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High Genetic Stability of Integrons in Clinical Isolates of *Shigella* spp. of Worldwide Origin▽

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Over a 12-year period, 68 *Shigella* strains (31 *S. sonnei*, 30 *S. flexneri*, 4 *S. dysenteriae*, and 3 *S. boydii* strains) were collected in a French University Hospital from the stools of patients who generally had a recent history of travel to various parts of the world (91%), particularly Africa (67%). These strains were often resistant (streptomycin, spectinomycin, trimethoprim, tetracycline, and sulfonamides, 66 to 84%; ampicillin and chloramphenicol, 34 to 38%; nalidixic acid, 4%) and even multiresistant (87%), and they generally carried integrons (81%) of class 1 (21%), class 2 (47%), or both (13%). Class 1 integrons were associated with ampicillin resistance due to the production of an OXA-30 β-lactamase in *S. flexneri* and *S. dysenteriae*. Class 2 integrons were associated with trimethoprim resistance in *S. sonnei*. Class 1 and class 2 integrons were inserted within transposons Tn*21* and Tn*7*, respectively, themselves located on the bacterial chromosome, except in one strain. Class 1 integrons showed an atypical organization consisting of the insertion sequence IS*1* at the 3′ end instead of the typical 3′ conserved segment and two bla_{OXA-30} and *aadA1* gene cassettes, despite the absence of epidemiological relationships between the strains, and an apparently functional integrase. Class 2 integrons showed the same albeit classical organization with the three *dfrA1*, *sat*, and *aadA1* gene cassettes. Occasionally, the 3′ end was deleted and the *aadA1* gene cassette was unexpressed. Thus, integrons contributed only in part to the multidrug resistance of the *Shigella* strains. The highly conserved organization of integrons might be related to their location within mobile genetic superstructures.

The four *Shigella* species *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, and *Shigella sonnei* are responsible for gastroenteritis that may progress to mucoid bloody diarrhea, also known as dysentery. The most severe forms, encountered with *S. dysenteriae* type 1 (the "Shiga bacillus") in children under 5 years of age, lead to a mortality rate of 10 to 30% during outbreaks. *Shigella* spp. can be transmitted by contaminated food and water and through person-to-person contact. In developing countries with unsafe water supplies and substandard hygiene, shigellosis is widespread and causes extensive outbreaks. In industrialized countries, this disease has become rare, and it currently occurs as sporadic cases in migrant workers or those who travel to developing countries and is limited to epidemic episodes among children in daycare centers, individuals in custodial institutions, and homosexual men (27). Consequently, although shigellosis is a major public health concern, there is a great disparity between developing countries (over 163.2 million cases each year) and developed ones (1.5 million cases) (15). In France, 941 cases of shigellosis was recorded in 1999, and the annual incidence has been stable in the past five years (2).

Shigellosis is one of the acute enteric disease for which antimicrobial therapy is generally required to manage infection and reduce fecal excretion of the bacterium to prevent further transmission. Although *Shigella* spp. are intrinsically susceptible to all antibiotics that are active against gram-negative bacilli, under antibiotic pressure, they have progressively acquired resistances to commonly recommended drugs (12, 19, 25, 28, 38, 41). Resistance dissemination among *Shigella* spp. is facilitated by the ability of this genus to acquire mobile genetic elements such as plasmids or transposons (15). Moreover, these elements may harbor integrons that can integrate resistance gene cassettes by site-specific recombination and then provide an efficient means for cumulating resistance determinants (3, 10, 34). Integrons and their association with multidrug resistance have been studied in most *Enterobacteriaceae* and gram-negative rods (16, 17, 32, 36, 37, 43), and several surveys have analyzed the distribution of the integrons in strains of *Shigella* spp. However, these studies were generally confined to a single species (5, 9, 22, 24, 28) or class of integron (9, 13, 22, 25, 26), in a restricted geographic area (5, 9, 13, 22, 24, 25, 28, 30, 31, 38), and in the course of outbreaks (5, 22, 24, 28), and the genetic location and organization of these structures have been rarely described, especially for class 1 integrons (30, 39).

