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Multiple Clones within Multidrug-Resistant *Salmonella enterica* Serotype Typhimurium Phage Type DT104

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Six distinct clones were present among Greek multidrug-resistant *Salmonella enterica* serotype Typhimurium phage type DT104, since isolates belonging to resistance phenotypes including the ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) core could be distinguished with respect to their pulsed-field gel electrophoresis patterns, *int*1 integron structures, and presence or absence of antibiotic resistance genes *ant*(3″)-Ia, *pse*-1, and *tem*-1.

In recent years, a marked increase in the number of multidrug-resistant *Salmonella enterica* serotype Typhimurium isolates belonging to definitive phage type 104 (DT104) and having a core pattern of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT) has been reported (3, 4, 13, 14). Molecular studies have demonstrated that in DT104 some of the resistance genes responsible for the ACSSuT phenotype are integron associated (8, 12).

To date, all reports have suggested that multidrug-resistant DT104 isolates are clonally related (R. Prager, A. Liesegang, W. Streckel, B. Gerike, G. Seltmann, R. Helmuth, W. Rabsch, and H. Tschape, Fourth Int. Meet. Bacterial Epidemiol. Markers, abstr. P40, 1997; 7, 8). In this study, we aimed to analyze the genetic relationships of multidrug-resistant serotype Typhimurium isolates from Greece, with respect to their chromosomal fingerprints and mechanisms of resistance.

During 1989 to 1997, a total of 1,005 *Salmonella* isolates of human, animal feed, animal, and food origins from various parts of Greece were referred to the National Reference Center for *Salmonella* and *Shigella*. They were identified as serotype Typhimurium by the API 20E system (BioMerieux S.A., Marcy l'Etoile, France) and the Kauffman serotyping scheme (5), using commercially obtained antisera (BioMerieux). Serotype Typhimurium represented the second most frequent serotype isolated from humans, as for 1987 to 1993 (10), standing at 17% in 1997. The organism was also an important serotype in isolates of animal and food sources.

Of the 1,005 serotype Typhimurium isolates, 328 (33%) were randomly selected for antimicrobial susceptibility testing by a disk diffusion method on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, United Kingdom), evaluated according to the standards of the National Committee for Clinical Laboratory Standards (6). Disks containing ampicillin, amoxicillin and clavulanic acid, cefalothin, cefamandole, ceftriaxone, ceftazidime, cefotaxime, streptomycin, kanamycin, gentamicin, chloramphenicol, doxycycline, nalidixic acid, ciprofloxacin, sulfonamides, sulfomethoxazole and trimethoprim, and nitrofurantoin were purchased from Oxoid. The isolates were grouped into 26 resistance phenotypes, the major ones being shown in Table 1. Eighty-four (26%) isolates were resistant to the ACSSuT core alone or to additional drugs as well. Thus, 11 distinct resistance phenotypes were compatible with the ACSSuT core resistance phenotype of multidrug-resistant serotype Typhimurium DT104. Fourteen isolates (17%) representing seven of these resistance phenotypes were therefore randomly selected for further analysis.

Phage typing was performed by the methods described by Callow (2). The scheme extended by Anderson et al. (1) uses 34 typing phages and differentiates in excess of 250 types. Of the 14 isolates, 11 belonged to phage type DT104 and 2 belonged to the related type DT104b, differing by a single phage reaction from DT104, while the remaining isolate belonged to the unrelated phage type DT193.

In conjugation experiments for the transfer of antibiotic resistance, carried out as previously described (16), only two DT104 isolates (195 and 1041) were able to transfer resistance to ampicillin, tetracycline, chloramphenicol, streptomycin, and cotrimoxazole.

