ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2000, p. 1568–1574 Vol. 44, No. 6
0066-4804/00/$04.00+0

Antibiotic Resistance in the ECOR Collection: Integrons and Identification of a Novel *aad* Gene

DIDIER MAZEL,[1] BRODERICK DYCHINCO,[2]† VERA A. WEBB,[2]‡ AND JULIAN DAVIES[2]*

Unité de Programmation Moléculaire et Toxicologie Génétique, Institut Pasteur, 75724 Paris cedex 15, France,[1] and Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada[2]

Received 23 September 1999/Returned for modification 17 December 1999/Accepted 13 March 2000

The 72 *Escherichia coli* strains of the ECOR collection were examined for resistance to 10 different antimicrobial agents including ampicillin, tetracycline, mercury, trimethoprim, and sulfonamides. Eighteen strains were resistant to at least one of the antibiotics tested, and nearly 20% (14 of 72) were resistant to two or more. Several of the resistance determinants were shown to be carried on conjugative elements. The collection was screened for the presence of the three classes of integrons and for the *sul1* gene, which is generally associated with class 1 integrons. The four strains found to carry a class 1 integron also had Tn*21*-encoded mercury resistance. One of the integrons encoded a novel streptomycin resistance gene, *aadA7*, with an *attC* site (or 59-base element) nearly identical to the *attC* site associated with the *qacF* gene cassette found in In40 (M.-C. Ploy, P. Courvalin, and T. Lambert, Antimicrob. Agents Chemother. 42:2557–2563, 1998). The conservation of associated *attC* sites among unrelated resistance cassettes is similar to arrangements found in the *Vibrio cholerae* superintegrons (D. Mazel, B. Dychinco, V. A. Webb, and J. Davies, Science 280:605–608, 1998) and supports the hypothesis that resistance cassettes are picked up from superintegron pools and independently assembled from unrelated genes and related *attC* sites.

The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements such as plasmids and transposons has been the major factor in the development of multiple drug resistance over the last 50 years. Integrons have been implicated in this spread among the gram-negative bacteria, especially in enteric bacteria and pseudomonads. Integrons contain gene expression elements that incorporate open reading frames (gene cassettes) and convert them to functional genes (45, 46). Variations of integrons that have been described include the multiresistant integrons (MRI), usually carried on mobile elements (for reviews see references 20, 21, and 45), and the chromosomal superintegrons, which have been identified in the chromosomes of several *Vibrio* species (36; for a review, see reference 35).

All known integrons have three key components necessary for the procurement of exogenous genes: (i) a gene coding for an integrase (*intI*), (ii) a primary recombination site (*attI*), and (iii) a promoter (19, 22, 46, 56). Integron integrases recombine gene cassettes downstream of the proximal *attI* site of the resident promoter, permitting expression of their encoded proteins. Most of the nearly 60 resistance cassettes known to date (20, 35) contain a single gene associated with a specific recombination sequence, the *attC* site (or 59-base element). The most common cassettes are those for aminoglycoside or trimethoprim resistance, of which 14 and 12, respectively, have been identified. The *attC* sites (59-base elements) comprise a family of diverse sequences which vary in size from 57 to 141 bp; only the boundaries are conserved sequences. Three classes of MRI have been defined based on the homology of the integrase genes, and each appears to be able to acquire the same gene cassettes. Some MRI carrying as many as five different resistance determinants have been characterized. Plasmid-borne integrons owe their mobility to association with transposable elements, and many class 1 integrons are found on Tn*21*-like transposons which also encode mercury and tetracycline resistances (29, 55). The class 1 integron found associated with Tn*21* contains the *aadA1* gene and has been designated In2 (for a review, see reference 29). The mechanism by which these multiple-drug-resistance cassette arrays are built up has been elucidated previously (10).

Several studies of integron distribution in clinically significant gram-negative isolates have been described (28, 33, 34); in all but one case, selection was for their aminoglycoside resistance phenotype. However, such an analysis has not, to our knowledge, been described for clinically unselected enterobacteria. The ECOR collection is a widely used set of 72 reference *Escherichia coli* strains isolated between 1973 and 1983 from a variety of animal hosts and a variety of geographic locations (41). The strains have been well characterized biochemically and represent the range of genotypic variation in the species as a whole (see, for example, references 1, 3, 5, 6, 11–18, 23–25, 27, 32, 37–40, 42, 44, 50, 52, 53, 57, 58, and 60). Pulsed-field gel electrophoresis studies have shown that the genome sizes of these natural isolates of *E. coli* vary considerably (4), and many carry plasmids (7, 47, 48); some of these carry colicins and colicin-related genes (47, 48). However, despite extensive examination of this collection, there are no reports of its overall antibiotic resistance patterns, although Summers and colleagues have investigated mercury resistance (30, 59).

We screened the ECOR strains for resistance to 10 different antimicrobial agents including ampicillin, tetracycline, mercury, trimethoprim, and sulfonamides. In addition, PCR was used to assay the collection for the presence of the three classes of mobile integrons and the *sul1* gene, which is gener-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of British Columbia, Room 300, Wesbrook Building, 6174 University Blvd., Vancouver, British Columbia, Canada, V6T 1Z3. Phone: (604) 882-9308. Fax: (604) 822-6041. E-mail: jed@unixg.ubc.ca.

† Present address: School of Public Health, University of Michigan, Ann Arbor, MI 48109-2029.

‡ Present address: Lookfar Solutions Inc., Tofino, British Columbia V0R2Z0, Canada.

