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ANALYSIS OF GENE CASSETTES OF STREPTOMYCIN-SPECTINOMYCIN RESISTANCE OF HUNGARIAN *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM STRAINS

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By PCR using the ant(3")-Ia primer pair the *aadA* gene was detected in 34 streptomycin- and spectinomycin-resistant *Salmonella enterica* serotype Typhimurium strains. Out of them 12 belonged to DT104 and 22 to non-DT104 phage type. Using different primer combinations it was demonstrated that this gene was integron-associated in all cases: in the DT104 strains it was generally contained by a 1 kb integron while in the majority of the non-DT104 strains by a 2.05 kb (less often by a 1.9 or 1 kb) integron. In the case of integrons carrying multiple cassettes the cassette containing the *aadA* gene was located closer to the 3' end of the integron. The *aadA* genes of DT104 and non-DT104 strains were different: in the former group the *aadA2* gene, while in the latter group (constituted by strains of five different phages types as well as unclassifiable and untypable strains) the *aadA1* gene could be identified. The RH50/RH51 primer pair described by Collis and Hall (1992) proved to be suitable for rapid discrimination between the *aadA1* and *aadA2* genes on the basis that the RH51 primer bound exclusively to the *aadA2* gene.

Key words: *Salmonella* Typhimurium, streptomycin/spectinomycin resistance, *aadA* gene cassettes, sequencing

Salmonella enterica serotype Typhimurium (*S.* Typhimurium) phage type DT104 has great epidemiological importance in Europe and in the United States (Threlfall et al., 1994; Low et al., 1997; Besser et al., 1997) and is usually characterised by the high prevalence of multidrug resistance to ampicillin (Amp), chloramphenicol (Chl), streptomycin (Sm), spectinomycin (Spc), sulphonamides (Su) and tetracycline (T) (Threlfall et al., 1996; Glynn et al., 1998; Ridley and Threlfall, 1998). In the 1990s that phage type spread widely also in Hungary, and

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it had become the dominant phage type already by 1991 (Szmollény et al., 2000; Pászti et al., 2001). Within the DT104 phage type, only in Felix and Callow's (1951) phage type 2 group did the incidence of multiresistant strains substantially exceed the average level, while in the phage type 2c group it did not (Pászti et al., 2001).

The *aadA* gene responsible for Sm/Spc resistance is the commonest integron-associated gene among isolates of *Enterobacteriaceae* in Europe (Martinez-Freijo et al., 1999). Its variants constitute a gene family the two most important members of which are *aadA1* sequenced by Hollingshead and Vapnek (1985), Fling et al. (1985) and Sundström et al. (1988), and *aadA2* sequenced by Tait et al. (1985) and Bito and Susani (1994). Further variants (*aadA3-A8, aadAsc*) have been described from *E. coli, Pseudomonas aeruginosa* and *Salmonella choleraesuis* isolates (Leung et al., 1992; Naas et al., 1999; Adrian et al., 2000; Mazel et al., 2000; White et al., 2000; Peters et al., 2001). The integron nature of the *aadA* gene and the plasmid- and/or transposon-associated location of the integrons may have played a role in the excessive horizontal spread of *aadA*.

In both of the above two groups of the DT104 strains analysed in this study, Sm/Spc resistance was closely associated with the presence of a 1 kb integron. For the majority of the other, non-DT104 *S.* Typhimurium strains it was associated with an integron of 2.05 kb size (Gadó et al., 2003).

The objective of this work was to identify and characterise the gene responsible for Sm/Spc resistance in a group of both DT104 and non-DT104 strains, to determine their association with integron(s) and to decide whether there exists an Sm/Spc resistance gene typical of the DT104 phage type. The existence of such a gene would support the notion that the DT104 phage type should be regarded as a distinct clone.

To attain the above objectives, PCR experiments were performed using the ant(3")-Ia F/ant(3")-Ia B (antF/antB) primer pair (Sandvang et al., 1998) suitable for the detection of *aadA* type genes. Amplicons were produced, besides the above primer pair, also with the RH50/RH51 primer pair (Collis and Hall, 1992), and with the combinations of these primers with each other and with the 5'-CS and 3'-CS primers (Sandvang et al., 1998). The integron nature of the *aadA* gene was demonstrated on the basis of the appearance of amplicons, while its presence in the individual integrons was shown by sequencing. The comparison of sequences indicated that the *aadA* genes of DT104 and non-DT104 strains were different.

Materials and methods

Salmonella *strains*

Forty-one *S.* Typhimurium strains were isolated and serotyped at the County Institutes of Public Health and Medical Officer Service in 1997–1999.

