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Multiple-Drug Resistance in D-Tartrate-Positive *Salmonella enterica* Serovar Paratyphi B Isolates from Poultry Is Mediated by Class 2 Integrons Inserted into the Bacterial Chromosome

Angelika Miko, Karin Pries, Andreas Schroeter, and Reiner Helmuth*

National Salmonella Reference Laboratory, Federal Institute for Risk Assessment, D-12277 Berlin, Germany

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The presence of integrons in 85 multiresistant German isolates of the predominating *Salmonella enterica* subsp. *enterica* serovar Paratyphi B dT$^+$ clone was investigated. All isolates possessed a chromosomally located Tn*7*-like class 2 integron carrying the same *dfrA1-sat1-aadA1* array of gene cassettes. Only four isolates (4.7%) revealed an additional class 1 integron with two strains each containing the *aadA1* or *dfrA1-aadA1* gene cassettes.

During the last decade multidrug-resistant D-tartrate-positive *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (*Salmonella* serovar Paratyphi B dT$^+$) isolates (formerly called *Salmonella* serovar Java) have increasingly been isolated from poultry and poultry products in Germany and The Netherlands (7, 18, 29). Recent studies by Brown et al. (1) strongly suggest that the same multiresistant clone found in German and Dutch poultry was responsible for 10 human cases of *Salmonella* serovar Paratyphi B dT$^+$ infections in Scotland.

By studying the phenotypic and molecular features of contemporary and old *Salmonella* serovar Paratyphi B dT$^+$ isolates from poultry, Miko et al. (18) could show that since the mid-1990s the majority of contemporary isolates have been multidrug resistant and belong to a particular clone defined by a distinct pulsed-field gel electrophoresis (PFGE) and IS*200* profile. Despite the multiple-drug resistance, class 1 integrons were found in only 1 of the 39 contemporary isolates, suggesting the minor importance of this integron class in the acquisition of multidrug resistance by the predominating *Salmonella* serovar Paratyphi B dT$^+$ clone.

The role of integrons and gene cassettes in the evolution and dissemination of multidrug resistance in gram-negative bacteria is well established (22, 23). Integrons are characterized by their ability to integrate and excise genes that are part of gene cassettes via a site-specific recombination event (2, 3, 5, 22). Integrons harbor a gene, *intI*, which encodes a site-specific recombinase (IntI integrase), and an attachment site, *attI*, into which individual gene cassettes are inserted (24). To date, five distinct integron classes associated with gene cassettes that contain antibiotic resistance genes have been described (4). The best-characterized group is class 1 integrons. They are the most widely disseminated ones among the members of the family *Enterobacteriaceae*, including many of the *Salmonella enterica* subsp. *enterica* serovars (10, 11, 15, 25, 26). For class 1 integrons, *intI1*, *attI1*, and promoter P_c are found within potentially mobile elements that are transposons, e.g., Tn*402*, or defective transposon derivatives. Class 2 integrons have been shown to contribute to the spread of antibiotic resistance genes in the family *Enterobacteriaceae* as well (9, 31). In *Salmonella* they have been identified so far only in the serovar Typhimurium (21). For class 2 integrons, the typical *intI2** gene, which includes a termination codon, and the *attI2* and P_c are found within transposons such as Tn*7* (27, 28). The remaining three integron classes are not well characterized, since to date only a single example of each of them has been detected.

The aim of this study was to determine whether class 2 integrons are common mediators of multidrug resistance in *Salmonella* serovar Paratyphi B dT$^+$ isolates of the predominating clone in Germany.

Characterization of *Salmonella* serovar Paratyphi B dT$^+$ isolates. A total of 85 contemporary multidrug-resistant strains isolated in Germany between 1995 and 2001 were included in the study. Thirty-nine of the isolates had been described in reference 18 and were already known to belong to the predominating clone (group 3 strains). The other 46 independent isolates from 2001 were selected by the same criteria given in reference 18. They originated from food products of poultry origin ($n = 31$) and from poultry ($n = 12$), cattle ($n = 1$), and feed ($n = 2$). Fourteen comparative strains described as group 1 and 2 strains in reference 18 were also analyzed. They did not belong to the contemporary clone (Table 1).

Serotyping, D-tartrate fermentation, plasmid profile typing, PFGE, and IS*200* profiling were done as described previously (18). All isolates from 2001 revealed the PFGE profile X8 and the IS*200* profile ISP9 as well and could consequently be assigned to the predominating German *Salmonella* serovar Paratyphi B dT$^+$ clone. Susceptibilities to a panel of antimicrobial agents (Table 1) were assessed by determining the MIC by the NCCLS broth microdilution method (19). Breakpoints given by the NCCLS and DANMAP (6) were used. All 85 isolates exhibited a core spectrum of antibiotic resistance determinants for trimethoprim, spectinomycin, and streptomycin. Additional resistances were found to sulfamethoxazole (in 71% of the strains), nalidixic acid (62%), ampicillin (51%), tetracycline (7%), kanamycin-neomycin (3%), and gentamicin (3%).