TABLE 1. Primer sequences for PCR analysis

Primer	Locus (direction)[a]	Nucleotide sequence (5′ to 3′)	GenBank accession no.	Position	Source or reference
int1.F	*intI1* (+)	GGG TCA AGG ATC TGG ATT TCG	U49101	786–766	This work
int1.R	*intI1* (−)	ACA TGC GTG TAA ATC ATC GTC G	U49101	303–324	This work
int2.F	*intI2* (+)	CAC GGA TAT GCG ACA AAA AGG T	L10818	219–240	This work
int2.R	*intI2* (−)	GTA GCA AAC GAG TGA CGA AAT G	L10818	1007–986	This work
int3.F	*intI3* (+)	GCC TCC GGC AGC GAC TTT CAG	D50438	71–91	This work
int3.R	*intI3* (−)	ACG GAT CTG CCA AAC CTG ACT	D50438	1050–1030	This work
qac.F	*qacΔE1* (+)	GGC TGG CTT TTT CTT GTT ATC G	U49101	1497–1518	This work
qac.R	*qacΔE1* (−)	TGA GCC CCA TAC CTA CAA AGC	U49101	1770–1750	This work
sul.F	*sul1* (+)	TGG TGA CGG TGT TCG GCA TTC	U49101	1833–1853	This work
sul.R	*sul1* (−)	GCG AAG GTT TCC GAG AAG GTG	U49101	2622–2602	This work
aadA.F	*aadA1a*[b] (+)	ATG AGG GAA GCG GTG ATC GCC	X12870	1299–1318	This work
aadA.R	*aadA1a* (−)	TCT TCC AAC TGA TCT GCG CGC	X12870	2041–2021	This work
IRI	In2 (+)[c]	TTT CAG AAG ACG GCT GCA CTG	AF071413	4046–4066	This work
IRT	In2 (+)[c]	TGG TGC AGT CGT CTT CTG AAA A	AF071413	15012–15033	This work
38/-	Tn*21* IR	GGG CAC CTC AGA AAA CGG AAA	AF071413	19669–19649	This work
merR1	*merR* (−)	GCG GAT TTG CCT CCA CGT TGA	K03089	458–477	30
merT1	*merT* (−)	CCA GGC AGC AGG TCG ATG CAA G	K03089	682–661	30
merA1	*merA* (+)	ACC ATC GGC GGC ACC TGC GT	K03089	2140–2159	30
merA5	*merA* (−)	ACC ATC GTC AGG TAG GGG AAC AA	K03089	3375–3357	30

[a] +, coding strand; −, noncoding strand.
[b] Originally named *aadA*; now called *aadA1a* (20).
[c] Tn*21* In2 IR_i and Tn*21* In2 IR_t (29).

ally associated with class 1 integrons. Four strains were found to carry a class 1 integron.

MATERIALS AND METHODS

Bacterial strains and plasmids. The 72 strains of the ECOR collection were kindly provided by H. Ochman. In conjugation experiments the recipient strain was the nalidixic acid-resistant *E. coli* K802NR (2). Products from PCR studies were cloned into the vector pCR2 (Invitrogen, Carlsbad, Calif.).

Antibiotic sensitivity testing. The antibiotic sensitivity profile of each ECOR strain was tested by spreading two Mueller-Hinton agar plates with 100 μl each of an overnight culture grown in Luria-Bertani medium (LB). Difco sensitivity disks for the following antibiotics were placed on the plates (six disks per plate): ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), oxacillin (1 μg), penicillin G (2 U), streptomycin (10 μg), sulfisoxazole (300 μg), tetracycline (30 μg), tobramycin (10 μg), and trimethoprim (5 μg). Disks containing mercuric chloride (0.125, 0.25, 0.5, or 1 μmol), ethidium bromide (50 μg), paromomycin (20 or 50 μg), astromycin (20 or 50 μg), lividomycin (20 or 50 μg), or butirosin integron (20 or 50 μg) were made from sterile blank disks. The plates were incubated at 37°C overnight. Antibiotic resistance was scored by comparing the zone of inhibition to those of sensitive strains of *E. coli*.

PCR procedures. PCR was performed in 50-μl volumes in 96-well plates. Reaction conditions were as follows: 9 μl of Ultratherm buffer containing 50 mM $MgCl_2$, each deoxynucleoside triphosphate (dNTP) to 2 mM, 25 pmol of each primer, 10 μl of template DNA, and 0.4 μl of Ultratherm enzyme (Bio/Can Scientific, Mississauga, Ontario, Canada). Template DNA was prepared as follows: a cell pellet from 1 ml of overnight culture was resuspended in 0.5 ml of water and boiled for 10 min. The primers for PCR are listed in Table 1. Conditions for amplification using the *intI1*, *intI2*, *intI3*, and *sul1* primers were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 60 s. Conditions for amplification using the *qac* primers were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Conditions for amplification using the primer combinations IRI and int1.F, IRT and merA1, and 38/- and merT1 were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min. In all experiments an additional 8-min extension at 72°C was included after the 30th cycle. Amplification using the *mer* primers was carried out as described previously (30).

Conjugation studies. All ECOR collection strains that exhibited resistance to any of the antibiotics tested were further examined for the ability to transfer resistance by conjugation. The recipient *E. coli* strain K802NR and each donor ECOR strain tested were grown to mid-log phase. Equal volumes (25 μl) of donor and recipient were spread on a Mueller-Hinton plate and incubated at 37°C overnight. The resulting biomass was harvested, plated on Mueller-Hinton agar plates containing nalidixic acid (30 μg/ml) and the selective antibiotic (indicated in Table 3), and incubated overnight at 37°C.