In this study, we examined the antibiotic resistance and the full integron content of all *Shigella* strains isolated over a 12-year period in a French University hospital for adults, where shigellosis essentially corresponds to unrelated episodes of traveler's diarrhea. The integrons' location and organization have been established and have revealed an unexpectedly high genetic stability over this prolonged period of time and despite the worldwide origin of the strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All isolates of *Shigella* spp. collected between January 1990 and November 2002 at the Saint-André Hospital of Bordeaux, France, which specializes in gastrointestinal diseases, were included in

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TABLE 1. Primers used in this study

Primer	Sequence (5′→3′)	Location	Reference or source
5′ Conserved segment	GGCATCCAAGCAGCAAG	5′CS integron 1	18
intI 1L	ACATGTGATGGCGACGCACGA	*intI1*	32
intI 1R	ATTTCTGTCCTGGCTGGCGA	*intI1*	32
intI 2L	CACGGATATGCGACAAAAAGGT	*intI2*	32
intI 2R	GTAGCAAACGAGTGACGAAATG	*intI2*	32
intI 3L	GCCTCCGGCAGCGACTTTCAG	*intI3*	32
intI 3R	ACGGATCTGCCAAACCTGACT	*intI3*	32
OXA1F	CACAATACATATCAACTTCGC	bla_{OXA-1}	29
OXA1R	GTGTGTTTAGAATGGTGATCGC	bla_{OXA-1}	29
TEM-A2	GTATCCGCTCATGAGACAATA	bla_{TEM-1}	8
TEM-EXT	TCTAAAGTATATATGAGTAAAC	bla_{TEM-1}	8
TnpM	GTCAGGGAAGACTCTATGACC	*tnpM* (Tn*21*)	This study
INT1FIN	CCTCACTAGTGAGAGGTAGGG	*intI1*	This study
OXA1Fin	TATGGGAAAACTGGTGCAGGA	bla_{OXA-1}	This study
AAD2DI	CAGGAACCGGATCAAAGAG	*aadA1*	This study
AADVD	AAGGTAGTCGGCAAATAATG	*aadA1*	This study
INSB	CTTTGTCATGCAGCTCCACC	IS*1* (*insb*)	This study
INSBR	GGTGGAGCATGACAAAG	IS*1* (*insb*)	This study
ORFINV	CCAGACTGCTCCTGTATGACC	IS*600* (ORFB)	This study
INT2INV	ACCTTTTTGTCGCATATCCGTG	*intI2*	This study
DFRA1-FIN	CATCGAGCCGGAAGGTGATG	*dfrA1*	This study
DFRA1R	GTTAGAGGCGAAGTCTTGGG	*dfrA1*	This study
ORFXINV	CCATTTATGACGACCAATGC	ORFX	This study
AP 12h	CGGCCCCTGT		44
ERIC1R	ATGTAAGCTCCTGGGGATTCAC		42
ERIC2	AAGTAAGTGACTGGGGTGAGCG		42

this study. They were identified to the genus and species levels using the API 20E system (bioMérieux, Marcy l'Etoile, France) and antisera provided by Bio-Rad (Marnes la Coquette, France). Serotyping was performed by the Institut Pasteur of Paris. Clinical cases were retrospectively analyzed to obtain epidemiological data, particularly recent travel (less than 1 month). *Escherichia coli* TOP10 and DH5α were the recipient strains in transformation experiments. *E. coli* Ec1484R (7), *Acinetobacter baumannii* BM4431 (32), and *E. coli* DH5α (p22K9) (4) were used as controls for the amplification of the *intI1*, *intI2* and *intI3* genes, respectively, and *E. coli* Ec1484R and *E. coli* Ec223 (6) were used for the amplification of the bla_{OXA-1} and bla_{TEM} genes, respectively. All bacterial strains were routinely cultured at 37°C on Mueller-Hinton (MH) agar (Diagnostics Pasteur, Marnes la Coquette, France) or in brain heart (Bio-Rad) or trypticase soy (Diagnostics Pasteur) broth.

Antibiotic susceptibility testing. Antibiotic susceptibility of the 68 isolates was determined for 27 antimicrobial agents (Bio-Rad) by the disk diffusion method in MH agar medium according to French guidelines (http://www.sfm.asso.fr).

β-Lactamase extraction and isoelectric focusing. β-Lactamases of the 26 isolates of *Shigella* spp. that are resistant to amino- and carboxypenicillins were released by ultrasonic treatment, and their pIs were determined by isoelectric focusing on a pH 3.5 to 10 ampholin polyacrylamide gel as described previously by Matthew et al. (23). Enzyme activities were detected by the iodine procedure in gel by using benzylpenicillin (75 μl/ml) as the substrate. β-Lactamases with known pIs, TEM-1 (pI 5.4) and OXA-1 (pI 7.4), were used as pI markers.

Transformation experiments and plasmid content analysis. Plasmid DNA was extracted from the 55 *Shigella* strains harboring an integron and *E. coli* DH5α carrying recombinant plasmids using an alkaline lysis method (1) and the QIAGEN Plasmid DNA Midi kit (QIAGEN, Courtaboeuf, France). Plasmid content was analyzed by electrophoresis on a 1% agarose gel and subsequent exposure to UV light in the presence of ethidium bromide. Plasmid DNA extracts obtained from the clinical strains were electroporated into *E. coli* TOP10 or *E. coli* DH5α cells with selection on MH agar plates containing either ampicillin (AMP) (100 μg/ml) or trimethoprim (TMP) (20 μg/ml).