Typing by pulsed-field gel electrophoresis (PFGE) of genomic DNA digested with *Xba*I (New England Biolabs, Beverly, Mass.) was performed as previously described (10). DNA fragment patterns were assessed visually and compared by the GelCompar software package (Applied Maths, Kortrijk, Belgium), using the Dice coefficient, UPGMA (unweighted pair group method using arithmetic averages) clustering, and 1% tolerance in band position differences. Isolates were considered as belonging to different types if differing by four or more DNA fragments (11). Given the genetic homogeneity of *Salmonella* populations (10), this criterion can be considered stringent. Five distinct types, A to D and F, were observed among DT104 isolates (Fig. 1). Four isolates belonged to type

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TABLE 1. Main resistance phenotypes of serotype Typhimurium (1989–1997)

Resistance phenotype[a]	No. of isolates for yr:						Total no.
	1989	1991	1993	1995	1996	1997	
Susceptible	10	7	16	8	7	6	54
S	2	1	2	1	7	12	25
Su	0	1	2	1	3	2	9
T	1	0	1	0	0	1	3
ST	2	3	1	3	6	6	21
SSu	2	5	6	4	14	19	50
SSuT	6	5	1	2	2	3	19
STFu	2	3	1	0	0	4	10
SSuTFu	0	1	3	1	1	2	8
SSuTSxt	0	1	3	4	3	7	17
SSuTGKNx	0	0	0	0	1	0	1
ASSu	0	1	1	4	0	0	6
ASSuT	0	4	1	2	2	4	13
ASSuTSxt	0	0	3	1	2	1	7
ASSuTGKNx	0	0	0	0	0	1	1
ACSSuT	1	1	2	5	0	0	9
ACSSuTAmc	0	1	1	4	1	0	7
ACSSuTFu	0	0	0	0	3	6	9
ACSSuTAmcFu	0	1	3	15	21	3	43
ACSSuTAmcFu+	0	1	2	5	5	3	16
Total	26	35	49	60	78	80	328

[a] A, ampicillin; T, tetracycline; S, streptomycin; Su, sulfonamides; Fu, nitrofurantoin; C, chloramphenicol; Sxt, cotrimoxazole; Amc, amoxicillin-clavulanic acid; Nx, nalidixic acid; G, gentamicin; K, kanamycin; +, any other antibiotic. Intermediate and high resistance are grouped together.

A, with the remaining seven distributed among types B (two isolates), C (two), D (two), and F (one).

For PCR amplification of integron-related and resistance gene sequences, DNA was prepared as previously described (17). PCR was carried out in 50-μl-total-volume reactions containing 1.5 mM $MgCl_2$, 100 μM (each) deoxynucleoside triphosphate, 0.1 U of *Taq* polymerase (Promega, Madison, Wis.), 1 μl of bacterial lysate, and 0.4 μM primers for *int*1 integron (8), *ant*(3″)-Ia (17), *pse*-1 (8), and *tem*-1 sequences (9). Under conditions used, three distinct amplicons were obtained from the DT104 isolates with the *int*1 primers (Fig. 2). Two PCR products of approximately 0.9 and 1.1 kb were generated from five isolates belonging to PFGE types A and D, while a product of approximately 1.4 kb was generated from the two DT104 isolates belonging to PFGE type C. No *int*1 PCR product could be obtained from the remaining four DT104 isolates belonging to PFGE types B, D, and F. All DT104 isolates that were positive for *int*1-type integron sequences also yielded an *ant*(3″)-I product of the expected size, 0.5 kb (Table 2). Of the four isolates (1308, 461, 385, and 1511) from which no *int*1 or *ant*(3″)-I products could be generated, all were intermediately resistant to streptomycin. Amplicons of the expected size, 0.3 kb, were obtained with the *pse*-1 primers from five DT104 isolates, while amplicons of the expected size for *tem*-1, 0.7 kb, were detected in four DT104 isolates (Table 2). In contrast to *ant*(3″)-Ia and *pse*-1 sequences, which were shown by PCR to be internal to *int*1 amplicons, the *tem*-1 gene was not contained in an integron gene cassette (data not shown). All strains positive for *int*1 sequences also contained *qac*E-1 and *sul*1 sequences (not shown).