Nucleotide sequencing. Nucleotide sequencing reactions were performed using the ABI PRISM Dye Terminator cycle sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin-Elmer [PE] Applied Biosystems, Foster City, Calif.) and electrophoresed on a 373 Stretch (PE Applied Biosystems).

Computer analysis. Initial nucleotide sequence analysis was performed by BLAST (National Center for Biotechnology Information [NCBI]). Alignments and phylogenetic analysis were performed using the PAUP and PAUPTREES programs of the Wisconsin package (Genetics Computer Group, Madison, Wis.) or CLUSTAL W1.7.

Nucleotide sequence accession numbers. The *aadA7* cassette nucleotide sequence has been deposited in GenBank under accession number AF224733. Nucleotide and amino acid sequences presented in Fig. 1 were retrieved from GenBank.

RESULTS

Antibiotic resistance profiles. The antibiotic resistance profiles of the ECOR collection strains are summarized in Table 2. Of the 14 antimicrobial agents tested, resistance to 8 was observed. One-quarter of the strains in the collection (18 of 72) were resistant to at least one antibiotic, and nearly 20% (14 of 72) were resistant to two or more antimicrobial agents. Strains 3, 31, 37, and 48 and their transconjugants were tested for resistance to both streptomycin and spectinomycin. All were resistant to both antibiotics (Tables 2 and 3). The most common resistances were to sulfisoxazole (14 strains), tetracycline (12 strains), and streptomycin (11 strains).

Conjugation studies. The 18 antibiotic-resistant strains were tested for their abilities to transfer their resistance phenotypes by conjugation as described in Materials and Methods. The results are summarized in Table 3.

Distribution of integrons. PCR-based examination for the presence of integrons was carried out on the entire collection. The results of this analysis, which used oligonucleotide primers specific for the three different *intI* genes, are summarized in Table 4. None of the strains showed an amplification product when the int2 or int3 primers were used. A class 1 integron, however, was found in four strains, strains 3, 31, 37, and 48, based on the presence of PCR products with the three primer sets specific for a class 1 integron, namely, int1, sul, and qac primers. All four of these strains were resistant to streptomycin and to the sulfonamide drug sulfisoxazole. When assayed with the int1.R and qac.R primers, all four produced similar 2.3-kb

TABLE 2. Antibiotic resistance patterns[a]

ECOR strain no.	Host	Site	Yr	Group	Antibiotic resistance phenotype[b]	Resistance or sensitivity[c] to:	
						Hg	EB
3	Dog	Massachusetts	1983	A	Str Spc Sul Tet	R	S
4	Human	Iowa	1973	A	Sul Tet	S	R
6	Human	Iowa	1973	A	Tet	S	R
10	Human	New York	1983	A	Sul Amp	S	S
16	Leopard	Washington Zoo[d]	1973	A	Str Sul Tet	S	S
17	Pig	Indonesia	1973	A	Str Sul Tet	S	S
18	Ape	Washington Zoo	1973	A	Str Sul Tet	S	R
19	Ape	Washington Zoo	1973	A	Str Sul Tet	S	R
24	Human	Sweden	1983	A	Str Sul	S	S
25	Dog	New York	1983	A	Str Sul	S	R
30	Bison	Alberta	1973	B1	Str Tet	S	R
31	Leopard	Washington Zoo	1973	E	Str Spc Sul Amp Kan Tet	R	R
37	Marmoset	Washington Zoo	1973	E	Str Spc Sul Cm Kan	S	S
38	Human	Iowa	1973	D	Tet	S	S
39	Human	Sweden	1983	D	Tet	S	S
48	Human	Sweden	1983	D	Str Spc Sul Tet	R	S
54	Human	Iowa	1973	B2	Sul	S	S
70	Gorilla	Washington Zoo	1973	B1	Sul	S	S

[a] Antimicrobial agents tested are listed in Materials and Methods.
[b] Str, streptomycin; Spc, spectinomycin; Sul, sulfonamide; Tet, tetracycline; Amp, ampicillin; Kan, kanamycin; Cm, chloramphenicol.
[c] Hg, mercuric chloride; EB, ethidium bromide; R, resistance; S, sensitivity.
[d] Seattle, Wash.

PCR products, indicating that they each harbored a single resistance cassette. When amplified with intI1.R and aadA.R (a primer specific for the aminoglycoside adenylyltransferase cassette *aadA1a*, formerly named *aadA* [20]), ECOR strains 3, 37, and 48 were positive in the amplification reaction, suggesting that they carried In2. Interestingly, strain 31 was negative in this assay.

For additional characterization of these integrons, the four strains carrying a class 1 integron were screened for the presence of a *mer* locus typical of Tn*21*-like transposons using *mer*-specific primers and primers specific for either the 5′ or 3′ ends of the inverted repeats of In2 (primers IRI and IRT) or the inverted repeat of Tn*21* IR_{mer} (primer 38/-). All four strains were positive with the *mer* primers (Table 4). In agreement with these results, ECOR strains 3, 31, and 48 were phenotypically mercury resistant (Hg^r); however, ECOR strain 37 was mercury sensitive (Hg^s). When primers specific for the inverted repeat regions of In2 and Tn*21* were used in combination with the int1.F and *mer* primers, only strains 3, 37, and 48 produced the expected products (Table 4). Thus, ECOR strains 3, 37, and 48 contain the typical Tn*21* carrying the In2 integron (59). The structure harboring the integron in ECOR strain 31 is similar to Tn*21* but differs from it in two ways: first, the cassette carried by the integron is not *aadA1* (see below),