Four strains were isolated from animals or from foods of animal origin at the County Veterinary and Food Control Stations and serotyped in the National Food Investigation Institute in 1998–2000 (Table 1) The strains were grown on nutrient agar (Oxoid No. 2) without any selection and stored on stock agar at room temperature. Their phage type was determined by the methods of Anderson et al. (1977) and Felix and Callow (1951). Their antibiotic susceptibility testing was done by disk diffusion method on Mueller-Hinton agar with sheep blood using antibiotic disks (Oxoid) as follows: ampicillin (Amp, 20 μg), chloramphenicol (Chl, 30 μg), streptomycin (Sm, 30 μg), tetracycline (T, 30 μg), gentamicin (Gm, 20 μg), kanamycin (Km, 3020 μg), Sumetrolim (Sxt, 25 μg), cefoperazone (Cfp, 75 μg), cephalexin (Cfl, 30 μg), cefuroxime (Cxm, 30 μg) and nalidixic acid (N, 30 μg). (The disks were Oxoid products.) Spectinomycin (Spc) sensitivity of Sm-resistant strains was tested with plating on nutrient agar (Oxoid No. 2) containing Spc (Sigma) dilution series, while the sulphamethoxazole (Su) resistance of integron carrier strains was checked with Oxoid disks (25 μg). Spc and Su resistance was not taken account in the R-types.

PCR

Fifty μl final volumes contained 25 pmol of both forward and reverse primers, 5 μl of 10× PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KCl, 1% Triton X-100], dATP, dCTP, dGTP, dTTP 200 μM each, 2.5 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (Promega Corp., Madison, USA) and 5 μl supernatant of cell suspension boiled for 10 min. Amplifications were performed in thermocycler 'Progene' (Techne, Cambridge, UK) using the following amplification cycles: an initial denaturation of 5 min at 94 °C was followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 5 min at 72 °C. After the last cycle, the mixture was incubated for 5 min at 72 °C.

For determination of integron patterns (IPs) primers 5'-CS (5'-GGC ATC CAA GCA GCA AG-3') and 3'-CS (5'-AAG CAG ACT TGA CCT GA-3'), (Lévesque et al., 1995), for detection of *aadA* genes primers ant (3")-Ia F (antF) (GTG GAT GGC GGC CTG AAG CC) and ant (3")-Ia B (antB) (ATT GCC CAG TCG GCA GCG) (Sandvang et al., 1998) and RH50 (CAT CAA GCT TTA CGC CCA CAG TAA CC) and RH51 (CGC AGA TCA CTT GGA AGA ATT CAT TCG C) (Collis and Hall, 1992) were used. The primer combinations used for producing various amplicons can be seen in Fig. 1.

The amplicons were detected by horizontal agarose gel electrophoresis in 2% agarose (Sigma type I) with Tris-acetate/EDTA buffer applying 140 V for 3 h. The pGEM (Promega) was used as molecular marker. The results were visualised by staining with 0.5 μg/ml ethidium bromide and evaluated over UV transillumination applying GelDoc (BioRad) with Quantity One software.

Table 1
S. Typhimurium strains used in the present work

Strain	Phage type		R-type	Isolation		IP[d]	Sm/Spc resist-ance
	A[a]	F–C[b]		Year	Source[c]		
A: DT 104 strains							
M81	104L	2c	SCTACfp	1998	H	3	+
M82	104L	2c	A	1998	H	2	–
M86	104L	2c	SCTAN	1998	H	3	+
M117	104L	2c	SCTAN	1998	H	3	+
M147	104H	2c	Sxt	1998	H	–	–
M168	104L	2	S	1998	H	1	+
M172	104B	2b	–	1998	H	–	–
M235	104L	2	SCTANCfp	1998	H	3	+
M256	104L	2	SKGN	1998	H	1	+
M278	104B	2	SCTACfp	1998	H	3	+
M279	104L	2c	SCTA	1998	H	3	+
M349	104L	2a	SCTACfp	1998	H	3	+
M593	104L	2	A	1998	H	2	–
M602	104B	2c	S	1998	H	4	+
M656	104L	2	S	1998	H	1	+
NB128	104L	2	SCTA	1998	A	3	+
B: non-DT104 strains							
M98	DT193	2b	SCTAKSxtCflCfpCxm	1998	H	9	+
M152	UNTY	35	SCTAKGCflCfpCxm	1998	H	9	+
M155	UNTY	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M179	UNTY	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M182	UNTY	35	SCTAKGCflCfpCxm	1998	H	9	+
M187	UNTY	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M189	UNTY	35	SCTAKGCflCfpCxm	1998	H	9	+
M193	UNTY	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M268	UNTY	4	SCTAKSxtCflCfpCxm	1998	H	9	+
M275	UNTY	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M345	UNTY	2b	CTK	1998	H	–	–
M347	UNTY	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M472	DT193	35	SCTAKSxtCflCfpCxm	1997	H	9	+
M519	RDNC	35	SCTAN	1999	H	3	+
M534	RDNC	2c	SCTAN	1999	H	3	+
M560	30	Nt	–	1999	H	–	–
M566	DT1	1	SKSxt	1999	H	6	+
M577	DT193	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M600	UNTY	35	–	1998	H	–	–
M626	DT193	35	SCTAKSxtCflCfpCxm	1998	H	9	+
M627	UNTY	35	–	1998	H	–	–
M630	DT193	2b	SCTASxtCflCfpCxm	1998	H	9	+
M641	RDNC	2a	A	1998	H	–	–
M651	RDNC	2d	SG	1998	H	5	+
M653	DT29	2d	TAK	1998	H	–	–
M676	RDNC	2c	SCTAN	1997	H	3	+
NB214	DT99	2b	ST	1998	A	9	+
NB215	DT120	35	SCAN	1998	A	3	+
NB227	DT193	35	STA	1998	A	–	–