PCR, sequencing, and cloning experiments. Total DNA of *Shigella* species strains was extracted as previously described (8). Gene detection was carried out under standard PCR conditions (35) using primers specific for the *intI1*, *intI2*, *intI3*, bla_{TEM}, and bla_{OXA-1} genes or laboratory-designed primers (Table 1) and 0.1 μg of whole-cell DNA of clinical isolates. A combination of laboratory-designed primers also served for the determination of the integrons' genetic organization in clinical strains and from recombinant plasmids and for the detection of the free circular gene cassette *aadA1* in cultures of *S. flexneri* Sf631 and *E. coli* DH5α containing plasmid pC631E (Table 1) grown overnight. Amplification of large fragments (more than 3 kb) was performed with the GeneAmp XL-PCR kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. The amplicons were revealed by electrophoresis on a 1.5% agarose gel and exposure to UV light in the presence of ethidium bromide. For sequencing purposes, the PCR products, purified through Sephacryl S400 spin columns (Amersham Biosciences, Orsay, France), and recombinant plasmids pC631E and pC2023B were used as a template in a single-cycle reaction by using laboratory-designed primers and the DYEnamic ET dye terminator kit (Amersham Biosciences). Sequences were analyzed with an ABI 310 automatic sequencer (Perkin-Elmer, Courtaboeuf, France) using the Sequencing Analysis software and were compared to each other and to homologous sequences with the Sequence Navigator software. The nucleotide and the deduced protein sequences were analyzed by using software available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

The whole-cell DNA of isolates Sf631 and Ss2023 was totally digested by the EcoRI or BamHI enzyme, respectively, and ligated into the corresponding site of the pBK-CMV cloning vector. *E. coli* DH5α strains harboring recombinant plasmids pC631E and pC2023B were selected on MH agar plates containing 100 μg/ml of ampicillin and 50 μg/ml of kanamycin or 20 μg/ml of trimethoprim and 50 μg/ml of kanamycin, respectively.

Hybridization. DNA-DNA hybridization was carried out as described previously by Sambrook et al. (35). To determine the number of each class of integron, the template was a Southern transfer of an agarose gel containing total DNA digested by the EcoRI restriction enzyme from the 23 isolates containing a class 1 integron and the HindIII restriction enzyme from the 41 isolates harboring a class 2 integron. Probes consisted of a 569-bp and a 789-bp PCR fragment generated from total DNA of isolates Sf631 and Ss2023, respectively, and corresponded to parts of the *intI1* and *intI2* genes. To assess the chromosomal location of the integrons, undigested total DNA and plasmid DNA for three representative strains and bla_{OXA-30}- and *dfrA1*-specific probes were used. Nonradioactive labeling of the probe and signal detection were achieved by following the manufacturer's instructions (Roche, Applied Science, Meylan, France).

Molecular typing. For the 19 isolates of *S. flexneri* and the 4 isolates of *S. dysenteriae* harboring an integron, random amplified polymorphic DNA assay and enterobacterial repetitive intergenic consensus PCR were performed with primers AP12h (44), 208, and 272 (21) and ERIC2 and ERIC1R (42), respectively. After a first cycle of denaturation for 10 min at 94°C, the 45 subsequent

TABLE 2. Distribution of *Shigella* strains

Continent[a]	No. of strains[b]				
	S. sonnei	*S. flexneri*	*S. dysenteriae*	*S. boydii*	Total
Africa	19	15	1	3	38
Europe	4	5			9
Asia	4	1	3		8
America	1	1			2
ND	3	8			11
Total	31	30	4	3	68

[a] Africa includes Algeria ($n = 3$), Burkina-Faso ($n = 2$), Cameroon ($n = 1$), Egypt ($n = 1$), Gabon ($n = 4$), Guinea ($n = 2$), Ivory Coast ($n = 4$), Madagascar ($n = 4$), Mali ($n = 2$), Mauritania ($n = 1$), Morocco ($n = 3$), Nigeria ($n = 1$), Senegal ($n = 7$), Sudan ($n = 1$), Togo ($n = 1$), and unknown country ($n = 1$); Europe includes France ($n = 5$), Portugal ($n = 1$), Romania ($n = 1$), and Spain ($n = 2$); Asia includes India ($n = 4$), Indonesia ($n = 1$), Syria ($n = 1$), Turkey ($n = 1$), and Vietnam ($n = 1$); America refers to Cuba ($n = 2$).

