the identification of class D β-lactamase genes most often associated with class 1 integrons (46). This is the first description of an integron that carries two β-lactamase genes belonging to two structurally unrelated molecular classes (1). In addition, this is the third description of a $bla_{\text{VEB-1}}$-containing integron that is different in size and structure from the $bla_{\text{VEB-1}}$ integrons previously described (44, 51, 67). These findings confirm the ability of the $bla_{\text{VEB-1}}$ gene to spread among clinically relevant species and highlight the considerable heterogeneity of the genetic environment in which the $bla_{\text{VEB-1}}$ alleles can be found in different clinical isolates. A similar condition likely reflects the intervention of various mechanisms, such as horizontal plasmid transfer and cassette excision or integration, in

the dissemination of the bla_{VEB-1} gene among different hosts and different replicons.

ACKNOWLEDGMENTS

This work was funded by the Ministère de l'Education Nationale et de la Recherche (grant UPRES, JE-2227), Université Paris XI, Paris-Sud, France.

REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. Lond. B **289:**321–331.
2. **Arcangioli, M. A., S. Leroy-Setrin, J. L. Martel, and E. Chaslus-Dancla.** 1999. A new chloramphenicol and florfenicol resistance gene flanked by two integron structures in Salmonella typhimurium DT104. FEMS Microbiol. Lett. **174:**327–332.
3. **Bissonnette, L., S. Champetier, J. P. Buisson, and P. H. Roy.** 1991. Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In*4* integron of Tn*1696*: similarity of the product to transmembrane transport proteins. J. Bacteriol. **173:**4493–4502.
4. **Bissonnette, L., and P. H. Roy.** 1992. Characterization on In0 of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. J. Bacteriol. **174:**1248–1257.
5. **Bito, A., and M. Susani.** 1994. Revised analysis of *aadA2* gene of plasmid pSA. Antimicrob. Agents Chemother. **38:**1172–1175.
6. **Bolton, L. F., L. C. Kelley, M. D. Lee, P. J. Fedorka-Cray, and J. J., Maurer.** 1999. Detection of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol. J. Clin. Microbiol. **37:**1348–1351.
7. **Briggs, C. E., and P. M. Fratamico.** 1999. Molecular characterization of the antibiotic gene cluster of *Salmonella typhimurium* DT104. Antimicrob. Agents Chemother. **43:**846–849.
8. **Bunny, K., R. M. Hall, and H. W. Stokes.** 1995. New mobile gene cassettes containing an aminoglycoside resistance gene, *aacA7*, and a chloramphenicol resistance gene, *catB3,* in an integron in pBWH301. Antimicrob. Agents Chemother. **39:**686–693.
9. **Cameron, F. H., D. J. Groot Obbink, V. P. Ackennan, and R. M. Hall.** 1986. Nucleotide sequence of the AAD(2′) aminoglycoside adenylyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538–1 and *dfrII* in R388. Nucleic Acids Res. **14:**8625–8635.
10. **Carrol, S. F., and R. J. Collier.** 1984. NAD binding site of diphtheria toxin: identification of a residue within the nicotinamide subsite by photochemical modification with NAD. Proc. Natl. Acad. Sci. USA **81:**3307–3311.
11. **Chandrasekaran, S., and D. Lalithakumari.** 1998. Plasmid-mediated rifampicin resistance in *Pseudomonas fluorescens.* J. Med. Microbiol. **47:**197–200.
12. **Collis, C. M., and R. M. Hall.** 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J. Bacteriol. **174:**1574–1585.
13. **Collis, C. M., and R. M. Hall.** 1992. Gene cassettes from the insert region of integrons are excised as covalently closed circles. Mol. Microbiol. **6:**2875–2885.
14. **Collis, C. M., and R. M. Hall.** 1995. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob. Agents Chemother. **39:**155–162.