[a]by the method of Anderson et al. (1977); [b]by the method of Felix and Callow (1951); [c]H: human, A: animal or food origin; [d]Integron patterns (Gadó et al., 2003); IP1: 1, IP2: 1.2, IP3: 1+1.2, IP4: (0.64)+2.05, IP5: 1+(2.2); IP6: 1.9, IP9: (0.7)+ 1.45+2.05+ (3.5) kb integrons; In parentheses: weak, unstable bands; RDNC = This culture reacts with the typing phages but does not conform to a recognised type; UNTY = untypable

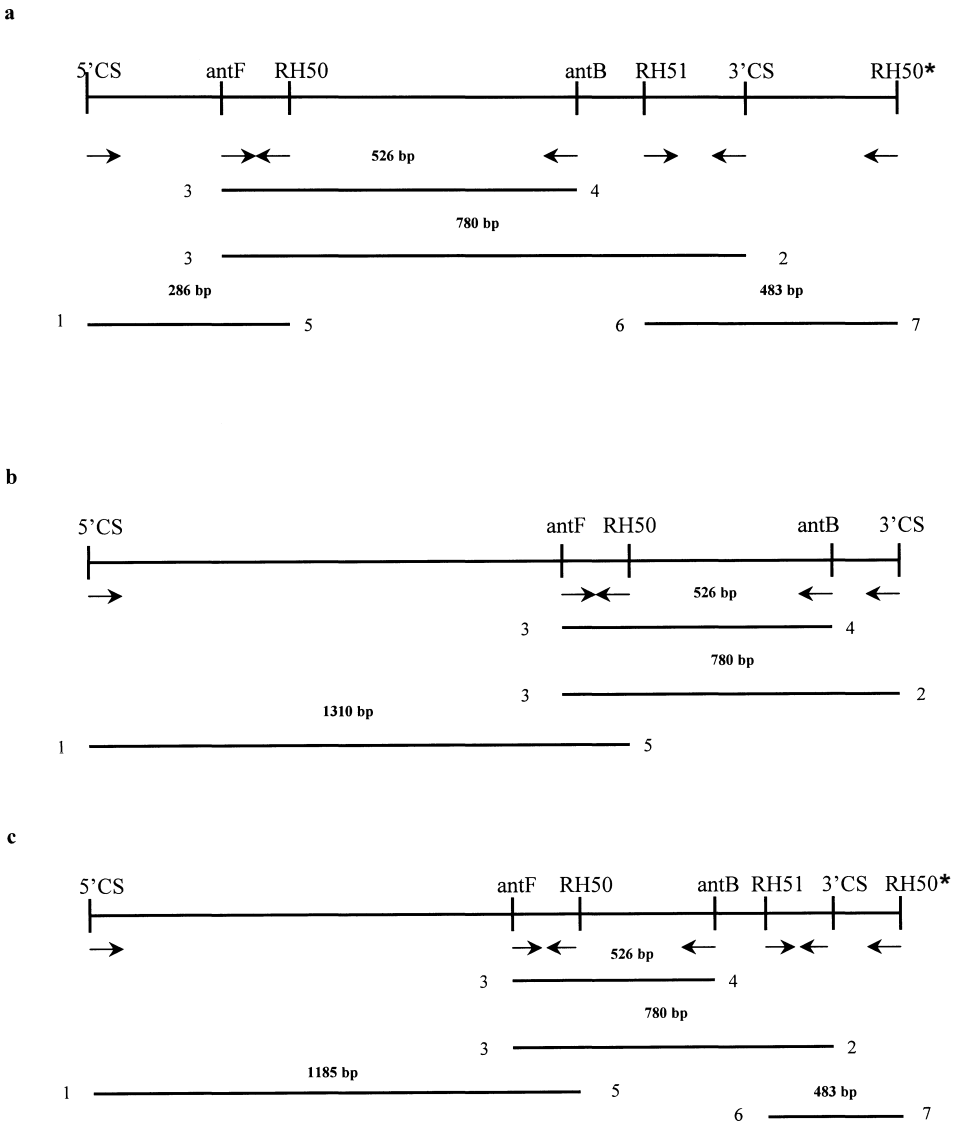

Fig. 1. Location and size of the sequenced amplicons obtained from strains representing different integrons. Amplicons obtained from strains having 1.0 kb (a), 2.05 kb (b) and 1.9 kb (c) integrons. Strains used: a: M117 (typical DT104), 256 (irregular DT104), 651 (typical non-DT104). (RH50/RH51 amplicon was obtained only in the case of strain M117). b: M182 (typical non-DT104), 602 (irregular DT104). c: M566 (irregular non-DT104). Primers and their binding sites: 5'-CS: 1, 3'-CS: 2, antF: 3, antB: 4, RH50: 5, 7*, RH51: 6. *second binding site in the *qacEΔ1* gene. Horizontal arrows indicate positions of primers and direction of synthesis

Sequencing

The amplicons to be sequenced were isolated from 1% agarose (Sigma type I) with QIAquick Gel Extraction kit (Qiagen Inc., USA) according to the instructions of the manufacturer. Cycle sequencing was performed in the Biological Research Center of the Hungarian Academy of Sciences with dye-terminator method using DNA sequencer model ABI 373.

Results

A PCR experiment using the antF/antB primer pair indicative of the presence of the *aadA* gene was performed with 10 Sm-sensitive and 35 Sm-resistant *S.* Typhimurium strains. (Four of the sensitive and 12 of the resistant strains were of phage type DT104.) Irrespective of the phage type, a close correlation was found between Sm resistance and the appearance of an amplicon of the size expected on the basis of the literature (526 bp). A single strain constituted an exception: unlike the other strains, this strain was Spc sensitive and integron free.