Therefore, of the five PFGE types within phage type DT104, type A was prevalent. Type A isolates contained two distinct *int*1 integrons, detected by PCR products of 0.9 and 1.1 kb, as well as *ant*(3″)-Ia and *pse*-1 sequences. Similar studies have found that the small integron harbors the aminoglycoside-resistant gene cassette *ant*(3″)-Ia, conferring resistance to streptomycin and spectinomycin, while the large amplicon harbors the *pse*-1 β-lactamase gene (7, 8). The variety in integron

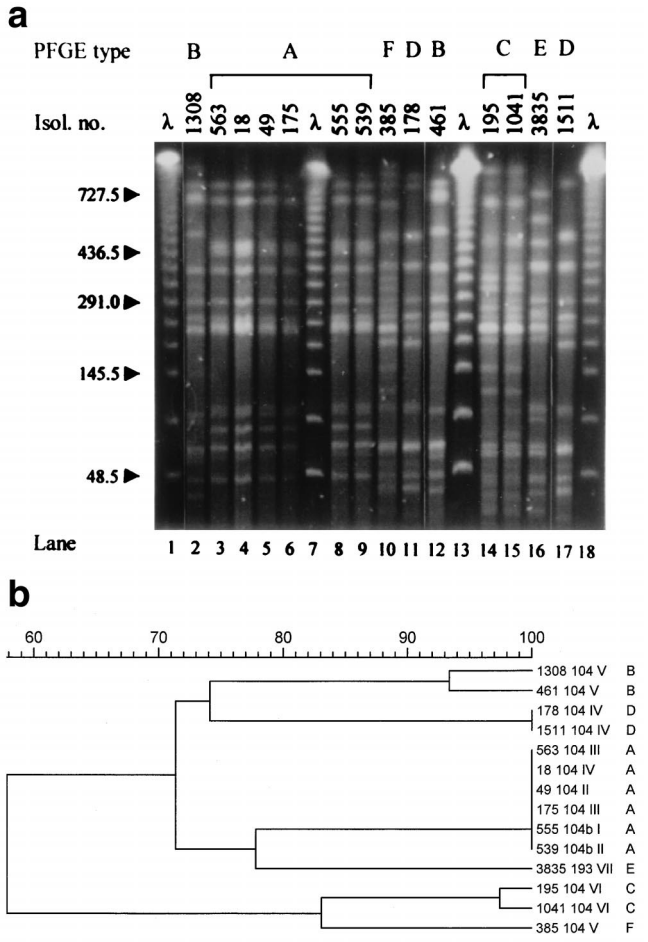

FIG. 1. (a) PFGE patterns of 14 multidrug-resistant serotype Typhimurium isolates. Isol. no., isolate number. The sizes, in kilobases, of lambda phage DNA concatemers (λ) are shown to the left of the gel. All lanes are from the same gel. (b) Dendrogram of isolate similarities based on the chromosomal fingerprints shown in panel a. Isolate number, phage type, resistance phenotype code, and PFGE type are shown to the right of the dendrogram. A percent scale of similarity is shown above the dendrogram.

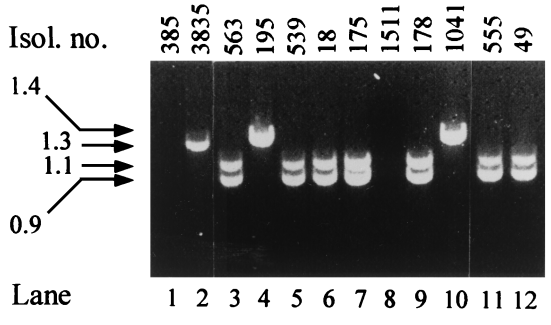

FIG. 2. PCR amplification products of class 1 integrons from representative multidrug-resistant serotype Typhimurium isolates. Isol. no., isolate number. The sizes, in kilobases, of the indicated amplification products are shown to the left of the gel. All lanes are from the same gel.

