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INTEGRON CONTENT OF *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM STRAINS ISOLATED IN HUNGARY IN THE YEARS 1997–1999

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The integron content of 52 DT104/U302 phage type strains and 53 non-DT104/U302 strains of *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) was studied in PCR experiments using a 5'-CS/3'-CS primer pair (Lévesque et al., 1995). Forty-three out of 44 streptomycin- and/or ampicillin-resistant DT104 and related phage type strains were found to carry a 1 kb and/or 1.2 kb long integron. The other resistance markers did not affect the number and size of integrons; no integron-free multidrug-resistant (MDR) DT104 strains were found. The two large groups of DT104 strains (Felix-Callow's phage types 2 and 2c) proved to be identical in respect of integron patterns (IPs), supporting the views of those authors who consider DT104 a single clone. Strains of human and animal origin did not differ from each other in their IPs. Within the non-DT104 phage types, ampicillin- and/or streptomycin-resistant, integron-free MDR strains were also found. Based on amplicons varying between 290 and 3500 bp an IP system was suggested. The commonest amplicon sizes in non-DT104 strains were 1450 and 2050 bp. The IPs of DT104 strains and of non-DT104 strains containing an integron of 1 and 1.2 kb size were stable. In contrast, the IPs of other non-DT104 strains showed a varying degree of instability. Integron loss was frequently associated with spontaneous plasmid elimination and changes of R-type among the descendants of a given strain.

Key words: Typhimurium, phage type, DT104, integron pattern, instability, PCR

Since the early 1990s, the DT104 phage type of *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) has spread all over the world (Threlfall et al., 1993; Threlfall et al., 1994; Besser et al., 1997; Sandvang et al., 1998; Metzer et al., 1998; Baggesen and Aerestrup, 1998; Ridley and Threlfall, 1998; Glynn et al., 1998), and it has become the second commonest salmonella isolated from human salmonellosis cases in Germany (Almuth et al., 1997) and in the United Kingdom (Threlfall et al., 1996). The very common multidrug-resistant (MDR) DT104 was typically resistant to ampicillin, chloramphenicol, streptomycin, spectinomycin, sulphonamides and tet-

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racyclines (ACSSpSuT R-type) (Threlfall et al., 1994; Threlfall et al., 1996; Low et al., 1997; Casin et al., 1999; Poirel et al., 1999). Selective pressure due to the wide use of antibiotics in animal production may have markedly contributed to the spread of these MDR strains (Williams and Heymann, 1998).

Resistance determinants are often located on mobile elements, through which they may disseminate not only vertically but also horizontally (Davies, 1994). Integrons, first described by Australian authors (Stokes and Hall, 1989), represent an important group of such mobile elements. They consist of resistance cassettes built in between two conservative segments (5'-CS and 3'-CS) (Stokes and Hall, 1989; Hall and Collis, 1995). The cassettes contain a resistance gene and a so-called 59 bp element at its 3' end. The integrase encoded by an *intI* gene of 5'-CS builds in the cassettes by site-specific recombination (Hall et al., 1991). Four classes of integrons were described till now, having different integrase genes (White et al., 2001). Most of the integrons in the clinical isolates of the family *Enterobacteriaceae* belong to class 1 (Brown et al., 1996).

It has been recently demonstrated that the multidrug resistance of DT104 can be attributed to a chromosomal locus, in which two integrons contain the resistance cassette of streptomycin and spectinomycin and that of ampicillin (Sandvang et al., 1998; Casin et al., 1999; Poirel et al., 1999; Arcangioli et al., 1999; Boyd et al., 2001). The *suII* gene responsible for sulphonamide resistance is located on the 3'-CS segment (Hall and Collis, 1995). The genes of chloramphenicol and tetracycline resistance are located in a Tn-like structure outside but close to two integrons (Briggs and Fratamico, 1999; Arcangioli et al., 1999).

In contrast to the above, for other phage types of *S.* Typhimurium the quantitative and qualitative characteristics of integrons are not known precisely. Reports published so far differ as regards the chromosomal (Threlfall et al., 1994; Casin et al., 1999; Martinez-Freijo et al., 1999; Markogiannikis et al., 2000) or plasmid (Tosini et al., 1998) location of integrons, as well as the number of IPs shown by *S.* Typhimurium strains and the size of integrons characterising the individual IPs (Daly et al., 2000; Guerra et al., 2000; Markogiannikis et al., 2000).