15. **Dabbs, E. R., K. Yazawa, Y. Mikami, M. Miyaji, N. Morisaki, S. lwasaki, and K. Furihata.** 1995. Ribosylation by mycobacterial strains as a new mechanism of rifampin inactivation. Antimicrob. Agents Chemother. **39:**1007–1009.
16. **Dabbs, E. R., K. Yazawa, Y. Tanaka, Y. Mikami, M. Miyaji, S. J. Andersen, N. Morisaki, S. Iwasaki, O. Shida, H. Takagi, and K. Furihata.** 1995. Rifampicin inactivation by *Bacillus* species. J. Antibiot. (Tokyo) **48:**815–819.
17. **Dorman, C. J., and T. J. Foster.** 1985. Posttranscriptional regulation of the inducible nonenzymatic chloramphenicol resistance determinant of IncP plasmid R26. J. Bacteriol. **161:**147–152.
18. **Gu, Z., R. Harrod, E. J. Rogers, and P. Loyett.** 1994. Anti-peptidyl transferase leader peptides of attenuation-regulated chloramphenicol-resistance genes. Proc. Natl. Acad. Sci. USA **91:**5612–5616.
19. **Guerineau, F., L. Brooks, and P. Mullineaux.** 1990. Expression of the sulfonamide resistance gene from plasmid R46. Plasmid **23:**35–41.
20. **Hall, L. C. M., D. M. Livermore, D. Gur, M. Akova, and H. E. Akalin.** 1993. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**1637–1644.
21. **Hall, R. M., D. E. Brookes, and H. W. Stokes.** 1991. Site-specific insertion genes into integrons: role of the 59-base element and determination of the recombination cross-over point. Mol. Microbiol. **5:**1941–1959.
22. **Hall, R. M., H. J. Brown, D. E. Brookes, and H. W. Stokes.** 1994. Integrons found in different locations have identical 5′ ends but variable 3′ ends. J. Bacteriol. **176:**6286–6294.
23. **Hall, R. M., and C. M. Collis.** 1995. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol. Microbiol. **15:**593–600.
24. **Hayashi, O., and K. Ueda.** 1985. ADP-ribosylation. Annu. Rev. Biochem. **54:**73–100.
25. **Honoré, N., and S. T. Cole.** 1993. Molecular basis of rifampin resistance in *Mycobacterium leprae.* Antimicrob. Agents Chemother. **37:**414–418.
26. **Huovinen, P., S. Huovinen, and G. A. Jacoby.** 1988. Sequence of PSE-2 β-lactamase. Antimicrob. Agents Chemother. **32:**134–136.
27. **Iida, S., B. Mollet, J. Meyer, and W. Arber.** 1984. Functional characterization of the prokaryotic mobile genetic element IS*26*. Mol. Gen. Genet. **198:**84–89.
28. **Imai, T., K. Watanabe, Y. Mikami, K. Yazawa, A. Ando, Y. Nagata, N. Morisaki, Y. Hashimoto, K. Furihata, and E. R. Dabbs.** 1999. Identification and characterization of a new intermediate in the ribosylative inactivation pathway of rifampin by *Mycobacterium smegmatis.* Microb. Drug Resist. **5:**259–264.
29. **Innis, A. M., D. H. Gelfand, J. J. Sninsky, and T. J. White.** 1990. PCR protocols. Academic Press, Inc., New York, N.Y.
30. **Kleckner, N.** 1989. Transposon Tn*10*, p. 227–268. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
31. **Laraki, N., M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J.-M. Frère, and G. M. Rossolini.** 1999. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. **43:**890–901.
32. **Levesque, C., S. Brassard, J. Lapointe, and P. H. Roy.** 1994. Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. Gene **142:**49–54.
33. **Liebert, C. A., R. M. Hall, and A. O. Summers.** 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. **63:**507–522.
34. **Littlejohn, T. G., D. Diberardino, L. J. Messerotti, S. J. Spiers, and R. A. Skurray.** 1992. Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. Gene **101:**59–66.
35. **Lovett, P. S.** 1990. Translational attenuation as the regulator of inducible *cat* genes. J. Bacteriol. **172:**1–6.