By PCR using CS/ant combined primer pairs it was demonstrated that the *aadA* gene was located on an integron in all cases. The gel electrophoretogram obtained with the DT104 and non-DT104 strains is shown in Fig. 2. Both CS/ant combinations produced an amplicon, but while the 3'-CS/ant F amplicon was approx. 780 bp irrespective of the phage type, the size of the 5'-CS/ant B amplicon was in average 723 bp for DT104 and non-DT104 strains having a 1 kb integron, and in average 1782 bp for the non-DT104 group carrying a 2.05 kb integron. For the DT104 strain designated M602 and having a different IP an amplicon 1690 bp in size was obtained. From the several amplicons obtained by the use of the 5'-CS/antB primer pair, the specific amplicon was selected by the comparison of *aadA* positive and negative strains, and this was confirmed also by sequencing the amplicon (data not shown).

Detection of the *aadA* gene was also attempted by the use of the RH50/RH51 primer pair for 34 Sm/Spc resistant strains which contained an *aadA* gene demonstrable by the antF/antB primer pair (Table 2). It can be seen that while 10 out of 12 DT104 strains were positive (producing a 483 bp amplicon), of the 22 non-DT104 (DT1, 29, 30, 120, 193, unclassifiable and untypable) strains only those 5 strains were positive which contained 1 + 1.2 kb or 1.9 kb integrons. Surprisingly, however, with the 5'-CS/RH50 primer combination all Sm/Spc resistant strains possessing the *aadA* gene produced an amplicon. The size of that amplicon was 286 bp for strains with a 1 kb integron and 1185–1310 bp for strains containing a 1.9–2.05 kb integron (Fig. 3). Using the other possible primer combination (3'-CS/RH51), only those strains produced a uniformly approx. 200 bp amplicon which were positive with the RH50/RH51 primer pair (Table 2).