In Hungary the spread of DT104 strains has not been followed continuously, as for practical considerations Felix–Callow's method (Felix and Callow, 1951; Milch et al., 1984) was used for routine phage typing instead of Anderson's method (Anderson et al., 1977). Comparative phage typing of the strains isolated in 1998–1999 revealed a relationship between Anderson's phage type DT104 and Felix–Callow's phage types 2 and 2c, with the exception of a negligible number of strains (Szmollény et al., 1998; Szmollény et al., 2000; Pászti et al., 2001).

Thus, in a retrospective manner it could be demonstrated that DT104 had started to spread already in 1989 also in Hungary, and had become the dominant phage type by 1991. The presence of DT104 in human material was detected also by an independent study (Szolyka and Füzi, 1999). Interestingly, however, the very high frequency of multiresistance considered typical of DT104 by the lit-

erature was only true for Felix–Callow's phage type 2, the incidence of which increased only later. Among strains of phage type 2c, the rate of multiresistance did not exceed that observed for non-DT104 strains, and the ratio of susceptible strains was also very high (Pászti et al., 2001). Thus, the question arose whether the two groups of DT104 were identical in respect of integrons. This identity would support the view that MDR DT104 strains belong to the same clonal lineage, in accordance with the majority of authors. In addition, the integrons of the non-DT104 strains was also studied to decide whether they fit in any of the IP systems described in the literature, and whether the DT104 phage type shows a definite character with respect to IP.

Materials and methods

Salmonella *strains.* Seventy-six *S.* Typhimurium strains were isolated and serotyped at the County Institutes of Public Health and Medical Officer Service in 1997–1999, 29 strains were isolated from animals or foods of animal origin at the County Veterinary and Food Control Stations. Fifty-two DT104/U302 (further on termed DT104) strains representing Felix-Callow's phage types 2 and 2c had been chosen in roughly equal number, 10 strains belonging exceptionally to other Felix–Callow's phage types – 2a, 2b, 4, 35 and untypable (UNTY) – was also added (Table 1). DT104L was represented by 33 strains, the other 19 strains distributed among subtypes A, B, H, I, and the related phage type U302. The group of non-DT104/U302 (further on termed non-DT104) strains consisted of DT1, 29, 30, 73, 85, 92, 99, 110B, 120, 192, 193, 194 phage types, RDNC (not conform to a recognised type) and UNTY strains, which belonged to Felix–Callow's phage types 2a, 2b, 2d, 4, 35 and UNTY. The strains were grown on nutrient agar (Oxoid No. 2) without any selection and stored on stock agar at room temperature.

Table 1

Distribution of *S.* Typhimurium strains investigated in the present work

Resistance	Numbers of strains							
	DT104[+]				Non-DT104[+]			
	2[*]	2c[*]	other[*]	total[*]	2[*]	2c[*]	other[*]	total[*]
Sensitive	0	4	3	7(0)	1	5	3	9(5)
Resistant to 1 or 2 drugs	4	4	0	8(2)	1	0	8	9(3)
Multidrug resistant (MDR)	15	15	7	37(12)	1	2	32	35(6)
Total	19	23	10	52(14)	3	7	43	53(14)

[+]Phage types were determined by the method of Anderson et al. (1977); [*]phage types were determined by the method of Felix and Callow (1951). For DT104 subtypes, phage types of non-DT104 group and the Felix–Callow's phage types see Materials and methods; In parentheses: strains from animal sources

Phage typing. Phage typing was performed by the method of Anderson et al. (1977). The phage type of all strains was determined also by the method of Felix and Callow (1951), as adapted by Milch et al. (1984).

Biotyping. Biotype determination was carried out according to Milch (1972). With a few negligible exceptions, the strains used belonged to biotype 3.