* Corresponding author. Mailing address: Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, D-12277 Berlin, Germany. Phone: 49-30-8412-2233. Fax: 49-30-8412-2953. E-mail: r.helmuth@bfr.bund.de.

TABLE 1. Integrons in *Salmonella* serovar Paratyphi B dT$^+$ strains

No. of strains	Yrs of isolation	Antibiotic resistance[a]	Integron (cassettes/location[b])		Profile[c]	
			Class 1	Class 2	PFGE	IS*200*
Multiresistant strains of the predominating clone						
81	1995–2001	**TMP, SPE, STR,** SMX, NAL, AMP, KAN-NEO		*dfrA1-sat1-aadA1*/C	X8	ISP9
4	1995–2001	**TMP, SPE, STR,** SMX, TET, NAL, AMP, GEN	*dfrA1-aadA1*/P or *aadA1*/P	*dfrA1-sat1-aadA1*/C	X8	ISP9
Comparative strains						
5	1994–1999	TMP, SPE, STR, SMX, TET, NAL, CHL, KAN-NEO	*dfrA12-aadA2*/P		X7	ISP8
9	1960–1993	Sensitive			X1–X6	ISP1–ISP7

[a] Boldface indicates resistance exhibited by all strains, and lightface indicates resistance exhibited by only some strains. Abbreviations: TMP, trimethoprim; SPE, spectinomycin; STR, streptomycin; SMX, sulfamethoxazole; NAL, nalidixic acid; AMP, ampicillin; KAN-NEO, kanamycin-neomycin; TET, tetracycline; GEN, gentamicin; CHL, chloramphenicol.
[b] P, high-molecular-weight plasmid; C, chromosome.
[c] Assignment according to reference 18. X, *Xba*I; ISP, IS*200* profile.

Identification of integrons. Integrons were detected in all 85 multiresistant isolates by using PCR with degenerate primers to amplify conserved regions of the integron integrase genes *intI1*, *intI2*, and *intI3* (30). In order to distinguish class 1 and class 2 integrons, PCR amplifications were carried out with *intI1* (20)- and *intI2* (17)-specific primers. All isolates carried class 2 integrons, and four of them revealed a class 1 integron as well (Table 1).

To detect inserted gene cassettes, the variable regions of class 1 integrons, carried by the four *intI1*-positive isolates, were amplified using primers 5′ CS and 3′ CS, which annealed with the DNA regions flanking the recombination site *attI* (16). Direct sequencing of the purified PCR products revealed the *dfrA1* and *aadA1* gene cassettes or the *aadA1* gene cassette alone in two isolates each.

Class 2 integron cassette arrays, carried by the 85 *intI2*-positive isolates, were amplified using primers hep74, which binds to *attI2*, and hep51, which binds to *orfX*, situated at the right end of the cassette region within transposon Tn*7* (12, 31). All of these isolates and two Tn*7*-containing positive control strains, *Escherichia coli* K-12 J62 (ColE1::Tn*7*) and *E. coli* K-12 χ6149 S17-1 (RP4-2), carried a class 2 integron characterized by a PCR product of about 2.2 kb. By contrast, no PCR product was observed in the comparative strains (Table 1).

The sequence similarity among the 2.2-kb amplicons was investigated and confirmed by restriction analysis with the restriction enzyme *Hin*cII, *Hin*fI, or *Ava*I. All amplicons gave identical restriction patterns, and the sum of the sizes of the restriction fragments was consistent with the size of the undigested amplicon (Fig. 1 and data not shown).

Tn*7* has been shown to carry three integrated resistance gene cassettes, namely, *dfrA1*, *sat1*, and *aadA1*, encoding resistance to trimethoprim, streptothricin, and spectinomycin-streptomycin, respectively (22, 27, 28). Recently, a fourth gene cassette, ORFX, coding for a protein of unknown function was described by Hansson et al. (12).

We detected the Tn*7*-borne antibiotic resistance genes in all *Salmonella* serovar Paratyphi B dT$^+$ isolates carrying class 2 integrons by PCR analysis. The gene-specific primers P3/P4 for *dfrA1* (8); sat1-F/B (GAAACATTGGATGCTGAG/GAACCAGTACCAGTACAT), designed for this work; and aadA-F/B (26) were used. To confirm the cassette content and the specific gene arrangement in the integron, PCR amplifications using primers flanking adjacent gene regions (hep74/P4, P3/sat1-B, sat1-F/aadA-B, and aadA-F/hep51) were performed. Partial sequencing of the amplicons corroborated the presence of the same array of cassettes, *dfrA1-sat1-aadA1*, in all class 2 integrons.