[b] Includes *S. sonnei* biotype g ($n = 31$); *S. flexneri* serotypes 2 ($n = 10$), 6 (Boyd88) ($n = 6$), 3 ($n = 3$), 1 ($n = 2$), y ($n = 2$), and 5 ($n = 1$) and undetermined ($n = 6$); *S. dysenteriae* serotypes 1 ($n = 2$) and 3 ($n = 2$); and *S. boydii* serotype 1 ($n = 1$). ND, not determined.

cycles of amplification consisted of a denaturation step for 1 min at 94°C, an annealing step for 1 min at 42°C, and an extension step for 1 min at 72°C, with a final extension step for 10 min at 72°C. The amplification products were analyzed by electrophoresis of 10-μl samples on 1.5% agarose gels in the presence of ethidium bromide.

Nucleotide sequence accession numbers. The sequences of the 5.4-kb insert of recombinant plasmid pC631E and part of the 16.6-kb insert of plasmid pC2023B are available in the GenBank nucleotide database under accession numbers DQ923619 and DQ975275, respectively.

RESULTS

Distribution of *Shigella* strains according to species and geographic origin. Over a 12-year period (1990 to 2002), 68 nonrepetitive isolates of *Shigella* spp. were collected at the Saint-André Hospital of Bordeaux exclusively from stools of individuals who presented symptoms of infectious diarrhea at the time of isolation. Most of the strains were *S. sonnei* (46%) and *S. flexneri* (44%) strains (Table 2). When their past history was available, it appeared that most of the patients (52/57; 91%) had recently traveled, often to developing countries, mainly Africa (38/57; 67%) (Table 2). The African strains, like the European strains (including five apparently autochthonous strains), belonged mainly to the *S. sonnei* and *S. flexneri* species, while the Asiatic strains were essentially *S. sonnei* and *S. dysenteriae* species. Finally, the three *S. boydii* strains were isolated from patients who had returned from Africa. Most often, the disease corresponded to isolated cases of shigellosis, except for two epidemic episodes concerning four isolates of *S. sonnei* collected from patients who had returned from travel to Gabon and three isolates of *S. sonnei* from patients who had returned from a humanitarian mission in the Ivory Coast.

Antimicrobial resistance of *Shigella* strains. Most *Shigella* strains (60/68; 88%) presented at least one acquired resistance phenotype (Table 3). The four species were affected but at variable frequencies: all *S. sonnei* and *S. dysenteriae*, 80% of the *S. flexneri*, and only one-third of the *S. boydii* strains were affected. The most frequent resistances were to streptomycin (STR), spectinomycin (SPT), TMP, tetracycline (TET), and sulfonamides (SUL) (66 to 84%), followed by AMP and chloramphenicol (CHL) (34 to 38%). Nalidixic acid-resistant strains (4%, two from India and one of unknown origin) were infrequent, and none of them were resistant to pefloxacin. All strains were fully susceptible to expanded-spectrum cephalosporins, kanamycin, gentamicin, tobramycin, netilmicin, and amikacin. Most strains (59/68; 87%) were resistant to at least three antibiotics (without taking spectinomycin into account). A total of 13 antibiotic resistance phenotypes were identified. The profile STR-SPT TET SUL TMP was most frequent in *S. sonnei* strains (18 strains), and AMP STR-SPT TET CHL SUL TMP was most frequent in *S. flexneri* strains (16 strains). Ampicillin resistance, analyzed by isoelectric focusing, PCR amplification, and sequencing, was due to the production of an OXA-30 β-lactamase (pI 7.4) in most *S. flexneri* and all *S. dysenteriae* strains and of a TEM-1 enzyme (pI 5.4) in a single *S. flexneri* and two *S. sonnei* strains.

Integron content and antibiotic resistance of the *Shigella* strains. Most *Shigella* strains (55/68; 81%) were found to harbor integrons of one or two classes: 23 strains possessed a class 1 integron (42%), 41 strains possessed a class 2 integron (75%), and 9 strains had both classes of integron; no class 3 integrons were detected (Table 4). All the strains harboring an integron were multidrug resistant (two to six resistance markers), and only five resistant strains of *S. flexneri* did not contain any integron. None of the eight susceptible strains possessed an integrase gene. The distribution of the integrons varied according to the species and the resistance phenotype. The *S. sonnei* and *S. boydii* strains contained a single integron of class 2, while the *S. flexneri* and *S. dysenteriae* strains carried a class 1 integron alone or associated with a class 2 integron. All strains harboring a class 1 integron were resistant to ampicillin due to the production of an OXA-30 enzyme and to streptomycin-spectinomycin (except for *S. dysenteriae* strain Sd629), tetracycline, and chloramphenicol, and the most prevalent phenotype was AMP STR-SPT TET CHL SUL TMP (17/23, 74%). All of the strains containing a class 2 integron were