TABLE 2. Genotypic and phenotypic characteristics of multidrug-resistant serotype Typhimurium isolates

Isolate no.	Isolate yr	Origin	Resistance phenotype[a]	Phage type	PFGE type	*int*1				*ant*(3)	*pse*-1	*tem*-1	Conj.[c]
						0.9 kb	1.1 kb	1.3 kb	1.4 kb				
555	1997	Frozen chicken	ACSSuT [I][b]	DT104b	A	+	+			+	+		
539	1997	Human feces	ACSSuTFu [II]	DT104b	A	+	+			+	+		
49	1996	Sewage filter	ACSSuTFu	DT104	A	+	+			+	+		
175	1996	Frozen pork	ACSSuTAmcSxt [III]	DT104	A	+	+			+	+		
563	1997	Ground chicken carcasses	ACSSuTAmcSxt [III]	DT104	A	+	+			+	+		
1308	1991	Human feces	AC(S)SuTAmcFu [IV]	DT104	B							+	
461	1993	Human feces	AC(S)SuTAmcFu	DT104	B							+	
385	1997	Sewage filter	AC(S)SuTAmcFu	DT104	F								
18	1995	Human feces	ACSSuTAmcFu [V]	DT104	A	+	+			+	+		
1511	1995	Pigeon liver	ACSSuTAmcFu	DT104	D								
178	1996	Sewage filter	ACSSuTAmcFu	DT104	D	+	+			+	+		
1041	1995	Human feces	ACSSuTAmc(Fu)Sxt [VI]	DT104	C				+	+		+	+
195	1997	Human feces	ACSSuTAmc(Fu)Sxt	DT104	C				+	+		+	+
3835	1996	Human feces	ACSSuTAmcCFCroCtxFuGKMa [VII]	DT193	E			+					+

[a] A, ampicillin; Amc, amoxicillin-clavulanic acid; T, tetracycline; S, streptomycin; C, chloramphenicol; Su, sulfonamides; Fu, nitrofurantoin; Sxt, cotrimoxazole; G, gentamicin; K, kanamycin; CF, cefalothin; Ma, cefamandole; Ctx, cefotaxime; Cro, ceftriaxone. Parentheses indicate intermediate resistance.

[b] Roman numerals in brackets indicate the resistance phenotype.

[c] Conj., transfer of resistance by conjugation.

size and presence or absence of aminoglycoside-modifying enzymes and β-lactamases, set against a background of different chromosomal types, resulting in six distinct types among Greek multidrug resistant DT104 isolates, is reminiscent of other published data on multidrug resistant serotype Typhimurium (15). In their study, Tosini et al. (15) found three distinct class 1 integrons located on two conjugative plasmids. In our study, transfer of resistance by conjugation was possible only with isolates in which the *tem*-1 gene was not inserted in a 1.4-kb integron. In contrast, resistance of isolates harboring the *ant*(3″)-Ia and *pse*-1 gene cassettes inserted in two integrons was not transferable by conjugation, presumably due to the chromosomal location of these integrons (12).

It therefore appears that common phenotypic characteristics of antibiotic resistance and phage type may have been acquired by genotypically distinct multidrug-resistant serotype Typhimurium DT104 strains in Greece. This differentiates them from those isolated in other countries (Prager et al., Fourth Int. Meet. Bacterial Epidemiol. Markers; 7, 8), where clonality of multidrug-resistant serotype Typhimurium DT104 has been suggested. While we cannot rule out that the presence of various clones within multidrug-resistant DT104 isolates may be characteristic of the Greek situation, additional molecular studies from regions with a high rate of multidrug resistance in serotype Typhimurium, among both human and nonhuman isolates, would help to elucidate the origin and extent of this problem.

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