TABLE 3. Summary of conjugation experiments

ECOR strain no.	Conjugation[a]	Marker transferred	Linkage[b]	Colicin type and F-related plasmids[c]
3	+	Sul Str Spc Tet	Sul Str Spc; Tet	ColIa; F-like
4	+	Sul	NA[d]	F-like
6	−	NA	NA	
10	+/−	Amp	NA	F-like
16	+	Str	None	F-like
17	+	Sul	None	F-like
18	−	NA	NA	
19	+	Sul Tet	None	F-like
24	+	Sul Str	Sul Str	ColE1; F-like
25	−	NA	NA	ColB; F-like (R1 type)
30	−	NA	NA	
31	+	Sul Str Spc	Sul Str Spc	ColE1; F-like
37	−	NA	NA	F-like (R1 type)
38	−	NA	NA	ColIa
39	−	NA	NA	ColE1
48	−	NA	NA	ColIb; F-like (R1 type)
54	−	NA	NA	
70	++	Sul	None	F-like (R1 type)

[a] The recipient was *E. coli* K802NR. −, no transfer; +/−, sporadic transfer; +, transfer frequency, $>10^{-4}$; ++, transfer frequency, $>10^{-1}$.
[b] Transconjugants were tested for sensitivity to all the antibiotics listed in Materials and Methods.
[c] Data for production of colicin are from the work of Riley and Gordon (47), and data for F-related plasmid content are from the work of Boyd et al. (7).
[d] NA, not applicable.

TABLE 4. Summary of PCR experiments[a]

ECOR strain no.	PCR result with the indicated primer pair[b]									
	int1.F-int1.R	sul.F-sul.R	qac.F-qac.R	merA1–merA5	merR1–merT1	int1.R-qac.F	int1.R-aadA.R	IRT-merA1	IRI-int1.F	merT1–38/-
3	+	+	+	+	+	+	+	+	+	+
4	−	−	−	ND	ND	ND	ND	ND	ND	ND
6	−	−	−	ND	ND	ND	ND	ND	ND	ND
10	−	−	−	ND	ND	ND	ND	ND	ND	ND
16	−	−	−	ND	ND	ND	ND	ND	ND	ND
17	−	−	−	ND	ND	ND	ND	ND	ND	ND
18	−	−	−	ND	ND	ND	ND	ND	ND	ND
19	−	−	−	ND	ND	ND	ND	ND	ND	ND
24	−	−	−	ND	ND	ND	ND	ND	ND	ND
25	−	−	−	ND	ND	ND	ND	ND	ND	ND
30	−	−	−	ND	ND	ND	ND	ND	ND	ND
31	+	+	+	+	+	+	−	−	+	+
37	+	+	+	+	+	+	+	+	+	+
38	−	−	−	ND	ND	ND	ND	ND	ND	ND
39	−	−	−	ND	ND	ND	ND	ND	ND	ND
48	+	+	+	+	+	+	+	+	+	+
54	−	−	−	ND	ND	ND	ND	ND	ND	ND
70	−	−	−	ND	ND	ND	ND	ND	ND	ND

[a] PCR regimens and antimicrobial agents tested are listed in Materials and Methods.
[b] +, PCR amplification product; −, no amplification product; ND, not determined.

and the IRT region is not identical to Tn*21* (as shown by the absence of PCR amplification with IRT plus merA1 primers) (29). The KH802NR streptomycin-resistant transconjugants obtained from mating with ECOR strain 3 and ECOR strain 31 were also assayed and gave results identical to those for donor strains (data not shown).

Characterization of the cassette carried by the ECOR strain 31 integron. As ECOR strain 31 carried an integron different from In2, the PCR product from the assay containing the int1.R and qac.R primers was cloned and further characterized. According to its location in the so-called 5′ and 3′ conserved segments (22), the full cassette array of a class 1 integron is theoretically contained in such an amplification product. The nucleotide sequence of the entire fragment was determined, and analysis revealed a single cassette inserted at the *attI1* site. This cassette encoded an aminoglycoside adenylyltransferase not described previously, which conferred streptomycin and spectinomycin resistance on a sensitive *E. coli* DH5α strain. This novel AAD(3″)-like enzyme is related to the known AAD(3″) enzymes, its closest relatives being those encoded by *aadA1a*, *aadA1b*, and *aadA2* (about 70% identity), which are also integron cassettes (see Fig. 1A). This gene has been named *aadA7*. The *attC* site carried by this cassette is located just downstream of the *aadA7* stop codon. This *attC* site is homologous to those found in six other antibiotic resistance cassettes (see Fig. 1), its closest relative being the one found in the *qacF* cassette (43).

DISCUSSION

We have screened the ECOR collection of *Escherichia coli* strains for antibiotic resistance phenotypes and their genetic organization (integrons). The 72 strains were collected between 1973 to 1983, from primarily healthy human and animal hosts, although 11 were isolated from infected human urinary tracts (41). Of the 18 strains found to be resistant to at least one antibiotic, all but 1 (ECOR strain 48) were from healthy individuals and most (10 strains) belonged to group A (Table 2). These results provide another striking example of the spread of resistance genes in bacterial populations. Nearly all of the animal hosts were bred under human control, either as domestic animals or in zoos. Animals may acquire resistant strains via many routes, e.g., through selection by antimicrobial therapy, drug supplements in the food, or contamination from a worker. Forty percent of the group A strains (10 of 25) were resistant to at least one antimicrobial agent.