TABLE 3. Antimicrobial resistance of *Shigella* strains

Species	No. of isolates (%)	No. of resistant isolates (%)[a]								
		AMP	STR	SPT	TET	CHL	SUL	TMP	NAL	Total
S. sonnei	31 (45.6)	2 (6.5)	31 (100)	28 (90.3)	23 (74.2)		23 (74.2)	31 (100)	2 (6.5)	31 (100)
S. flexneri	30 (44.1)	20 (66.7)	22 (73.3)	19 (63.3)	22 (73.3)	19 (63.3)	20 (66.7)	18 (60)	1 (3.3)	24 (80)
S. dysenteriae	4 (5.9)	4 (100)	3 (75)	3 (75)	4 (100)	4 (100)	1 (25)	2 (50)		4 (100)
S. boydii	3 (4.4)		1 (33.3)	1 (33.3)	1 (33.3)		1 (33.3)	1 (33.3)		1 (33.3)
Total	68 (100)	26 (38.2)	57 (83.8)	51 (75)	50 (73.5)	23 (33.8)	45 (66.2)	52 (76.5)	3 (4.4)	60 (88.2)

[a] NAL, nalidixic acid.

TABLE 4. Antimicrobial resistance of *Shigella* strains harboring an integron[a]

Species (no. of strains)	Integrase 1 gene		Integrase 2 gene		Integrase 1 + 2 genes		Total no. of resistant strains
	No. of strains	Resistance phenotype (no. of isolates)	No. of strains	Resistance phenotype (no. of isolates)	No. of strains	Resistance phenotype (no. of isolates)	
S. sonnei (31)	0		31	STR-SPT TMP (8) STR TET SUL TMP (1) STR TET SUL TMP NAL (2) STR-SPT TET SUL TMP (18) AMP STR-SPT TET SUL TMP (2)	0		31
S. flexneri (30)	12	AMP STR-SPT TET CHL (3) AMP STR-SPT TET CHL SUL TMP (9)	0		7	AMP STR-SPT TET CHL SUL TMP (7)	19
S. dysenteriae (4)	2	AMP STR-SPT TET CHL (2)	0		2	AMP TET CHL TMP (1) AMP STR-SPT TET CHL SUL TMP (1)	4
S. boydii (3)	0		1	STR-SPT TET SUL TMP (1)	0		1
Total (68)	14		32		9		55

[a] NAL, nalidixic acid.

resistant to trimethoprim and to streptomycin-spectinomycin (except for four strains), and the predominant phenotype was STR-SPT TET SUL TMP (19/41; 46%). Southern blot experiments carried out using the total DNA digested by EcoRI (class 1 integrons) or HindIII (class 2 integrons) produced, in all cases, a single band at a variable size (Fig. 1), indicating that a single copy of each class of integron was present.

Genetic location and structure of integrons. Plasmid content analysis by electrophoresis in agarose gels yielded several bands for all *Shigella* strains harboring an integron. However, transformation experiments using these plasmid DNA extracts failed to give any transformants except for a single strain of *S. flexneri* (Sf2032), which contained a plasmid that was found to be conjugative and to harbor a class 2 integron. These results suggested a chromosomal location of the integrons in nearly all cases. Southern blot hybridization with undigested total DNA and plasmid DNA of three representative strains (Sf631, Ss2023, and Sf633 containing both classes of integrons) and using bla_{OXA-30}- or *dfrA1*-specific probes also argued for the chromosomal location of these elements (data not shown).

In order to determine the gene cassette arrays of the class 1 integrons, cloning of ampicillin resistance from *S. flexneri* strain Sf631 exhibiting the prevalent phenotype was undertaken. The obtained clone carried recombinant plasmid pC631E, and sequencing of the 5.4-kb insert revealed the presence of an atypical class 1 integron encompassing the *intI1* gene, the *attI* recombination site, two cassettes including the bla_{OXA-30} and *aadA1* genes associated with their *attC* recombination sites, and the insertion sequence IS*1* instead of the conventional 3′ conserved segment end (Fig. 2). This element was inserted at its 5′ end downstream from the *tnpM* gene of the transposon Tn*21* and at its 3′ end in the *orfB* of IS*600.* PCR experiments with the 22 remaining strains of *Shigella* containing the *intI1* gene (18 *S. flexneri* and 4 *S. dysenteriae* isolates) showed the same atypical integron structure up to IS*1*, except for *S. flexneri* strain Sf2016, in which the *aadA1* gene cassette and the IS*1*