Antibiotic susceptibility testing was done using the disk diffusion method on Mueller–Hinton agar with sheep blood as described by Bán (1999). The disks used were ampicillin (A, 20 μg), chloramphenicol (C, 30 μg), streptomycin (S, 30 μg), tetracycline (T, 30 μg), gentamicin (G, 20 μg), kanamycin (K, 30 μg), Sumetrolim (Sxt, 25 μg), cefoperazone (Cfp, 75 μg), cephalexin (Cfl, 30 μg), cefuroxime (Cxm, 30 μg), nalidixic acid (N, 30 μg). (The disks were products of Oxoid.) Spectinomycin (Sp) resistance of S resistant strains was tested with plating on nutrient agar (Oxoid No. 2) containing Sp (Sigma) dilution series. The sulphamethoxazole (Su) resistance of integron carrier stains was controlled with Oxoid disks (25 μg). A strain resistant at least to three antibiotics was considered to be MDR. Sp and Su resistance was not taken account into the R-types.

Plasmid profile. The plasmid preparation was done by the method of Kado and Liu (1981), and agarose gel electrophoresis was performed as described by Meyers et al. (1976) using 0.75% agarose in vertical system with Tris-borate/EDTA buffer applying 60 mA current. The DNA was stained with 0.5 μg/ml ethidium bromide. The plasmids used as molecular mass standards were the eight plasmids of *E. coli* V517 (1.4–35.6 MDa) and plasmids RI (62 MDa) and R27 (112 MDa).

PCR for integron detection was performed as described by Daly et al. (2000). Briefly, 50 μl final volumes contained 25 pmol of both forward (5'-CS, 5-GGC ATC CAA GCA GCA AG-3) and reverse (3'-CS, 5-AAG CAG ACT TGA CCT GA-3) primers (Lévesque et al., 1995), 5 μl of 10 × PCR buffer (100 mM Tris-HCl [pH 9.0], 500 mM KCl, 1% Triton X-100), dATP, dCTP, dGTP and dTTP 200 μM each, 2.5 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (Promega Corp., Madison, USA) and 5 μl of cell suspension boiled for 10 min. Int1 (5'-CCT CCC GCA CGA TGA TC-3') and int2 (5'-TCC ACG CAT CGT CAG GC-3') primers (Bolton et al., 1999) specific to class 1 integrase gene were also used to prove that the integrons belong to class 1. Amplifications were performed using the following amplification cycles: an initial denaturation of 5 min at 94 °C was followed by 35 cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 5 min. After the last cycle, the mixture was incubated for 5 min at 72 °C. The amplification was performed in thermocycler 'Progene' (Techne, Cambridge, UK).

The amplification products were detected in horizontal agarose gel electrophoresis in 2% agarose (Sigma type I) with Tris-acetate/EDTA buffer applying 140 V for 3 h. pGEM (Promega) was used as molecular marker. The results were visualised by staining with 0.5 μg/ml ethidium bromide and photographed

over UV transillumination. The measurements of amplicons were analysed with GelDoc (Bio-Rad) with Quantity One software.

Analysis of descendant cultures. Stored DT104 and non-DT104 strains were plated on nutrient agar (Oxoid No. 2). Colonies from the first generations were subcultured and examined for IPs, R-types and plasmid profiles.

Results

The integron content of 52 *S.* Typhimurium DT104 strains of human and animal origin was studied in PCR experiments using a 5'-CS/3'-CS primer pair (Table 2). The 7 susceptible strains and one Sxt-resistant strain gave negative results. S resistance was usually associated with the appearance of a 1 kb amplicon, while A resistance with that of a 1.2 kb amplicon. The resistance of MDR strains to other drugs (T, C, K, G, Cfp, N) was not related to the IP. Integrons other than the above were observed in a single case only: an S-resistant strain of DT104B phage type contained two integrons of irregular size (0.64 and 2 kb, respectively). No integron-free MDR strain was found among the 37 strains examined.

Table 2

Integron content and antibiotic resistance of 52 DT104 strains[a]

Resistance	Strains tested	Number of strains				
		–	1 kb	1.2 kb	1 + 1.2 kb	other amplicons
Sensitive	7	7 (0,4,3)	–	–	–	
Sxt	1	1 (0,1,0)	–	–	–	
S	5	–	4 (3,1,0)	–	–	1[b] (0,1,0)
A	2	–	–	2 (1,1,0)	–	
MDR	37	–	2 (2,0,0)[c]	–	35[d] (13,15,7)	
Total	52	8 (0,5,3)	6 (5,1,0)	2 (1,1,0)	35[d] (13,15,7)	1 (0,1,0)