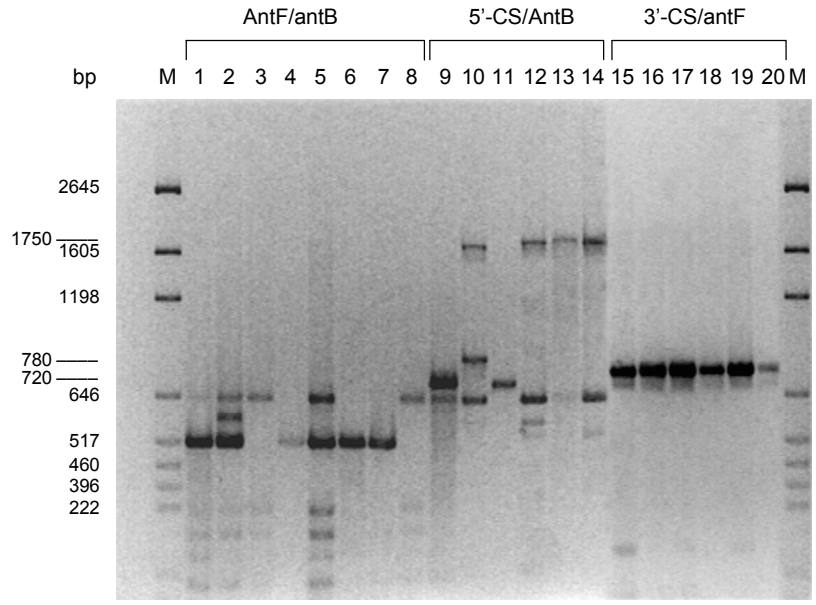

Fig 2. Detection of gene *aadA* by PCR applying antF/ant B primers. Strains used: M81 (DT104, IP 3), M593 (DT104, IP 2), M602 (DT104, IP 4), M98, M182, M187 (non-DT104, IP 9), M534 (non-DT104, IP 3). All of them was Sm/Spc resistant, except M593 which was Sm/Spc sensitive. NB227: non-DT104 (integron-free, Sm resistant, Spc sensitive. Lanes: 1, 9, 15: M81; 2, 10, 16: M602; 3: M593; 4, 11, 17: M534; 5, 12, 18: M98; 6, 13, 19: M182; 7, 14, 20: M187; 8: NB227. Marker: pGEM (Promega)

Table 2

The effectiveness of RH50 and RH51 primers in the presence of *aadA* gene

Phage type	Integron		Primer pairs		
	content (kb)[a]	pattern[b]	RH50/RH51	5'-CS/RH50	3'-CS/RH51
DT 104 n = 12	1	1	2/3	3/3	2/3
	1 + 1.2	3	8/8	8/8	8/8
	(0.64) + 2	4	0/1	1/1	0/1
Non-DT 104 n = 22	1 + 1.2	3	4/4	4/4	4/4
	1 + (2.2)	5	0/1	1/1	0/1
	1.9	6	1/1	1/1	1/1
	(0.7) + 1.45 + 2.05 + (3.5)	9	0/16	14/16	0/16

[a]amplicons obtained with 5'-CS/3'-CS primers; [b]according to Gadó et al., 2003; ( ): integrons lost during storage; Numbers: ratio of amplicon-producing strains