36. **Lowery, R. G., and P. W. Ludden.** 1990. Endogenous ADP ribosylation in procaryotes, p. 459–468. *In* J. Moss and M. Vaughan (ed.), ADP-ribosylating toxins and G proteins: insights into signal transduction. American Society for Microbiology, Washington, D.C.
37. **Mahillon, J., and M. Chandler.** 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. **62:**725–774.
38. **Martinez-Freijo, P., A. C. Fluit, F. J. Schmitz, J. Verhoef, and M. E. Jones.** 1999. Many class I integrons comprise distinct stable structures occurring in different species of *Enterobacteriaceae* isolated from widespread geographic regions in Europe. Antimicrob. Agents Chemother. **43:**686–689.
39. **Mazel, D., B. Dychinco, V. Webb, and J. Davies.** 1998. A distinctive class of integron in the *Vibrio cholerae* genome. Science **280:**605–608.
40. **Middlebrook, J. L., and R. B. Dorland.** 1984. Bacterial toxins: cellular mechanisms of action. Microbiol. Rev. **48:**199–221.
41. **Mollet, B., S. Iida, J. Shepherd, and W. Arber.** 1983. Nucleotide sequence of IS*26*, a new prokaryotic mobile genetic element. Nucleic Acids Res. **11:**6319–6330.
42. **Morisaki, N., Y. Hashimoto, K. Furihata, T. Imai, K. Watanabe, Y. Mikami, K. Yazawa, A. Ando, Y. Nagata, and E. R. Dabbs.** 2000. Structures of ADP-ribosylated rifampicin and its metabolite: intermediates of rifampicin-ribosylation by *Mycobacterium smegmatis* DSM43756. J. Antibiot. (Tokyo) **53:**269–275.
43. **Naas, T., L. Philippon, L. Poirel, E. Ronco, and P. Nordmann.** 1999. An SHV-derived extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**1281–1284.
44. **Naas, T., L. Poirel, A. Karim, and P. Nordmann.** 1999. Molecular characterization of In50, a class 1 integron encoding the gene for the extended-spectrum β-lactamase VEB-1 in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **176:**411–419.
45. **Naas, T., L. Poirel, and P. Nordmann.** 1999. Molecular characterization of In51, a class 1 integron containing a novel aminoglycoside adenylyltransferase gene cassette, aadA6, in Pseudomonas aeruginosa. Biochim. Biophys. Acta **1489:**445–451.
46. **Naas, T., and P. Nordmann.** 1999. OXA-type β-lactamases. Curr. Pharm. Design **5:**865–879.
47. **Ohno, K., H. Koga, S. Kohno, T. Tashiro, and K. Hara.** 1996. Relationship between rifampin MICs and *rpob* mutations of *Mycobacterium tuberculosis* strains isolated in Japan. Antimicrob. Agents Chemother. **40:**1053–1056.
48. **Paulsen, I. T., T. G. Littlejohn, P. Radström, L. Sundström, O. Sköld, G. Swedberg, and R. A. Skurray.** 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. **37:**761–768.
49. **Paulsen, I. T., R. A. Skurray, R. Tam, M. H. Saier, R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius.** 1996. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol. Microbiol. **19:**1167–1175.
50. **Ploy, C. M., P. Courvalin, and T. Lambert.** 1998. Characterisation of In40 of

Enterobacter aerogenes BM2688, a class 1 integron with two novel gene cassettes, *cmlA2* and *qacF*. Antimicrob. Agents Chemother. **42:**2557–2563.
51. **Poirel, L., T. Naas, M. Guibert, E. B. Chaibi, R. Labia, and P. Nordmann.** 1999. Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum β-lactamase encoded by an *Escherichia coli* integron gene. Antimicrob. Agents Chemother. **43:**573–581.
52. **Poirel, L., I. Le Thomas, T. Naas, A. Karim, and P. Nordmann.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **44:**622–632.
