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Human Infections Attributable to the D-Tartrate-Fermenting Variant of *Salmonella enterica* Serovar Paratyphi B in Germany Originate in Reptiles and, on Rare Occasions, Poultry

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In this study, the population structure, incidence, and potential sources of human infection caused by the D-tartrate-fermenting variant of *Salmonella enterica* serovar Paratyphi B [*S.* Paratyphi B (DT+)] was investigated. In Germany, the serovar is frequently isolated from broilers. Therefore, a selection of 108 epidemiologically unrelated *S. enterica* serovar Paratyphi B (DT+) strains isolated in Germany between 2002 and 2010 especially from humans, poultry/poultry meat, and reptiles was investigated by phenotypic and genotypic methods. Strains isolated from poultry and products thereof were strongly associated with multilocus sequence type ST28 and showed antimicrobial multiresistance profiles. Pulsed-field gel electrophoresis XbaI profiles were highly homogeneous, with only a few minor XbaI profile variants. All strains isolated from reptiles, except one, were strongly associated with ST88, another distantly related type. Most of the strains were susceptible to antimicrobial agents, and XbaI profiles were heterogeneous. Strains isolated from humans yielded seven sequence types (STs) clustering in three distantly related lineages. The first lineage, comprising five STs, represented mainly strains belonging to ST43 and ST149. The other two lineages were represented only by one ST each, ST28 and ST88. The relatedness of strains based on the pathogenicity gene repertoire (102 markers tested) was mostly in agreement with the multilocus sequence type. Because ST28 was frequently isolated from poultry but rarely in humans over the 9-year period investigated, overall, this study indicates that in Germany *S. enterica* serovar Paratyphi B (DT+) poses a health risk preferentially by contact with reptiles and, to a less extent, by exposure to poultry or poultry meat.

Salmonellosis is still one of the major global causes of gastroenteritis in humans and animals. The underlying bacterium *Salmonella enterica* is divided into more than 2,570 serovars (16). The D-tartrate-fermenting variant (DT+) of *Salmonella enterica* subsp. *enterica* (referred to as *S. enterica*) serovar Paratyphi B, formerly called *S. enterica* serovar Java, is recognized as one important cause of gastroenteritis worldwide (6, 36). According to the White-Kauffmann-Le Minor scheme, the seroformula is 4,[5],12:b:1,2, optionally expressing or not expressing the O:5 antigen (16). Since the end of the 1990s, the serovar has become established in poultry, especially in Germany, Netherlands, and Belgium (12). It was shown that this variant is multidrug resistant, carrying a class 2 integron-associated *dfrA1-sat1-aadA1* gene cassette conferring resistance to trimethoprim, streptomycin, and spectinomycin (27), and that it possesses a particular pathogenicity gene repertoire (21). Outbreaks caused by *S. enterica* serovar Paratyphi B (DT+) have been associated with goat's milk cheese in France (10), alfalfa sprouts or aquariums in Canada (15, 32), tropical fish aquariums in Australia (25), turtle exposure in the United States (17), and poultry in several European countries (27, 33).

Multilocus sequence typing (MLST) data indicated that *S. enterica* serovar Paratyphi B, similar to *S. enterica* serovar Newport, is polyphyletic (31). Altogether, 17 distinct sequence types (STs) have been previously described, clustering in three distantly related groups (1).

The aim of this study was to get a better understanding of the clonality and genetic relatedness of *S. enterica* serovar Paratyphi B (DT+) in Germany and to identify the potential sources of human infection caused by this serovar and its potential transmission by poultry meat to humans. Therefore, a representative collection of 108 *S. enterica* serovar Paratyphi B (DT+) strains isolated in Germany from humans, the environment, reptiles, and poultry/poultry meat during the years 2002 to 2010 was investigated using phenotypic and genotypic methods. Moreover, the pathogenicity and resistance gene repertoire of selected strains were determined.