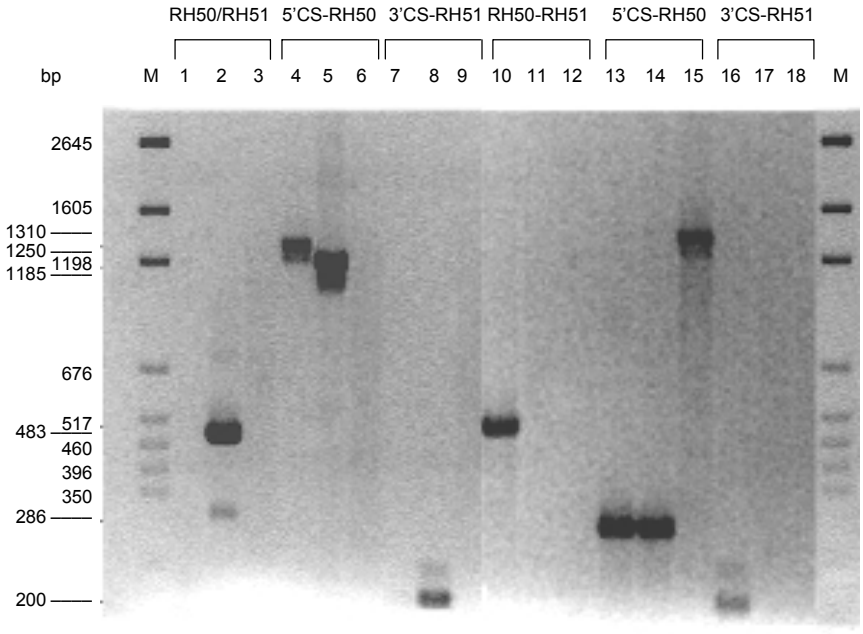

Fig. 3. Detection of gene *aadA* by PCR applying RH50/RH51 primers. Strains used: M182 (non-DT104, IP 9), M566 (non-DT104, IP 6), NB227 (non-DT104, integron-free), M117 (DT104, IP 3), M651 (non-DT104, IP 5), M602 (DT104, IP 4). All of them were Sm/Spc resistant, except NB227, which was Sm resistant and Spc sensitive. Lanes: 1, 4, 7: M182; 2, 5, 8: M566; 3, 6, 9: NB227; 10, 13, 16: M117; 11, 14, 17: M651; 12, 15, 18: M602. Marker: pGEM (Promega)

Subsequently the typical representative strains of the DT104 and non-DT104 groups were selected, together with some 'irregular' strains which reacted with the RH50/RH51 primer pair in a manner different from the behaviour shown by their group (Table 3). In the case of these six strains, the location of the *aadA* gene was demonstrated by partially sequencing the 1, 1.2, 1.45, 1.9 and 2.05 kb amplicons obtained using the 5'-CS/3'-CS primer pair, and it was found that it occurred exclusively in the 1, 1.9 and 2.05 kb integrons: in the 1 and 1.9 kb integrons of strain M117 (typical DT104) and strain M566 (irregular non-DT104), and in the 1 and 2.05 kb integrons of the other four strains (typical non-DT104 and irregular DT104) (unpublished data).

To identify the *aadA* gene, the sequences of amplicons obtained by the use of different primer pairs and covering the entire *aadA* gene were compared with the *aadA1* and *aadA2* sequences of the DataBank. The amplicons tested are shown in Fig. 1 and the results in Table 4. It can be seen that in the case of strains M117 (typical DT 104) and M566 (non-typical non-DT 104) three amplicons (antF/antB, 3'-CS/antF and 5'-CS/RH50) gave 96–100% homology with the *aadA2* standard (accession No. AF 071555) and only 87–91% homology with

the *aadA1* sequence (accession No. AJ 009819). The other four representative strains (M182, M256, M602 and M651) showed 97–100% homology with AJ 009819 (*aadA*1) and only 86–92% homology with AF 071555 (*aadA2*).

Table 3

Representative strains used for sequencing

Phage type[c]	Integron		Strains	Amplicon with primers RH50/RH51
	content (kb)[a]	pattern[b]		
DT 104	1 + 1.2	3	M117[x]	+
	1	1	M256[xx]	–
	2	4	M602[xx]	–
Non-DT 104	1.45 + 2.05	9	M182[x]	–
	1	5	M651[x]	–
	1.9	6	M566[xx]	+

[a]amplicons obtained with 5'-CS/3'-CS primers (present status); [b]according to Gadó et al. (2003); [c]according to Anderson et al. (1977); [x]typical; [xx]irregular strains

Figure 4/a shows the sequence of the primary binding site of RH50 within the antF/antB amplicon. When strains M117 and M566 having a sequence identical with the *aadA2* standard are compared with sequences of the other four strains, a difference in the RH50 binding site can be seen at 3 bp. This, however, did not prevent the functioning of this primer (the 5'-CS/RH50 amplicon of the expected size was produced in all *aadA*-positive strains tested; Table 2). The antF/3'-CS amplicon contained the binding site of RH51 (Fig. 4/b), which differed from the *aadA2* standard at 3 and 4 points, respectively, thus rendering dysfunctional the RH51 primer.

Table 4

Identities with the deposited DataBank sequences

Amplicon	Strain	Identity (%) with	
		AF 071555 (*aad A2*)	AJ009819 (*aadA1*)
antF/antB[a]	117, 566	96–99	88–91
	182, 256, 602, 651	90–91	97–99
3'CS/antF[b]	117, 566	96–99	87–89
	182, 256, 602, 651	87–88	98–100
5'CS/RH50[a]	117	100	87
	256, 651	91–92	98
	566	98	88
	182, 602	86	98–99