The most frequent phenotypes were sulfonamide resistance, tetracycline resistance, and streptomycin resistance. Because sulfonamide has been used since the 1930s, resistance to this compound is now widespread. Tetracyclines were the first major group of "broad-spectrum" antibiotics (49), and they have been used worldwide in both human and animal medicine. Streptomycin has also been used for a considerably long period of time, and even if it has been largely abandoned for the treatment of infections caused by gram-negative bacteria, resistance is still frequent in enteric bacteria, particularly in *E. coli*. Two independent studies of *Enterobacteriaceae* isolated in the 1990s showed an average of 23% streptomycin resistance for *E. coli* isolates (9, 31).

Since many resistance genes are carried on conjugative plasmids, we assayed the transferability of the different resistance markers found in the ECOR strains in a simple conjugation assay. Natural isolates of *E. coli* harbor an average of four plasmids (54), and many of the ECOR strains have been shown to carry colicinogenic and IncF-related plasmids (7, 47, 48). In tests of conjugative transfer at 37°C, we observed at least one transferable resistance determined in half of the resistant strains. Notably, all of these conjugative elements were carried by strains that had been shown to contain an IncF plasmid (see Table 3), some belonging to the R1 subclass (7). Further analysis of the transconjugants is required to show if they carry the F-like plasmids of the donor strains.

Integron acquisition is considered the major cause of multiple resistance in gram-negative species, mainly in enteric bacteria and pseudomonads. Several studies have shown that 43 to 50% of recent European clinical isolates carried detectable integrons and that these strains were statistically more likely to be resistant to antibiotics than integron-negative strains (26, 33). Previously, Roy and colleagues found that about 75% of aminoglycoside-resistant clinical isolates carried an integron (28). Integrons are less frequently represented in the ECOR

A

```
AadA1a          MREAVIAEVSTQLSEVVGVIERHLEPTLLAVHLYGSAVDGGLKPHSDIDLLVTVTVRLDE
AadA1b          MREAVIAEVSTQLSEVVGVIERHLEPTLLAVHLYGSAVDGGLKPHSDIDLLVTVTVRLDE
AadA2           ----MTIEISNQLSEVLSVIERHLESTLLAVHLYGSAVDGGLKPYSDIDLLVTVAVKLDE
AadA7           MSEKVPAEISVQLSQALNVIGRHLESTLLAVHLYGSALDGGLKPYSDIDLLVTVAAPLND
AadA3           MGEFFPAQISEQLSHARGVIERHLAATLDTIHLFGSALDGGLKPDSDIDLLVTVSAAPND
AadA.Sch        MTLSIPPSIQCQTEAACRLITRVTGDTLRAIHLYGSAVAGGLKPNSDIDLLVTICQPLTE
                     .  .:. * .  .  :* *    ** ::**:***: ***** ********:     :

AadA1a          TTRRALINDLLETSASPGESEILRAVEVTIVVHDDIIPWRYPAKRELQFGEWQRNDILAG
AadA1b          TTRRALINDLLETSASPGESEILRAVEVTIVVHDDIIPWRYPAKRELQFGEWQRNDILAG
AadA2           TTRRALLNDLMEASAFPGESETLRAIEVTLVVHDDIIPWRYPAKRELQFGEWQRNDILAG
AadA7           AVRQALLVDLLEVSASPGQNKALRALEVTIVVHSDIVPWRYPARRELQFGEWQRKDILAG
AadA3           SLRQALMLDLLKVSSPPGDGGPWRPLEVTVVARSEVVPWRYPAYVSFSSVSGSGHNILSG
AadA.Sch        AQRATLMQELLALSSPPGASAEKRALEVTVVLYSQLVPWCFPPSREMQFGEWLREDICQG
                : * :*: :*:  *: ** .   *.:***:*  .:::** :*.  .:.  .   .:*  *

AadA1a          IFEPATIDIDLAILLTKAREHSVALVGPAAEELFDPVPEQDLFEALNETLTLWNSPPDWA
AadA1b          IFEPATIDIDLAILLTKAREHSVALVGPAAEELFDPVPEQDLFEALNETLTLWNSPPDWA
AadA2           IFEPAMIDIDLAILLTKAREHSVALVGPAAEEFFDPVPEQDLFEALRETLKLWNSQPDWA
AadA7           IFEPATTDSDLAILLTKAKQHSVVLAGSAAKDLFSSVPESDLFKALADTLKLWNSPPDWA
AadA3           TFEPSVLDHDLAILLTKARQHSLALLGPSAVTFFEPVPNEHFSKALFDTIAQWNSESDWK
AadA.Sch        IYEPAQQDWDIVLLITQILETSIPLKGERAERLFTPAPVAQLLKALRYPLDLWQSTADVQ
                 :**:  * *:.:*:*:  : *: * *  *  :* ..*  .: :**  .:  *:* .*

AadA1a          GDERNVVLTLSRIWYSAVTGKIAPKDVAADWAMERLPAQYQPVILEARQAYLGQEEDRLA
AadA1b          GDERNVVLTLSRIWYSAVTGRIAPKDVAADWAMERLPASISPVILEARQAYLGQEEDRLA
AadA2           GDERNVVLTLSRIWYSAITGKIAPKDVAADWAIKRLPAQYQPVLLEAKQAYLGQKEDHLA
AadA7           GDERNVVLTLSRIWYTAATGKIAPKDVAATWAMARLPAQHQPILLNAKRAYLGQEEDYLP
AadA3           GDERNVVLALARIWYSASTGLIAPKDVAAAWVSERLPAEHRPIICKARAAYLGSEDDDLA
AadA.Sch        GDEYHIVLTLARIWYTLSTGRFTSKDAAADWLLPQLPEEYAATLRAAQREYLGLEQQDWH
                *** ::**:*:****:  ** ::.**.** *   :** .  . :  *:  *** :::

AadA1a          SRADQLEEFVHYVKGEITKVVGK--
AadA1b          SRADQLEEFVHYVKGEITKVVGK--
AadA2           SRADHLEEFIRFVKGEIIKSVGK--
AadA7           ARADQVAALIKFVKYEAVKLLGASQ
AadA3           MRVEETAAFVRYAKATIERILR---
AadA.Sch        ILLPAVVRFVDFAKAHIPTQFT---
                       :: :.*      .
```