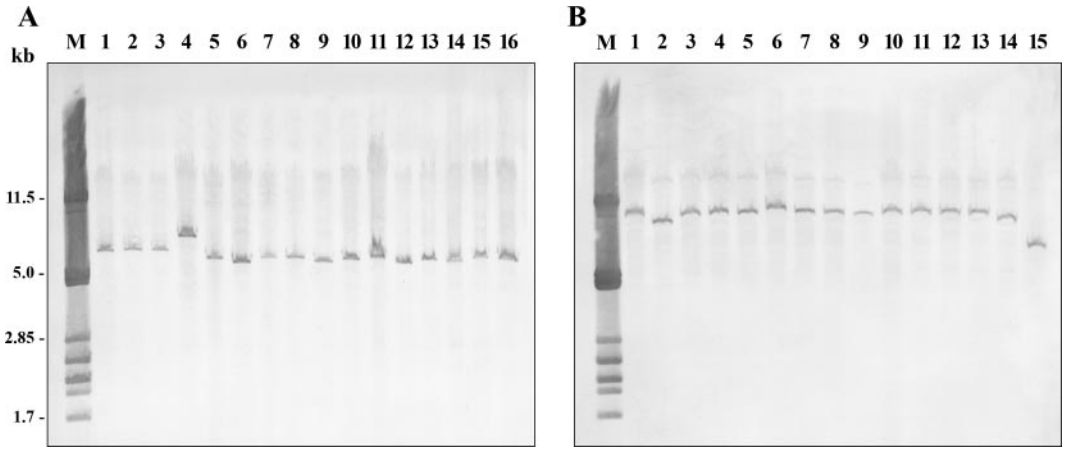

FIG. 1. Southern blotting and hybridization with *intI1* (A)- or *intI2* (B)-specific probes. Lane M, size marker consisting of λ phage DNA digested by PstI. (A) DNA of 14 strains of *S. flexneri* (lanes 1 to 14) and 2 strains of *S. dysenteriae* (lanes 15 and 16) digested with EcoRI. (B) DNA of 14 strains of *S. sonnei* (lanes 1 to 14) and 1 strain of *S. boydii* (lane 15) digested with HindIII.

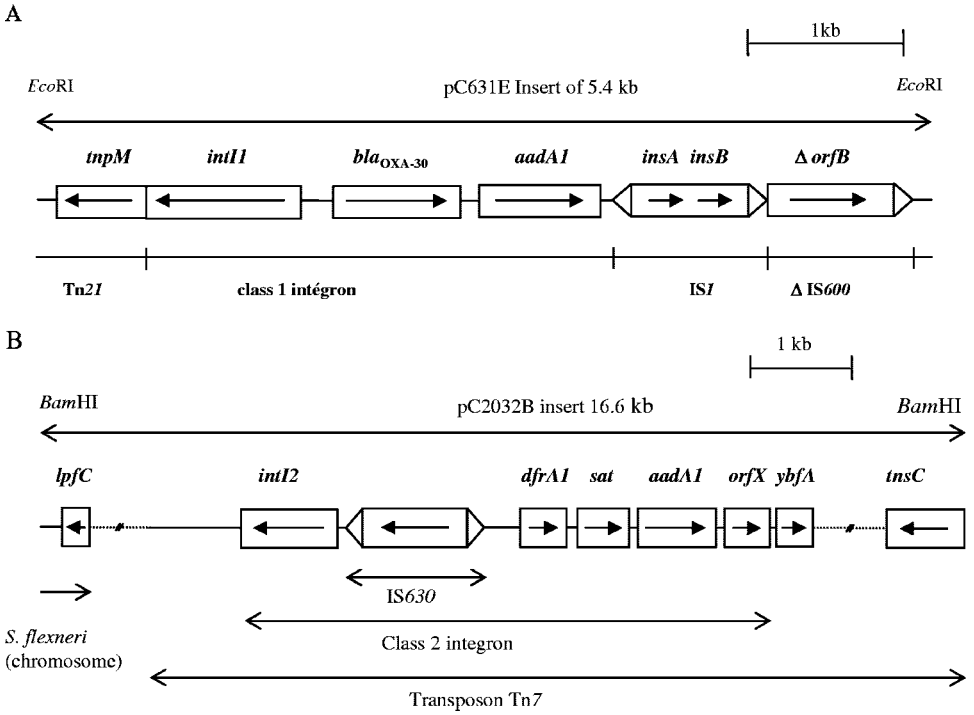

FIG. 2. Schematic representation of part of recombinant plasmid pC631E containing the atypical class 1 integron in strain Sf631 (A) and of recombinant plasmid pC2023B containing the class 2 integron in strain Ss2023 (B). The horizontal arrows indicate the translation orientation. The solid lines represent the sequenced fragments, with the different genes boxed, and the dotted lines indicate the unanalyzed sequences.

element were absent. Likewise, for all strains, the insertion sites in Tn*21* were identical.