[a]sequencing with both primers; [b]sequencing with 3'-CS primers

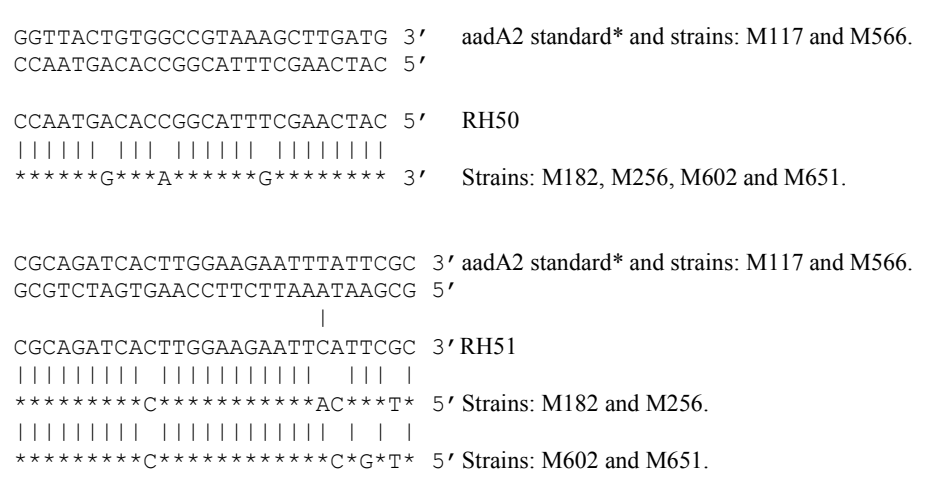

Fig. 4. Differences in the binding sites of RH50 and RH51 primers in case of DT104 and non-DT104 strains. *accession number AF 071555. a: position of RH50 primer in the antF/ant B amplicons (2113–2138 bp). Sequencing was done with both the antF and antB primers. Length of the amplicon: 526 bp. b: position of RH51 primer in the 3'-CS/antF amplicons (2686–2713 bp). Sequencing was done with both the 3'-CS and antF primers. Length of the amplicons: 780 bp

The RH50/RH51 amplicon produced with the M117 and M566 strains contained a second binding site of the RH50 oligonucleotide in a way that rendered possible partially a precise and partially an imprecise pairing within the *qacEΔ1* gene (Fig. 5/a). The sequence of this amplicon showed 100% identity with the segment located between 2686 bp and 3168 bp in the AF 07155 sequence (Fig. 5/b). This amplicon could not be produced with the other four strains. Finer sequence analysis made possible further subcategorisation (according to Peters et al., 2001) of strains with *aadA1* homologous genes: while M602 and M651 strains showed 100% homology with the *aadA1a* subgroup, M182 and M256 strains differed in 1 bp substitution (C to T substitution in position 750) from the *aadA1a* subgroup (data not shown).

Discussion

Of the possible mechanisms of resistance to antibiotics (impermeability, efflux, change of the target and modification of the active agent), integron cassettes mostly contain the genes responsible for the modifying enzymes. Sm/Spc resistance is mediated by the adenylating enzymes designated AAD(3") or ANT(3") and encoded by the *aadA* gene cassette family (Shaw et al., 1993; Fluit and Schmitz, 1999; Naas et al., 1999; Sandvang, 1999; Adrian et al., 2000; White and Rawlinson, 2001). In *S.* Typhimurium DT104 the *aadA2* gene is re-

sponsible for Sm/Spc resistance (Arcangioli et al., 1999; Briggs and Fratamico, 1999; Casin et al., 1999; Ng et al., 1999; Poirel et al., 1999; Guerra et al., 2000; Boyd et al., 2001; Frana et al., 2001), which is located in an approx. 1 kb integron. According to an alternative nomenclature this corresponds to the *ant(3")-Ib* gene (Adrian et al., 2000). In the 2 kb integron of a DT104 strain the *aadA1a* gene was found by Guerra et al. (2000).

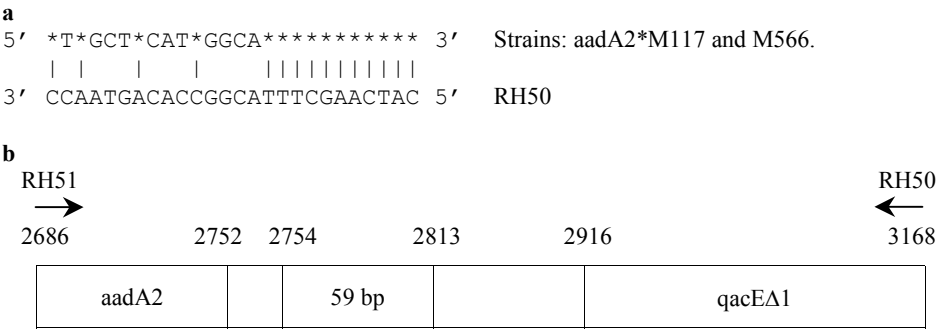

Fig. 5. Amplicon synthesis initiated by RH50/RH51 primers (RH50 bound to the *qacEΔ1* gene). *accession number AF 071555. a: position of RH50 primer in the *qacEΔ1* gene (3142–3168). Sequencing was done with both the RH50 and RH51 primers. Length of the amplicons: 483 bp. b: relation between integron structure and PCR products obtained using RH50/RH51 primers. Arrows indicate the positions of primers. Numbers above the schematic picture correspond to sequence positions in Genbank accession number AF 071555

It is a source of confusion that Sandvang et al. (1998), Daly et al. (2000) and Markogiannikis et al. (2000) have described the Sm/Spc resistance gene of DT104 strains as *ant(3")-Ia*, which would be *aadA1* according to this nomenclature.