B

```
aacA            TAACAACTCATTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAATTCAGGCG tta
qacF            TAACAATTCATTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAATTCAGGCG tta
aadA7           TAACAATTCATTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAATTCAAGCG tta
aadA1a          TAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCG tta
aadB            TAACAATTCGTCCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAGGTG tta
aadA2           TAACAATTCGTTCAAGCCGACCGCGCTACGCGCGGCGGCTTAACTCCGGCG tta
aadA1b          TAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTACCTTGGCCG tta
                ****** ** * *********  **** ****  ********  *     * ***
```

FIG. 1. (A) Sequence alignment of the AAD(3″)-like enzyme encoded by the *aadA7* gene cassette with its homologues; (B) sequence alignment of the different *attC* sites related to the *aadA7*-associated *attC* site. Only the bases of the inverse core site (ICS) and core site (CS) which are conserved in all of the *attC* sites are shown, i.e., the TAAC of the ICS and the GTTA of the CS. The lowercase letters of the CS (G tta) correspond to the functional CS of the cassette, i.e., the one that is located upstream of the *aadA* gene and is linked to the G in the circularized cassette. Invariable positions in sequences are indicated by asterisks. Dashes, gaps; asterisks, invariable positions; periods and colons, different levels of similarity. The sequences used came from the following sources (given as GenBank accession numbers): AadA1a, X12870; AadA1b, M95287; AadA2, X68227; AadA3, Z50802; AadA.Sch, X68089; *aacA*, M29695; *aadB*, X04555; *qacF*, AF034958.

strains; only 4 out of the 11 aminoglycoside-resistant strains carry a class 1 integron (Table 4), and all 4 contain an *aadA* cassette encoding streptomycin and spectinomycin resistance. These strains were found in groups A, D, and E (Tables 2 and 4).

Three of these strains (ECOR strains 3, 37, and 48) carried an In2 integron, while the fourth (ECOR strain 31) carried an integron containing a single *aadA* cassette not previously described (see below). All four gave positive results in PCR screening for the *mer-1* locus carried by the family of Tn*21*-like transposons. These transposons also contain a class 1 integron (8, 29). We have confirmed the presence of Tn*21* in strains 3, 37, and 48; however, only three strains (3, 31, and 48), were found to be resistant to mercuric chloride (Table 2). Our results differ from those obtained by Summers and colleagues, who found seven Hg^r strains in the ECOR collection (ECOR strains 3, 31, 34, 37, 41, 48, and 65) (30, 59). This discrepancy is likely due to differences in the protocols followed in the two studies.

The integron of ECOR strain 31 has been found to contain a novel resistance cassette, *aadA7*, encoding an aminoglycoside adenylyltransferase. Its product is closely related to other

AAD(3″) enzymes found in gram-negative bacteria; it has about 70% identity with the enzymes encoded by *aadA1a*, *aadA1b*, and *aadA2*, 55% identity with that encoded by *aadA3*, and 44% identity with that encoded by *aadA*(Sch) (Fig. 1A). Notably, with the exception of *aadA*(Sch) from *Salmonella enterica* serovar Choleraesuis, all the gram-negative AAD(3″) enzymes are carried on integron cassettes. The *attC* site associated with *aadA7* is closely related to those found in six other resistance cassettes (Fig. 1B). Three correspond to the *aadA1a*, *aadA1b*, and *aadA2* cassettes, whose genes are also highly similar to *aadA7*. However, the *qacF*-associated *attC* site (43) is most similar, with only one difference out of 51 nucleotides (from the inverse core site to the G of the core site; see Fig. 1B). Other related *attC* sites are those found in the *aacA* cassette, which encodes an aminoglycoside 6′-acetyltransferase, and the *aadA1a* cassette (Fig. 1B). This family of related *attC* sites seems to be the type most represented among resistance cassettes. Conservation of associated *attC* sites among unrelated cassettes is typical of the situation found in the *Vibrio cholerae* and related superintegrons (36), supporting the hypothesis that resistance cassettes are picked up from superintegron cassette pools. Furthermore, this suggests that such cassettes are independently assembled from unrelated genes and related *attC* sites, probably from the wide variety of bacterial species which contain superintegrons (51).