[a]included 5 U302 strains; [b]0.64 + 2 kb integrons; [c]with R-type SKGN; [d]R-types: SCA, SCTA, SCAN, SCTAN, SCTACfp, SCTANCfp; Abbreviations for antibiotics as in Materials and methods. In parentheses: distribution of strains on the basis of their phage types determined by the method of Felix and Callow (1951) in the order of 2, 2c and others (2a, 2b, 4, 35, UNTY)

DT104 strains of 2, 2c or other Felix–Callow's phage types did not contain different integrons and did not exhibit different correlations between resistance and integron content (Table 2). Similar results were obtained with strains of subtypes of DT104 (A, B, I, H and L) and of U302 phage type as well as with strains of human and animal origin.

Subsequently the integron content of 53 non-DT104 strains was studied in PCR experiments using the CS primer pair (Table 3). No integron was found in 18 strains, which included not only susceptible strains and strains resistant to one or two drugs but also those resistant to three drugs including S and A. Among 35 integron-carrier strains 31 were MDR with 12 R-types.

Table 3

Resistance and integron carriers among 53 non-DT104 strains[a]

Resistance	Integron-free		Integron carrier	
	No. of strains	R-types	No. of strains	R-types
Sensitive	9	–	0	–
Resistant to 1 or 2 drugs	5	A, K, N, TSxt	4	SG, ST
MDR	4	TAK, CTK, STA, TASxt	31	12 R-types[b]
Total	18		35	

[a]Phage types (by the method of Anderson et al., 1977): see Materials and methods; [b]SCTA resistance was completed with G, K, N, Sxt, Cfr, Cfp and Cxm resistance in various combinations. In case of 3 strains A, or C or T resistance was absent

All integron carrier strains, independently of their phage types, proved to be Su resistant and S and Sp resistance appeared in parallel among them. The integron-free S resistant strains were Sp sensitive.

All strains which exhibited amplicons with 5'-CS/3'-CS primer pair gave positive results (an amplicon with a size of 280 bp was produced) also with int1/int2 primers (data not shown). Strains producing amplicon with the latter primer pair only were not found.

Table 4

Integron patterns of *S.* Typhimurium strains

IP	Amplicons (bp)	No. of strains	
		DT104[a]	Non-DT104
1	1000	6	
2	1200	2	
3	1000, 1200	35	6
4	640, (2000)	1	
5	1000, (2200)		3
6	1900		1
7	550, (650), 1950		1
8	290, (550), (650), 690, 1600, 2050		1
9	700, 1450, 2050, (3500)		23
Total		44	35

( ): very weak band; [a]U302 strains included

The IP system established on the basis of PCR studies is summarised in Table 4, while the amplicons of resistant strains representing different IPs can be seen in Fig. 1. The IPs of DT104 strains belonged to IP1-3 and that of the irregular DT104 strain was classified to IP4. One and 1.2 kb integrons were found also in some non-DT104 strains classified into IP3, while 1 and 2.2 kb integrons were present in IP5. Except for three strains belonging to IP6, IP7 and IP8, the other non-DT104 strains belonged to IP9, which was characterised by the partial or complete presence of four amplicons (700, 1450, 2050 and 3500 bp).