MATERIALS AND METHODS

Selection of strains. Between 2002 and 2010 the National Reference Laboratory for Salmonella (NRL-BfR) received for diagnostic serotyping 35,925 *Salmonella* strains, isolated by public or private diagnostic laboratories across Germany, from livestock, animals, food, feed, and the environment. Of these, 2.1% (751 strains) were assigned to *S. enterica* serovar Paratyphi B (DT+) (Table 1). Only 33 of these strains (4.4%) exhibited the O:5 antigen in addition to the 4,12 antigen. Similarly, between 2002 and 2010 the National Reference Centre for Salmonella and other Enterics (NRZ-RKI), Wernigerode, Germany, received 33,935 *Salmonella* strains isolated from humans with *Salmonella* infection in Germany. Of these, 211 strains (0.6%) were serotyped as *S. enterica* serovar Paratyphi B (DT+) (Table 1). Most of the *S. enterica* serovar Paratyphi B (DT+) strains (195 strains, 92%) expressed the O:5 antigen. Based on both collections, altogether 56 *S. enterica* serovar Paratyphi B (DT+) strains from humans and

Received 29 May 2012 Accepted 1 August 2012
Published ahead of print 10 August 2012
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doi:10.1128/AEM.01732-12

TABLE 1 Number and source of *S. enterica* serovar Paratyphi B (dT+) isolates in Germany received by the NRL-BfR and NRZ-RKI

Yr of isolation	No. of isolates from:		4,5,12:b:1,2 isolates		4,12:b:1,2 isolates	
	NRL	NRZ	Total no.	Composition of group (no. [source])	Total no.	Composition of group (no. [source])
2002	4,411	6,300	20	15 (human), 4 (reptile), 1 (other)[a]	108	2 (human), 36 (poultry/meat), 1 (poultry/organ), 57 (poultry/feces), 2 (food), 3 (environment), 7 (other)
2003	3,630	3,930	12	12 (human)	79	4 (human), 26 (poultry/meat), 4 (poultry/organ), 26 (poultry/feces), 3 (food), 5 (environment), 11 (other)
2004	3,604	3,691	18	17 (human), 1 (reptile)	84	1 (human), 15 (poultry/meat), 3 (poultry/organ), 40 (poultry/feces), 1 (cattle), 7 (environment), 17 (other)
2005	4,090	3,655	28	26 (human), 2 (reptile)	56	2 (human), 26 (poultry/meat), 2 (poultry/organ), 21 (poultry/feces), 4 (food), 1 (other)
2006	3,887	3,333	34	34 (human)	76	42 (poultry/meat), 10 (poultry/organ), 5 (poultry/feces), 6 (food), 9 (environment), 4 (other)
2007	3,955	3,855	21	16 (human), 2 (reptile), 3 (food)	84	1 (human), 67 (poultry/meat), 8 (poultry/organ), 3 (food), 1 (environment), 4 (other)
2008	3,606	3,205	19	17 (human), 2 (reptile)	84	59 (poultry/meat), 9 (poultry/organ), 1 (poultry/feces), 1 (food), 2 (cattle), 5 (environment), 7 (other)
2009	4,111	3,646	34	23 (human), 9 (reptile), 2 (food)	73	6 (human), 34 (poultry/meat), 13 (poultry/organ), 3 (poultry/feces), 3 (food), 9 (environment), 5 (other)
2010	4,631	2,320	42	35 (human), 7 (reptile)	90	31 (poultry/meat), 28 (poultry/organ), 2 (poultry/feces), 11 (food), 2 (cattle), 9 (environment), 7 (other)
Total	35,925	33,935	228	195 (human), 27 (reptile), 5 (food), 1 (other)	734	16 (human), 336 (poultry/meat), 78 (poultry/organ), 155 (poultry/feces), 33 (food), 5 (cattle), 48 (environment), 63 (other)

[a] Other, rare isolates or source of isolation not specified.

52 from animals, food, and the environment were chosen for a deeper molecular typing procedure (Table 2). Within the selection, all 16 strains isolated from humans and not exhibiting the O:5 antigen were included. The remaining 40 strains (71%) isolated from humans and expressing the O:5 antigen were randomly selected. Twenty-seven of the 52 strains (52%) selected from animals, food, and the environment were O:5 antigen positive. They represent 79% of all O:5-antigen-expressing *S. enterica* serovar Paratyphi B (dT+) strains isolated between 2002 and 2010. The remaining 25 strains (48%) lacked the O:5 antigen and were selected with a focus on poultry and chicken meat. Two strains originated from cattle, and one strain each was from the environment (compost), ice cream, and fish.