REFERENCES

1. **Anton, A. I., A. J. Martinez-Murcia, and F. Rodriguez-Valera.** 1998. Sequence diversity in the 16S–23S intergenic spacer region (ISR) of the rRNA operons in representatives of the *Escherichia coli* ECOR collection. J. Mol. Evol. **47:**62–72.
2. **Bachman, B.** 1996. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 2460–2488. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 2. ASM Press, Washington, D.C.
3. **Barcus, V. A., A. J. B. Titheradge, and N. E. Murray.** 1995. The diversity of alleles at the *hsd* locus in natural populations of *Escherichia coli*. Genetics **140:**1187–1197.
4. **Bergthorsson, U., and H. Ochman.** 1995. Heterogeneity of genome sizes among natural isolates of *Escherichia coli*. J. Bacteriol. **177:**5784–5789.
5. **Boyd, E. F., and D. L. Hartl.** 1998. Chromosomal regions specific to pathogenic isolates of *Escherichia coli* have a phylogenetically clustered distribution. J. Bacteriol. **180:**1159–1165.
6. **Boyd, E. F., and D. L. Hartl.** 1997. Nonrandom location of IS*1* elements in the genomes of natural isolates of *Escherichia coli*. Mol. Biol. Evol. **14:**725–732.
7. **Boyd, E. F., C. W. Hill, S. M. Rich, and D. L. Hartl.** 1996. Mosaic structure of plasmids from natural populations of *Escherichia coli*. Genetics **143:**1091–1100.
8. **Brown, H. J., H. W. Stokes, and R. M. Hall.** 1996. The integrons In0, In2, and In5 are defective transposon derivatives. J. Bacteriol. **178:**4429–4437.
9. **Chiew, Y. F., S. F. Yeo, L. M. Hall, and D. M. Livermore.** 1998. Can susceptibility to an antimicrobial be restored by halting its use? The case of streptomycin versus *Enterobacteriaceae*. J. Antimicrob. Chemother. **41:**247–251.
10. **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.
11. **Desjardins, P., B. Picard, B. Kaltenböck, J. Elion, and E. Denamur.** 1995. Sex in *Escherichia coli* does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism. J. Mol. Evol. **41:**440–448.
12. **Ferat, J. L., M. Le Gouar, and F. Michel.** 1994. Multiple group II self-splicing introns in mobile DNA from *Escherichia coli*. C. R. Acad. Sci. Ser. III **317:**141–148.
13. **Garcia-Martinez, J., A. Martinez-Murcia, A. I. Anton, and F. Rodriguez-Valera.** 1996. Comparison of the small 16S intergenic spacer region (ISR) of the rRNA operons of some *Escherichia coli* strains of the ECOR collection and *E. coli* K-12. J. Bacteriol. **178:**6374–6377.
14. **Goullet, P., and B. Picard.** 1989. Comparative electrophoretic polymorphism of esterases and other enzymes in *Escherichia coli*. J. Gen. Microbiol. **135:** 135–143.
15. **Green, L., R. D. Miller, D. E. Dykhuizen, and D. L. Hartl.** 1984. Distribution of DNA insertion element IS*5* in natural isolates of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81:**4500–4504.
16. **Guttman, D. S., and D. E. Dykhuizen.** 1994. Clonal divergence in *Escherichia coli* as a result of recombination, not mutation. Science **266:**1380–1383.
17. **Hall, B. G., L. L. Parker, P. W. Betts, R. F. DuBose, S. A. Sawyer, and D. L. Hartl.** 1989. IS*103*, a new insertion element in *Escherichia coli*: characterization and distribution in natural populations. Genetics **121:**423–431.
18. **Hall, B. G., and P. M. Sharp.** 1992. Molecular population genetics of *Escherichia coli*: DNA sequence diversity at the *celC*, *crr* and *gutB* loci of natural isolates. Mol. Biol. Evol. **9:**654–665. (Erratum, **9:**1174.)
19. **Hall, R. M.** 1997. Mobile gene cassettes and integrons: moving antibiotic resistance in Gram-negative bacteria. Ciba Found. Symp. **207:**192–205.
20. **Hall, R. M., and C. M. Collis.** 1998. Antibiotic resistance in Gram-negative bacteria: the role of gene cassettes and integrons. Drug Resist. Updates **1:**109–119.
21. **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.
22. **Hall, R. M., and H. W. Stokes.** 1993. Integrons: novel DNA elements which capture genes by site-specific recombination. Genetica **90:**115–132.
23. **Hartl, D. L., and S. A. Sawyer.** 1988. Why do unrelated insertion sequences occur together in the genome of *Escherichia coli*? Genetics **118:**537–541.
24. **Herzer, P. J., S. Inouye, and T. S. Whittam.** 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. J. Bacteriol. **172:**6175–6181.
25. **Hill, C. W., G. Feulner, M. S. Brody, S. Zhao, A. B. Sadosky, and C. H. Sandt.** 1995. Correlation of Rhs elements with *Escherichia coli* population structure. Genetics **141:**15–24.
26. **Jones, M. E., E. Peters, A. M. Weersink, A. Fluit, and J. Verhoef.** 1997. Widespread occurrence of integrons causing multiple antibiotic resistance in bacteria. Lancet **349:**1742–1743. (Letter.)
27. **Lecointre, G., L. Rachdi, P. Darlu, and E. Denamur.** 1998. *Escherichia coli* molecular phylogeny using the incongruence length difference test. Mol. Biol. Evol. **15:**1685–1695.
28. **Lévesque, C., L. Piché, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel operons of resistance genes. Antimicrob. Agents Chemother. **39:**185–191.
29. **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.
30. **Liebert, C. A., J. Wireman, T. Smith, and A. O. Summers.** 1997. Phylogeny of mercury resistance (*mer*) operons of gram-negative bacteria isolated from the fecal flora of primates. Appl. Environ. Microbiol. **63:**1066–1076.
31. **London, N., R. Nijsten, A. van der Bogaard, and E. Stobberingh.** 1994. Carriage of antibiotic-resistant *Escherichia coli* by healthy volunteers during a 15-week period. Infection **22:**187–192.
32. **Marklund, B. I., J. M. Tennent, E. Garcia, A. Hamers, M. Baga, F. Lindberg, W. Gaastra, and S. Normark.** 1992. Horizontal gene transfer of the *Escherichia coli pap* and *prs* pili operons as a mechanism for the development of tissue-specific adhesive properties. Mol. Microbiol. **6:**2225–2242.
33. **Martinez-Freijo, P., A. C. Fluit, F. J. Schmitz, V. S. C. Grek, J. Verhoef, and M. E. Jones.** 1998. Class 1 integrons in gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. J. Antimicrob. Chemother. **42:**689–696.
34. **Martinez-Freijo, P., A. C. Fluit, F. J. Schmitz, J. Verhoef, and M. E. Jones.** 1999. Many class 1 integrons comprise distinct stable structures occurring in different species of *Enterobacteriaceae* isolated from widespread geographic regions in Europe. Antimicrob. Agents Chemother. **43:**686–689.
35. **Mazel, D., and J. Davies.** 1999. Antibiotic resistance in microbes. Cell. Mol. Life Sci. **55:**742–754.
36. **Mazel, D., B. Dychinco, V. A. Webb, and J. Davies.** 1998. A distinctive class of integron in the *Vibrio cholerae* genome. Science **280:**605–608.
37. **McKane, M., and R. Milkman.** 1995. Transduction, restriction, and recombination patterns in *Escherichia coli*. Genetics **139:**35–43.
38. **Milkman, R.** 1994. An *Escherichia coli* homologue of eukaryotic potassium channel proteins. Proc. Natl. Acad. Sci. USA **91:**3510–3514.
39. **Milkman, R., and M. M. Bridges.** 1993. Molecular evolution of the *Escherichia coli* chromosome. IV. Sequence comparisons. Genetics **133:**455–468.
40. **Nelson, K., F. S. Wang, E. F. Boyd, and R. K. Selander.** 1997. Size and sequence polymorphism in the isocitrate dehydrogenase kinase/phosphatase gene (*aceK*) and flanking regions in *Salmonella enterica* and *Escherichia coli*. Genetics **147:**1509–1520.
41. **Ochman, H., and R. K. Selander.** 1984. Standard reference strains of *Escherichia coli* from natural populations. J. Bacteriol. **157:**690–693.
42. **Picard, B., J. S. Garcia, S. Gouriou, P. Duriez, N. Brahimi, E. Bingen, J. Elion, and E. Danamur.** 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect. Immun. **67:**546–553.
43. **Ploy, M. C., P. Courvalin, and T. Lambert.** 1998. Characterization of In40 of *Enterobacter aerogenes* BM2688, a class 1 integron with two new gene cassettes, *cmlA2* and *qacF*. Antimicrob. Agents Chemother. **42:**2557–2563.
44. **Pupo, G. M., D. K. Karaolis, R. Lan, and P. R. Reeves.** 1997. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. Infect. Immun. **65:**2685–2692.
45. **Recchia, G. D., and R. M. Hall.** 1995. Gene cassettes: a new class of mobile