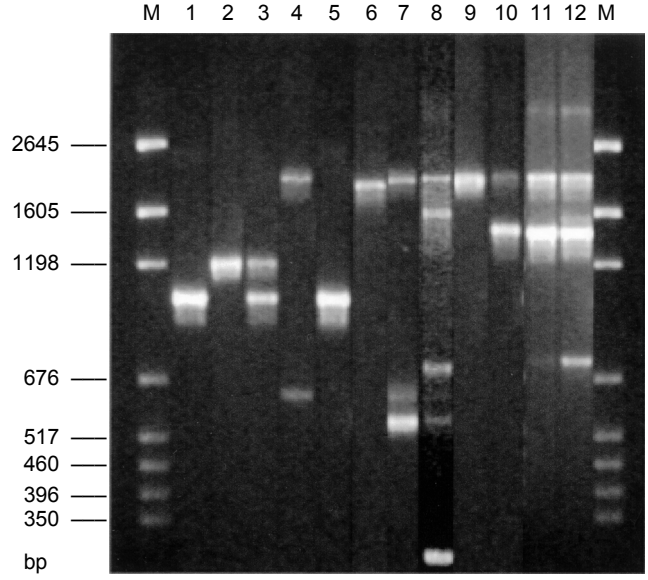

Fig. 1. Amplicons of antibiotic resistant *S.* Typhimurium strains representing different IPs. Representative strains, their R-types, phage types determined by the method of Anderson et al. (1977) and IPs: lane 1: strain 168, S, DT104L, IP 1; lane 2: strain 593, A, DT104L, IP2; lane 3: strain 86, SCATN, DT104L, IP3; lane 4: strain 602, S, DT104B, IP4; lane 5: strain 651, SG, RDNC, IP5; lane 6: strain 566, SKSxt, DT1, IP6; lane 7: strain 214, ST, DT99, IP7; lane 8: strain 332, CTKSxtN, DT30, IP8; lanes 9–12: variants of strain 152, SCTAKGCflCfpCXm, UNTY, IP9 with partial (lanes 9–11) and complete (lane 12) stock of amplicons. These strains descended from human sources except strain 214. For abbreviations of resistance see Materials and methods

The IPs 1-3 of DT104 and non-DT104 strains were well reproducible after storage or passage. In contrast, IPs 4–9 were unstable. The 3500 bp, 700 bp and shorter amplicons disappeared more frequently. The strain with IP8 exhibited maximal instability, the amplicons mean only a possible variant.

Subsequently, in the case of 32 DT104, and non-DT104 strains 3–6 parallel descendant cultures isolated from individual colonies were compared with each other and with the parent culture for IP, resistance and plasmid profile (Table 5). It can be seen that the descendant cultures of 12 DT104 and non-DT104

strains of IPs 1–3 were identical with each other and with the parent strains in all respects. In contrast, such stability was observed only for one out of the 20 strains of IPs 4–9, while the other strains showed changes with respect to the IP. In most cases the descendant cultures were heterogeneous and/or different from the parent culture also as regards resistance, and in 11 cases they were not uniform in plasmid profile either.

Table 5

The instability of *S.* Typhimurium strains in respect of IP, resistance and plasmid profile

Group of strains			Instability in respect of		
Phage type	IP	No. of strains	IP	resistance	plasmid profile
DT104	1–3	6	–	–	–
	4	1	+	+	+
Non-DT104	3	6	–	–	–
	5	1	+	+	+
	6–9	1	–	–	–
		1	+	–	–
		7	+	+	–
		9	+	+	+
Total		32			

Comparison of 3–6 cultures isolated from single colonies with the parental cultures and with each other; There were (+) differences or no (–) differences among descendants and/or between descendants and parental culture

Table 6 shows the details of the above analysis for 7 non-DT104 strains. A certain correlation seems to exist between integron content and resistance: integron loss was frequently associated with a reduction in the number of resistance markers, e.g. the loss of the 2 kb integron with the disappearance of S resistance. On the other hand, descendants with the same IP may have different R-type (566 a-b), and those with the same R-type may have different IPs (332 c-f). Integron-free descendants may show C, A, T, K, Cfl, Cfp and Cxm resistance.

If a descendant became negative with CS-PCR, amplicon production with int-PCR also did not occur (data not shown).

The correlation between the plasmid profile and integron content of the descendant cultures is also of similar nature: the loss of plasmids was often associated with integron loss, but strains with equal plasmid profile might have different IPs (182 c-d, 347 b-c) and vice versa (566 a-b, 347 c-d) (Table 6).

Table 6

Instability of seven non-DT104 *S.* Typhimurium strains in respect of IP, resistance and plasmid profile (detailed)