The strains selected have no epidemiological link (i.e., they were not isolated at the same place or time or from the same animals or foods). They cover various geographical origins and potential sources in Germany isolated between 2002 and 2010, with an emphasis on the years 2009 and 2010.

To study the pathogenicity gene repertoire using DNA microarrays and to compare *sop* gene sequence typing (*sop*-ST) (18) with MLST (23), a subset of 35 *S.* Paratyphi B (dT+) strains was further chosen to reflect the diversity of pulsed-field gel electrophoresis (PFGE) XbaI profiles within the set of 108 epidemiologically unrelated strains (Table 2).

Serotyping. Serotyping was performed according to the White-Kauffmann-Le Minor scheme (16) by slide agglutination with O- and H-antigen-specific sera (Sifin Diagnostics, Berlin, Germany).

Antimicrobial susceptibility testing. Antimicrobial susceptibility of strains was tested against 14 antimicrobials or antimicrobial combinations by determining the MICs using the CLSI broth microdilution method (8) in combination with the semiautomatic Sensititre system (TREK Diagnostic Systems, Cleveland, OH). Cutoff values to be used to determine susceptibility to 10 antimicrobials were applied as described in the Commission Decision on a harmonized monitoring of antimicrobial resistance in poultry and pigs [(EG) 2007/407], as follows (mg/liter): cefotaxime (FOT), >0.5; nalidixic acid (NAL), >16; ciprofloxacin (CIP), >0.06; ampicillin (AMP), >4; tetracycline (TET) >8; chloramphenicol (CHL) >16; gentamicin (GEN) >2; streptomycin (STR) >32; trimethoprim (TMP) >2; and sulfamethoxazole (SMX) >256. Cutoff values for the remaining four antimicrobials were adopted from the European Committee on Antimicrobial Susceptibility Testing ([EUCAST] 2011 breakpoints [http://www.eucast.org/clinical_breakpoints/]), as follows (mg/liter): colistin (COL), >2; florfenicol (FFN), >16; kanamycin (KAN) >32; and ceftazidime (TAZ), >2.

Genomic DNA purification. DNA for PCRs and DNA microarray experiments was isolated from strains grown in Luria-Bertani broth (Merck, Darmstadt, Germany) at 37°C for 16 to 18 h. A 1.6-ml aliquot was used for purification using an RTP Bacteria DNA Mini Kit (Stratec Molecular GmbH, Berlin, Germany) according to the manufacturer's protocol with one additional step. After the cell lysis step at 95°C for 5 to 10 min, 5 μl of 100 mg/ml RNase (Qiagen GmbH, Hilden, Germany) was added, and the sample was incubated at room temperature for 30 min. The quality and quantity determinations of DNA were performed spectrophotometrically. For DNA labeling with fluorophores, a minimum of 4 μg of DNA was used, and for PCRs 1-ng/μl DNA dilutions in Tris-EDTA (TE) buffer were used.

Multilocus sequence typing (MLST) and *sop*-ST typing. MLST was carried out as previously described including partial sequences of the seven housekeeping genes: *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*

TABLE 2 *S. enterica* serovar Paratyphi B (dT+) isolates used for phenotypic and molecular analysis in this study