In *S.* Typhimurium strains of non-DT104 phage type Sm/Spc resistance was encoded by *aadA1a*, which occurred in 1, 1.6 and 2 kb integrons (Guerra et al., 2000). The same gene was present also in 2 and 3.2 kb integrons (Tosini et al., 1998).

For the DT104 strains analysed in the present study, the presence of the *aadA* gene in the 1 kb integron has been demonstrated by PCR using the antF/antB primer pair, thus confirming the data published in the literature on a larger number of strains.

Our Sm/Spc resistant non-DT104 strains also gave positive result with the above primer pair. The integron localisation of this *aadA* gene was proved by the use of CS/ant primer combinations while its presence in the 1, 1.9 and 2.05 kb integrons was demonstrated by sequencing.

From the size of the CS/ant amplicons obtained with non-DT104 strains containing 1.9 and 2.05 kb integrons (i.e. that 3'-CS/antF is shorter than 5'-CS/antB) it could be established that the *aadA* gene was located closer to the 3'-CS segment. This was supported also by sequencing (unpublished data).

As ampicillin and chloramphenicol resistance has been found to be encoded by different genes in DT104 and in non-DT104 strains (bla_{pse-1} for ampicillin and *flo* for chloramphenicol in DT104 and *oxa-1* for ampicillin and *cat* for chloramphenicol in non-DT104 strains; unpublished data), it has been suggested that the two types of strains may carry different members of the *aadA* gene family and, thus, one or more minor but consistent differences typical of DT104 may exist. Comparing the homology of specific segments of the *aadA* gene, obtained using different primer combinations, with the *aadA1* and *aadA2* standards of DataBank, interpretable differences were obtained between DT104 and non-DT104 typical strains, and it could be established that the 1 and 2.05 kb integrons of the non-DT104 strains contained the *aadA1a* gene. Some irregular members of both strain groups carried the gene typical of the other strain group. In the 1.9 kb integron of the irregular non-DT104 strain designated M566 an *aadA2* gene was found, which is consistent with the integron of similar size described by Guerra et al. (2001).

The above findings support the notion that DT104 is a distinct clone. The appearance of a small number of 'irregular' strains does not refute this; it only suggests the transfection of integrons through mobile elements.

Because of the – partly cumulative – difference in the 3'-CS/antF amplicons the RH51 primer did not find a suitable binding site in the non-DT104 strains, and therefore the RH50/RH51 primer pair did not induce amplicon production. In contrast, the mismatch pairing that showed scattered occurrence at the RH50 binding site did not inhibit the functioning of the primer.

However, the positive result obtained for DT104 strains with the RH50/RH51 primer pair could not be explained even on the basis of the above assumptions. Namely, according to Collis and Hall (1992) this primer pair sets off the amplification of the *aadA2* gene only if the latter is present in a free cassette, or if the integron contains two identical cassettes of tandem arrangement, as from these primers the synthesis starts in divergent directions. At the same time, in the present case there was no reason to suppose either of the above two conditions, the more so as the integrons of DT104, unlike the non-DT104 strains, showed striking stability (Gadó et al., 2003), and thus the presence of several free cassettes could not be expected. This contradiction was explained by the sequence of the RH50/RH51 amplicon. Namely, this amplicon spread over to the 3'-CS segment where it ended in the *qacEΔ1* gene, and there the RH50 primer found an additional binding site. RH50 bound to that site and RH51 bound to the *aadA2* gene started the DNA synthesis towards each other, and this made possible the partial amplification of the integrated *aadA2* cassette also by this primer pair.

Since the use of the RH50/RH51 primer pair enabled the rapid analysis of a larger number of strains, the results of the present study – in line with the few data existing in the literature – allow us to draw the conclusion that the vast majority of strains belonging to phage types other than DT104 and those untypable

by Anderson's method contained the *aadA1a* gene. At the same time, two groups of the DT104 strains (phage types 2 and 2c according to the method of Felix and Callow) showed no difference in this respect.

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