Strains		Amplicons (kb)	Resistance	Plasmid profile (MDa)
98	P	1.5, 2, (3.5)	CATSKSxt Cef	5.5, 10.1, 18.5, 30, 95
	a	1.5, 2, (3.5)	CATSKSxt Cef	5.5, 10.1, 18.5, 30, 95
	b	1.5, 2, (3.5)	CATSKSxt Cef	5.5, 10.1, 18.5, 30, 95
	c	2	CATS Cef	5.5, 10.1, 18.5, 30, 95
189	P	1.5, 2	CATS Cef	5, 33, 62, 82
	a	(2)	CATS Cef	5, 33, 103
	b	–		33, 82
	c	–	A Cef	5, 33, 62
566	P	1.95	S K Sxt	1.7, 26, 67, 78
	a	1.95	S Sxt	26, 67, 78
	b	1.95	S K Sxt	1.7, 26, 67, 78
	c	–	K	50, 110
577	P	1.5, 2	CATS Cef	5.3, 11.5, 16, 28, 88
	a	–	CAT Cef	5.3, 11.5, 22.5, 29, 58
	b	2	CATS Cef	5.3, 11.5, 16, 28, 88
	c	2	CATS Cef	5.3, 11.5, 16, 28, 88
182	P	1.5, 2, (3.5)	CATSK Sxt Cef	5, 30, 73
	a	–	AT Cef	5, 30, 73
	b	2	CATSK Cef	5, 30
	c	–	AT Cef	5, 30, 73
	d	1.5, (3.5)	CAT Cef	5, 30, 73
	e	1.5, 2, (3.5)	CATSK Sxt Cef	5, 30, 95
	f	–	AT	5, 30, 73
332	P	0.29, 0.55, 0.65, 1.5, 1.9	CTK Sxt N	75
	a	0.65	–	–
	b	0.65	–	–
	c	0.29, 0.55, 1.9	K Sxt	75
	d	0.95, 1.5	C Sxt N	–
	e	0.29, (0.44), 0.55, 0.65	–	75
	f	0.29, (0.44), 0.55, 0.65, 1.9	K Sxt	75
347	P	1.5, 2, (3.5)	CATSK Sxt Cef	5, 32, 97
	a	1.5, 2, (3.5)	CATSK Sxt Cef	5, 32, 97
	b	1.5	CAT K Cef	5, 32, 97
	c	1.5, 2, (3.5)	CATSK Sxt Cef	5, 32, 97
	d	1.5, 2, (3.5)	CATSK Sxt	32, 97
	e	1.5, 2, (3.5)	CATSK Sxt	32, 97
	f	1.5	CAT K Cef	5, 32, 97

( ): very weak band; Cef: cephaperazone, cephalexin and cefuroxime resistance; P: parent culture; a–f: subcultures of parallel descendant colonies

Discussion

PCR starting from two conservative segments of integrons results in the production of as many amplicons as the number of integrons of different length present per cell (Sandvang et al., 1998; Daly et al., 2000; Guerra et al., 2000). This allows the typing of resistant strains by establishing an 'integron pattern' (IP). On that basis, Daly et al. (2000) established 6 IPs among *S.* Typhimurium strains. All DT104, DT104B and U302 strains were classified into IP-I, which was characterised by 0.2, 1.0 and 1.2 kb long amplicons. The 1 kb and 1.2 kb amplicons could be indistinctly demonstrated in three other IPs as well. Integron-free resistant strains occurred only among the non-DT104 strains. A partially different IP system was constructed by Daly and Fanning (2000) in a later paper.

Using CS primer pair, Markogiannikis et al. (2000) obtained, besides 0.9 and 1.1 kb amplicons, also a 1.4 kb amplicon for a certain proportion of Greek DT104 strains, and they also found integron-free MDR strains.

Guerra et al. (2000) also established 6 IPs, consisting of 1, 1.2, 1.6, 2.0 and 2.3 kb integrons, for 10 *Salmonella* serotypes. *S.* Typhimurium strains occurred in four IPs (in IP-I, II, IIIa and IV) and DT104 primarily belonged to IP-I, characterised as having a size of 1 and 1.2 kb integrons. These chromosomal integrons were present in only 76% of the MDR DT104 strains.

Three integrons (1.5, 2 and 3.1 kb) were localised in two conjugative large plasmids of *S.* Typhimurium (Tosini et al., 1998), but the phage types of their strains were not published.

According to the results obtained in this study, DT104 and the related phage types were characterised by a close association of S and A resistance with the 1 and 1.2 kb integrons, only a single exception was found among the 44 strains (Table 2). This fact is in harmony with the literature (e.g. with data reported by Sandvang et al., 1998; Ridley and Threlfall, 1998; Briggs and Fratamico, 1999; Poirel et al., 1999; Daly et al., 2000; Heir et al., 2002; Izumiya et al., 2001 and many others). At the same time, in contrast to the Greek strains (Markogiannikis et al., 2000), in the strains examined in this study neither 1.4 kb integron nor integron-free MDR strains were found.