Strain no.	Yr of isolation	Origin	Resistance[a]	PFGE cluster	PFGE profile no.	MLST	*sop*-ST	Microarray (PAT PT)[b]	O antigen
02-04534	2002	Fertilizer	Susceptible	B	44	149	12-34-25	9	4,5,12
02-00729	2002	Reptile, Mississippi turtle	Susceptible	B	32	43	11-25-25	5	4,5,12
02-04564	2002	Reptile, snake	Susceptible	A	15	88	NT	NT	4,5,12
02-04565	2002	Reptile, snake	Susceptible	A	28	88	NT	NT	4,5,12
02-04626	2002	Reptile, corn snake	TET	A	19	88	NT	NT	4,5,12
04-01793	2004	Reptile, snake	TET	A	14	88	39-28-24	2	4,5,12
05-01473	2005	Reptile, snake	STR TET TMP	A	3	88	39-28-24	2	4,5,12
05-05074	2005	Reptile, snake	Susceptible	A	28	88	NT	NT	4,5,12
07-01916-1	2007	Fish, pintado	Susceptible	B	58	88	41-28-24	2	4,5,12
07-03272	2007	Reptile, snake	Susceptible	A	1	88	NT	NT	4,5,12
07-04003	2007	Reptile, unkown	Susceptible	A	20	88	NT	NT	4,5,12
08-01440	2008	Reptile, turtle	Susceptible	B	54	88	NT	NT	4,5,12
08-04475	2008	Reptile, snake	NAL CIP	A	16	88	NT	NT	4,5,12
09-01458	2009	Reptile, snake	Susceptible	A	12	88	NT	NT	4,5,12
09-02038	2009	Reptile, snake	Susceptible	A	2	88	39-28-24	4	4,5,12
09-02329	2009	Reptile, snake	TET	A	13	88	NT	NT	4,5,12
09-02447	2009	Reptile, snake	Susceptible	A	10	88	NT	NT	4,5,12
09-02452	2009	Reptile, turtle	Susceptible	A	10	88	39-28-24	2	4,5,12
09-02772	2009	Reptile, snake	Susceptible	A	26	88	NT	NT	4,5,12
09-03579	2009	Reptile, snake	Susceptible	A	11	88	NT	NT	4,5,12
09-03667	2009	Chicken, meat	Susceptible	B	47	149	12-34-25	9	4,5,12
09-04735	2009	Reptile, snake	Susceptible	A	17	88	NT	NT	4,5,12
10-00628	2010	Reptile, unkown	Susceptible	A	18	88	NT	NT	4,5,12
10-02147	2010	Reptile,snake	Susceptible	A	22	88	39-28-24	2	4,5,12
10-03633	2010	Reptile,snake	Susceptible	A	27	88	39-28-24	2	4,5,12
10-04378	2010	Reptile, snake	Susceptible	A	8	88	39-28-24	3	4,5,12
10-04870	2010	Reptile, snake	Susceptible	A	25	88	NT	NT	4,5,12
02-04177	2002	Chicken, feces	CIP NAL STR TMP	C	60	28	38-27-0	1	4,12
03-01517	2003	Fish	STR TMP	C	60	28	NT	NT	4,12
03-02942	2003	Turkey	AMP STR SMX TET TMP	C	60	28	NT	NT	4,12
04-03840	2004	Cattle, organ	CIP KAN NAL STR SMX TET TMP	C	60	28	NT	NT	4,12
05-01581	2005	Compost	AMP CIP NAL STR SMX TMP	C	60	28	NT	NT	4,12
06-02092	2006	Ice cream	CIP NAL STR TMP	C	60	28	NT	NT	4,12
06-05076	2006	Chicken, meat	AMP CHL STR SMX TET TMP	C	60	28	NT	NT	4,12
08-00451	2008	Chicken, meat	AMP CIP NAL STR TMP	C	60	28	NT	NT	4,12
08-01554	2008	Chicken, meat	SMX STR TET TMP	C	62	28	NT	NT	4,12
08-02848	2008	Cattle, organ	CIP NAL TMP	C	65	28	38-27-0	1	4,12
08-04157	2008	Chicken, meat	AMP CIP GEN NAL SMX TMP	C	68	28	NT	NT	4,12
08-04806	2008	Chicken, meat	CIP NAL SMX TMP	C	60	28	NT	NT	4,12
09-02946	2009	Chicken, meat	AMP CHL CIP GEN KAN NAL SMX STR TET TMP	C	73	28	38-27-0	1	4,12
09-03610	2009	Chicken, meat	CHL CIP NAL SMX STR TET TMP	C	69	28	NT	NT	4,12
09-04217	2009	Chicken, meat	AMP FOT TAZ TMP	C	60	28	38-27-0	1	4,12
10-00908	2010	Turkey	CIP KAN NAL SMX TET TMP	C	60	28	NT	NT	4,12
10-01119	2010	Chicken, meat	AMP CHL CIP NAL SMX STR TET TMP	C	66	28	NT	NT	4,12
10-01673	2010	Chicken, meat	AMP CIP NAL SMX TET TMP	C	60	28	NT	NT	4,12
10-01782	2010	Chicken, meat	CIP NAL TMP	C	70	28	38-27-0	1	4,12
10-03460	2010	Cattle, organ	AMP CHL CIP NAL SMX TET TMP	C	60	28	NT	NT	4,12
10-04808	2010	Turkey	AMP CHL CIP GEN KAN NAL SMX STR TET TMP	C	60	28	38-27-0	1	4,12
10-05176	2010	Chicken	CIP NAL STR TMP	C	71	28	NT	NT	4,12
11-01472	2002	Human	AMP CHL FFN SMX STR TET	B	34	43	NT	NT	4,5,12
11-01474	2003	Human	STR TET	A	5	88	NT	NT	4,5,12
11-01475	2003	Human	SMX STR	B	33	43	NT	NT	4,5,12
11-01476	2004	Human	Susceptible	B	45	149	NT	NT	4,5,12
11-01478	2004	Human	Susceptible	B	52	43	NT	NT	4,5,12
11-01479	2005	Human	STR	B	46	149	NT	NT	4,5,12
11-01480	2005	Human	COL	B	40	110	NT	NT	4,5,12
11-01481	2005	Human	Susceptible	B	50	43	11-25-25	5	4,5,12
11-01482	2006	Human	Susceptible	B	42	149	NT	NT	4,5,12