In order to determine the gene cassette arrays of the class 2 integrons, cloning of trimethoprim resistance from *S. sonnei* strain Ss2023, presenting the predominant phenotype, was achieved. The clone obtained carried recombinant plasmid pC2023B, and sequencing of a part of the 16.6-kb insert revealed the presence of a classical class 2 integron comprising the *dfrA1*, *sat*, and *aadA1* gene cassettes and *orfX*, located in the Tn*7* transposon (Fig. 2). However, in pC2023B, the class 2 integron was interrupted by IS*630*, inserted between *intI2* and the *attI2* recombination site, and there was a 42-bp deletion within *orfX*. PCR experiments with the 40 remaining strains of *Shigella* containing the *intI2* gene (30 *S. sonnei* strains, 7 *S. flexneri* strains, 2 *S. dysenteriae* strains, and 1 *S. boydii* strain) showed the same organization of the class 2 integron as in Ss2023 (but without IS*630* and the *orfX* deletion) for 34 strains (83%). The lack of *orfX* was observed once, and the absence of a fragment encompassing the *aadA1* gene cassettes and *orfX* was detected in five cases (three streptomycin-resistant *S. sonnei* isolates, one *S. flexneri* isolate harboring a class 1 integron, and *S. dysenteriae* Sd629).

Epidemiological relationship between *Shigella* strains harboring class 1 integrons. The surprising conservation of the genetic organization of the class 1 integrons led us to check the relationship between the carrying strains. Serotyping, plasmid profile analysis, and molecular typing by random amplified polymorphic DNA and enterobacterial repetitive intergenic consensus PCR (data not shown) demonstrated an absence of an epidemiological link between the isolates of *S. flexneri* or *S. dysenteriae*. Moreover, the set of primers amplifying the junction between IS*1* and IS*600* for the 23 strains containing the *intI1* gene gave fragments of variable sizes (619 bp for Sf631 and another strain and ca. 750 bp for 10 strains), several fragments (five strains), or no amplicons at all (six strains), confirming that these strains were unrelated. Sequencing of the 750-bp fragment in an *S. dysenteriae* strain showed that the atypical integron was inserted within IS*600*, 125 bp upstream compared to Sf631.

Integrase and cassette gene expression. With regard to class 1 integrons, integrase expression was tested by PCR detection (primers AAD2DI and AADVD) of the free circular gene cassette *aadA1* in Sf631 and *E. coli* DH5α containing pC631E. An expected fragment of 550 bp was obtained only with *E. coli* strain DH5α harboring the recombinant plasmid, and sequencing confirmed the presence of the free circular gene cassette in three independent assays. The first bla_{OXA-30} gene cassette was always expressed in *E. coli* DH5α containing pC631E and in the clinical strains harboring the atypical class 1 integron, whereas the streptomycin-spectinomycin resistance conferred by the second gene cassette was lacking in *S. dysenteriae* strain Sd629 despite the presence of the *aadA1* gene.

Concerning the class 2 integrons, the IS*630* element between *intI2* and *attI2* in Sf2023 did not affect the expression of the gene cassette in *E. coli* DH5α containing pC2023B. When the genes were present, the clinical strains were resistant to trimethoprim and to streptomycin-spectinomycin, suggesting that the gene cassettes were regularly expressed. However, the conjugative plasmid of strain Ss2032 conferred only trimethoprim resistance despite the presence of the *aadA1* gene, but this

strain also harbored a chromosomally encoded class 1 integron likely responsible for its streptomycin-spectinomycin resistance.

DISCUSSION

Analysis of antibiotic resistance and its integron basis in *Shigella* spp. isolated in an industrialized country offers the opportunity to compare strains from various geographic origins, as observed in our study. The endemic species *S. sonnei* and *S. flexneri* were more prevalent than the epidemic species *S. dysenteriae*, as expected (2, 26). The *S. boydii* strains came from Africa rather than from India, as usually reported (27), but the geographic origin of the *Shigella* strains in industrialized countries is influenced by the preferential destinations of tourists and migrants.

Antibiotic resistance of *Shigella* spp. is usually frequent, particularly towards streptomycin, trimethoprim, tetracycline, and sulfonamides (14, 28). Data on ampicillin and chloramphenicol resistance rates and their distribution according to species vary greatly (5, 14, 22, 24, 25, 28, 31). Ampicillin, chloramphenicol, and cotrimoxazole are inexpensive antibiotics that have been used extensively for the treatment of shigellosis. Our study confirmed that they are now generally inefficient in the empirical treatment of this disease, even in industrialized countries. Quinolone-resistant strains were rare, in contrast with some recent Asiatic surveys (12, 14, 28), but they came from India.

Most *Shigella* strains harbored one or two classes of integron. Variable but often lower rates were reported elsewhere previously (5, 13, 26, 38). The apparent correlation between multidrug resistance and integron carriage was not related to the presence of these elements on conjugative plasmids, since they were chromosomally mediated in nearly all strains. When it has been documented, the genetic locations of antibiotic resistance genes and integrons in *Shigella* spp. have been found to be variable. Thus, class 2 integrons and bla_{OXA} genes were usually located on the chromosome, while the bla_{TEM} genes were commonly carried by conjugative plasmids (14, 28, 30, 31, 33, 38).