53. **Purewal, A. S.** 1991. Nucleotide sequence of the ethidium efflux gene from *Escherichia coli*. FEMS Microbiol. Lett. **82:**229–232.
54. **Radström, P., O. Sköld, G. Swedberg, J. Flensburg, P. H. Roy, and L. Sundström.** 1994. Transposon Tn*5090* of plasmid R751, which carries an integron, is related to Tn*7*, Mu, and the retroelements. J. Bacteriol. **176:** 3257–3268.
55. **Quan, S., H. Venter, and E. R. Dabbs.** 1997. Ribosylative inactivation of rifampin by *Mycobacterium smegmatis* is a principal contributor to its low susceptibility to this antibiotic. Antimicrob. Agents Chemother. **41:**2456–2460.
56. **Recchia, G. D., and R. M. Hall.** 1997. Origins of the mobile gene cassettes found in integrons. Trends Microbiol. **5:**389–394.
57. **Rendenbach, M., H. M. Kieser, D. Denapaite, A. Eichner, J. Cullum, H. Kinashi, and D. A. Hopwood.** 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb Streptomyces coelicolor A3(2) chromosome. Mol. Microbiol. **21:**77–96.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **Steers, E., E. I. Foltz, B. S. Graves, and J. Riden.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. (Basel) **9:**307–311.
60. **Stokes, H. W., and R. M. Hall.** 1991. Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn*1696* integron suggests regulation by translational attenuation. Plasmid **26:**10–19.
61. **Stokes, H. W., D. B. O'Gorman, G. D. Recchia, L. Parsekhian, and R. M. Hall.** 1997. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol. Microbiol. **26:**731–745.
62. **Sundström, L., P. Radström, G. Swedberg, and O. Sköld.** 1988. Site-specific recombination promotes linkage between trimethoprim and sulfonamide resistance genes. Sequence characterization of *dfrV* and *sulI* and a recombination active locus of Tn*21*. Mol. Gen. Genet. **213:**191–201.
63. **Tanaka, Y., K. Yazawa, E. R. Dabbs, K. Nishikawa, H. Komaki, Y. Mikami, M. Miyaji, N. Morisaki, and S. Iwasaki.** 1996. Different rifampicin inactivation mechanisms in *Nocardia* and related taxa. Microbiol. Immunol. **40:**1–4.
64. **Tenover, F. C., D. Filpula, K. L. Phillips, and J. J. Plorde.** 1988. Cloning and sequencing of a gene encoding an aminoglycoside 6′-*N*-acetyltransferase from an R factor of *Citrobacter diversus*. J. Bacteriol. **170:**471–473.
65. **Thorsted, P. B., N. A. Macartney, P. Akhtar, A. S. Haines, N. Ali, P. Davidson, T. Stafford, M. J. Pocklington, W. Pansegrau, B. M. Wilkins, E. Lanka, and C. M. Thomas.** 1998. Complete sequence of the IncP beta plasmid R751: implications for evolution and organisation of the IncP backbone. J. Mol. Biol**. 282:**969–990.
66. **Tolmasky, E. M., and J. H. Crosa.** 1993. Genetic organization of antibiotic resistance genes *(aac(6′)-Ib, aadA, and oxa9)* in the multiresistance transposon Tn*1331*. Plasmid **29:**31–40.
67. **Tribuddharat, C., and M. A. Fennewald.** 1999. Integron-mediated rifampin resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:** 960–962.
68. **Williams, D. L., C. Waguespack, K. Eisenach, J. T. Crawford, F. Portaels, M. Salfinger, C. M. Nolan, C. Abe, V. Sticht-Groh, and T. P. Gillis.** 1994. Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob. Agents Chemother. **38:**2380–2386.
69. **Yazawa, K., Y. Mikami, A. Maeda, N. Morisaki, and S. Iwasaki.** 1994. Phosphorylative inactivation of rifampicin by *Nocardia otitidiscaviarum.* J. Antimicrob. Chemother. **33:**1127–1135.