Localization of the class 2 integrons. In order to map the class 2 integrons, PFGE after digestion of genomic DNAs with *Xba*I and subsequent Southern blot analysis with *intI2*-, *dfrA1*-, *sat1*-, and *aadA1*-specific probes (which were not cut by *Xba*I) was carried out. Plasmid DNA extracted by the method of Kado and Liu (14) was hybridized with these probes as well, in order to map these genes on the plasmids. Results of the experiments revealed that the *intI2*-, *dfrA1*-, *sat1*-, and *aadA1*-specific probes hybridized to the same two chromosomal bands of about 78 and about 410 kb in all *Salmonella* serovar Para-

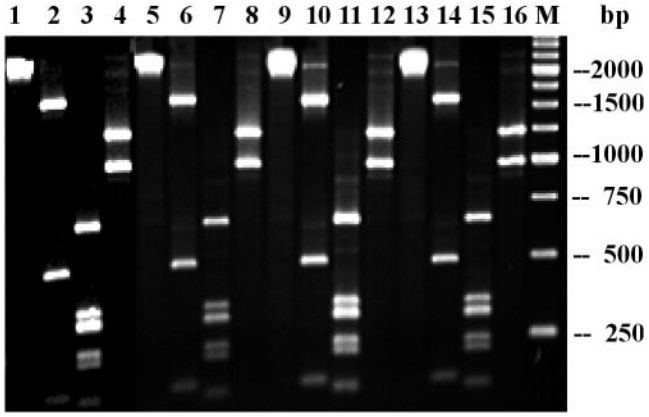

FIG. 1. Results of the restriction analysis of the 2.2-kb amplicons of ColE1::Tn*7*, RP4-2, and two representative class 2 integron-positive *Salmonella* serovar Paratyphi B dT$^+$ strains with *Hin*cII (lanes 2, 6, 10, and 14), *Hin*fI (lanes 3, 7, 11, and 15), and *Ava*I (lanes 4, 8, 12, and 16). Lanes 1, 5, 9, and 13 contain the undigested amplicons. Lane M, 250-bp ladder (Roche Diagnostics, Mannheim, Germany).

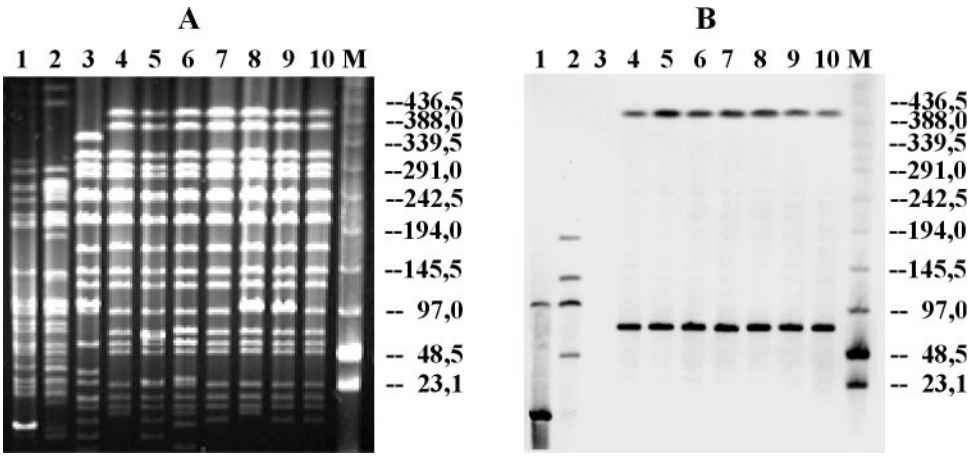

FIG. 2. Localization of class 2 integrons by PFGE mapping. Shown are PFGE patterns of representative *Salmonella* serovar Paratyphi B dT$^+$ strains (A) and results of a Southern blot analysis performed on the same gel with the *intI2*- (or *dfrA1*-, *sat1*-, or *aadA1*-) specific probe (B). Lanes 1, ColE1::Tn*7*; lanes 2, RP4-2; lanes 3, class 2 integron-negative comparative strain; lanes 4 to 10, representative class 2 integron-positive *Salmonella* serovar Paratyphi B dT$^+$ strains; lanes M, low-range PFGE markers (New England Biolabs, Frankfurt, Germany). The control strain ColE1::Tn*7* revealed the expected strong reactivity with the 20-kb plasmid fragment and with at least one chromosomal fragment (13). In the control strain RP4-2 the probes recognized four chromosomal fragments of different sizes, indicating the presence of four copies of the integron-specific genes.

typhi B dT$^+$ isolates carrying class 2 integrons (Fig. 2). This suggests the presence of two copies of the integrase 2 gene and the other integron-specific genes on the bacterial chromosome. In contrast to class 1 integrons, which could be localized on high-molecular-weight plasmids (data not shown), none of the class 2 integron genes was plasmid encoded.

In conclusion, this study shows that all isolates of the predominating German *Salmonella* serovar Paratyphi B dT$^+$ clone carry two Tn*7*-like class 2 integrons and that these integron structures are located on the bacterial chromosome. The chromosomal occurrence of class 2 integrons in this *Salmonella* serovar is a novel finding and in agreement with the hypothesis that the multiresistance in *Salmonella* serovar Paratyphi B dT$^+$ has evolved during the past decade by insertion of the transposon Tn*7* into the bacterial chromosome. The subsequent vertical transmission within the serotype explains why the resistance has become so widespread and persistent in European *Salmonella* serovar Paratyphi B dT$^+$ isolates.

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