As the resistance of MDR DT104 strains to other drugs (T, C, K, G, N, Cfp) did not affect the IP (neither more nor larger integrons appeared), the corresponding genes must be located outside the integrons. The exclusivity of the 1 and 1.2 kb integrons suggests that S and A resistance cassettes did not occur within the same integron. This fact which is in accordance with the literature cannot be explained.

According to Martinez-Freijo et al. (1999), most isolates contain only a single integron, and 2–3 integrons are only rarely present. The majority of our non-DT104 strains, however, carried more than one integron in contrast with the DT104 strains, and in most of them there were more than one cassette, taking the

average length of a cassette as 800 bp (Daly et al., 2000). Our strains could not be classified into either Daly's or Guerra's systems, differing from them also in size of integrons and in the number of IP variants.

The correlation between the results of PCRs performed with primer pairs 5'-CS/3'-CS and int1/int2 refers to the lack of 'empty' integrons (CSs without cassettes, when the distance between 5'-CS and 3'-CS is too small), integrons without 3'-CS or integrons in which the distance between 5'-CS and 3'-CS is too large. In these cases the int-PCR would give positive results, while the CS-PCR would be unsuccessful.

The determination of resistance genes is in progress. According to the preliminary results, 1 and 1.2 kb integrons contain genes *aadA2* and *pse-1*, respectively, while the 1.45 and 2.05 kb integrons contain genes *aadB* + *catB3*, and *oxa-1* + *aadA1*, respectively (unpublished data). The 700 bp or smaller integrons cannot carry a cassette capable of expression. This supposition is consistent with the observation that the descendants derived from strain 332 containing only integrons of such sizes proved to be sensitive (Table 6).

The instability of integrons especially in the absence of selective pressure was suggested by Recchia et al. (1994) and Seward and Towner (1999). In contrast, high stability was found among integrons of bacteria isolated from the same patients at different times (Maguire et al., 2001) or from various European hospitals (Martinez-Freijo et al., 1999). The deviations between the IP systems of Daly et al. (2000) and Daly and Fanning (2000) and some badly reproducible amplicons in their later paper also refer to the instability of integrons in non-DT104 strains.

The IP of numerous DT104 and related (U302) phage type strains proved to be highly stable in repeated experiments and on the basis of the examination of descendant strains. The non-DT104 strains containing also a 1 and 1.2 kb integron were characterised by a similarly high stability. This fact may have a role in the spread of DT104. The majority of the non-DT104 strains, however, exhibited an IP instability of varying degree in reproduction experiments with different generations and by analysis of the descendants colonies. A striking instability was observed also in respect of spontaneous plasmid elimination. These two phenomena do not appear to be casually related, because losses of integrons were found beside unchanged plasmid profiles. The only association was that both aspects of instability frequently occurred in the same strains.

Most of the authors consider the decisive majority of the MDR DT104 strains to be clonally related (Sandvang et al., 1998; Ridley and Threlfall, 1998; Casin et al., 1999; Baggesen et al., 2000; Heir et al., 2002). This is supported by the fact that their DNA amplification fingerprints (Daly et al., 2000) and their PFGE data (Baggesen et al., 2000; Izumiya et al., 2001) also show a uniform picture. The clonal identity of about 95% of DT104 isolates was found to occur in sensitive as well as a range of MDR variants (Prager et al., 1999). In contrast,

Markogiannikis et al. (2000) supposed the existence of 6 clones among Greek DT104 strains, primarily on the basis of PFGE; the dominant type 'A' was carrying the two integrons typical of DT104 (0.9 and 1.1 kb). Various clones of DT104 have been reported also by Walker et al. (2001).

According to our earlier observations (Pászti et al., 2001), the two Felix–Callow phage types (2c and 2) constituting the majority of DT104 strains spread in Hungary with a time difference of several years. An important difference between these two groups is that only one of them (i.e. phage type 2 which spread later) was characterised by a high frequency of MDR strains. The above results concerning the 1 and 1.2 kb integrons, however, applied to all DT104 strains tested, irrespective of their Felix–Callow's phage type. This observation is consistent with the possible existence of an originally single clone which later separated into the two Felix–Callow's phage types, in which phage type 2 may represent a new subclone.

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