The distribution of the integrons correlated to some extent with the species and the resistance phenotype. Such a relationship has been inconstantly observed. Indeed, the predominance of class 2 integrons in *S. sonnei* has been previously observed, but in studies focusing on genetically related strains responsible for outbreaks (5, 9, 22, 24, 28, 30, 31), no class 2 integrons were detected in *S. sonnei* in one study (38), and integrons of class 1 have sometimes been reported in this species. For *S. flexneri*, the data concerning the integron content were still more discrepant: in a study from Brazil, class 2 integrons predominated (31); in other studies low (26) or high (25) class 1 integron rates were reported, and in the latter works, high frequencies of class 1 and 2 integrons were detected (30, 38). Finally, little information on the integron content of *S. boydii* and *S. dysenteriae* strains and on the absence of class 3 integrons in *Shigella* spp is available. The higher prevalence of the OXA-1-type β-lactamase in *S. flexneri* and *S. dysenteriae* and the higher prevalence of the TEM-1-type enzyme in *S. sonnei* have been reported previously (14, 25, 30, 31, 38), but the reason why the OXA-30 enzyme is widespread in some *Shigella* species although the TEM-1 enzyme is by far the most common β-lactamase responsible for ampicillin resistance in the taxonomically related species *E. coli* and in other *Enterobacteriaceae* remains unexplained.

Cloning and PCR experiments showed that almost all class 1 integrons of *Shigella* spp. exhibited the same atypical structure. The lower rates of class 1 integrons in *S. flexneri* mentioned previously in some studies (26, 31) might be due to the use of primers amplifying classical integrons with the 3′ conserved segment fragment. A similarly atypical class 1 integron on the chromosome of *S. flexneri* 2a strain YSH6000 isolated in Japan was described previously (33). This integron was located in the *Shigella* resistance locus (SRL) of 16.7 kb, which encompasses chloramphenicol and tetracycline resistance determinants within a 66-kb pathogenicity island (PAI) designated the SRL PAI (20). The insertion of the class 1 integrons within the SRL might explain the chloramphenicol and tetracycline resistances observed in all our *S. flexneri* and *S. dysenteriae* strains containing a class 1 integron. The sequence of the 5.4-kb insert of pC631E shared 98.8% identity with the corresponding sequence of YSH6000 (GenBank accession number AF326777), with the major difference being the deletion of a 57-bp fragment in the central region of IS*600* in strain YSH6000. Similar fragments containing *intI1*, $bla_{\mathrm{OXA\text{-}30}}$, and *aadA1* genes and part of the IS*1* sequence have also been found in strains of various *Shigella* species from Japan and Australia (39) and the People's Republic of China (30). Thus, this chromosomal structure of resistance determinants previously observed in Asia and Oceania (30, 39) was also present in strains isolated in Africa, Europe, and Central America. The major sequence difference among our strains was the insertion of IS*1* within *orfB* of IS*600*, suggesting that the integration of the IS*1*-flanked fragment into the PAI might be due to independent events in these strains, especially in different species such as *S. flexneri* and *S. dysenteriae*. Indeed, the PAI involves several insertion sequence-flanked elements that can be lost under specific conditions (40). Thus, it is possible that insertion sequence regions may be deleted or inserted independently at hot spots, such as *orfB* of IS*600*, as is the case for the integration of class 1 integrons within the transposons of the Tn*3* family. The absence of a relationship between the strains led us to question about the functionality of the integrase to explain such conserved genetic organization. Preliminary experiments on the excision capability of the enzyme have suggested that IntI1 might be functional but repressed in its chromosomal location. Similarly, the absence of expression of the second gene cassette in one strain of *S. dysenteriae*, corroborated by previous reports (30), raises the question of a regulatory mechanism.

Class 2 integrons in *Shigella* strains, in contrast, had a classical organization (11) despite some variations, particularly a more frequent 3′-end deletion than in class 1 integrons. Since an internal stop codon within the *intI2* gene renders the type 2 integrase nonfunctional, the cassette arrays in class 2 integrons are usually constant (5, 9, 24, 30, 31). The absence of expression of the *aadA1* gene cassette was observed once, suggesting the existence of a regulatory process, as for class 1 integrons, but the literature does not provide much information on this aspect.

In conclusion, antibiotic resistance of *Shigella* spp. in industrialized countries appeared to be frequent and related to their

epidemiology in developing countries. Most strains carried class 1 and/or class 2 integrons that contributed only in part to the multidrug resistance of *Shigella* spp., and their chromosomal location should limit resistance dissemination. This highly conserved organization of the class 1 integron in unrelated *Shigella* strains might be linked to the insertion and stabilization of these elements within mobile genetic superstructures such as SRL and PAI, depending on mechanisms that should be explored further, as the expression of the distal gene cassettes.

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