element. Microbiology **141:**3015–3027.
46. **Recchia, G. D., and R. M. Hall.** 1997. Origins of the mobile gene cassettes found in integrons. Trends Microbiol. **5:**389–394.
47. **Riley, M. A., and D. M. Gordon.** 1992. A survey of Col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of Col-plasmid lineages. J. Gen. Microbiol. **138:**1345–1352.
48. **Riley, M. A., Y. Tan, and J. Wang.** 1994. Nucleotide polymorphism in colicin E1 and Ia plasmids from natural isolates of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **91:**11276–11280.
49. **Roberts, M. C.** 1994. Epidemiology of tetracycline-resistance determinants. Trends Microbiol. **2:**353–357.
50. **Rolland, K., N. Lambert-Zechovsky, B. Picard, and E. Denamur.** 1998. *Shigella* and enteroinvasive *Escherichia coli* strains are derived from distinct ancestral strains of *E. coli*. Microbiology **144:**2667–2672.
51. **Rowe-Magnus, D. A., A.-M. Guerout, and D. Mazel.** 1999. Super-integrons. Res. Microbiol. **150:**641–651.
52. **Saluta, M. V., and I. N. Hirshfield.** 1995. The occurrence of duplicate lysyl-tRNA synthetase gene homologs in *Escherichia coli* and other procaryotes. J. Bacteriol. **177:**1872–1878.
53. **Sawyers, S. A., E. E. Dykhuizen, R. F. DuBose, L. Green, T. Mutangadura-Mhlanga, D. F. Wolczyk, and D. L. Hartl.** 1987. Distribution and abundance of insertion sequences among natural isolates of *Escherichia coli*. Genetics **115:**51–63.
54. **Selander, R. K., D. A. Caugant, and T. S. Whittam.** 1987. Genetic structure and variation in natural populations of *Escherichia coli*, p. 1625–1648. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
55. **Summers, A. O.** 1986. Organization, expression, and evolution of genes for mercury resistance. Annu. Rev. Microbiol. **40:**607–634.
56. **Sundstrom, L.** 1998. The potential of integrons and connected programmed rearrangements for mediating horizontal gene transfer. APMIS Suppl. **84:** 37–42.
57. **Wang, F. S., T. S. Whittam, and R. K. Selander.** 1997. Evolutionary genetics of the isocitrate dehydrogenase gene (*icd*) in *Escherichia coli* and *Salmonella enterica*. J. Bacteriol. **179:**6551–6559.
58. **Wang, Y. D., S. Zhao, and C. W. Hill.** 1998. Rhs elements comprise three subfamilies which diverged prior to acquisition by *Escherichia coli*. J. Bacteriol. **180:**4102–4110.
59. **Wireman, J., C. A. Liebert, T. Smith, and A. O. Summers.** 1997. Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates. Appl. Environ. Microbiol. **63:**4494–4503.
60. **Zhao, S., and C. W. Hill.** 1995. Reshuffling of Rhs components to create a new element. J. Bacteriol. **177:**1393–1398.