(Continued on following page)

TABLE 2 (Continued)

Strain no.	Yr of isolation	Origin	Resistance[a]	PFGE cluster	PFGE profile no.	MLST	*sop*-ST	Microarray (PAT PT)[b]	O antigen
11-01483	2007	Human	Susceptible	B	58	88	NT	NT	4,5,12
11-01484	2007	Human	Susceptible	A	6	88	39-28-24	3	4,5,12
11-01485	2009	Human	Susceptible	A	23	88	NT	NT	4,5,12
11-01486	2009	Human	Susceptible	B	55	88	41-28-24	2	4,5,12
11-01487	2009	Human	Susceptible	B	59	88	NT	NT	4,5,12
11-01488	2009	Human	Susceptible	B	55	88	NT	NT	4,5,12
11-01489	2009	Human	Susceptible	A	24	88	NT	NT	4,5,12
11-01490	2009	Human	Susceptible	A	7	88	NT	NT	4,5,12
11-01491	2009	Human	Susceptible	A	7	88	NT	NT	4,5,12
11-01492	2009	Human	AMP CHL FFN SMX STR TET	B	34	43	11-25-25	6	4,5,12
11-01493	2009	Human	Susceptible	B	42	149	NT	NT	4,5,12
11-01494	2009	Human	Susceptible	B	42	149	12-34-25	10	4,5,12
11-01495	2009	Human	Susceptible	B	49	149	NT	NT	4,5,12
11-01496	2009	Human	Susceptible	B	31	896	12-24-25	8	4,5,12
11-01497	2009	Human	Susceptible	B	42	149	NT	NT	4,5,12
11-01498	2009	Human	Susceptible	B	42	149	NT	NT	4,5,12
11-01499	2009	Human	Susceptible	B	58	88	NT	NT	4,5,12
11-01500	2009	Human	Susceptible	B	38	43	NT	NT	4,5,12
11-01501	2009	Human	AMP CHL FFN SMX STR TET	B	34	43	NT	NT	4,5,12
11-01502	2009	Human	Susceptible	B	36	307	11-25-25	11	4,5,12
11-01503	2010	Human	Susceptible	B	51	43	NT	NT	4,5,12
11-01504	2010	Human	Susceptible	B	29	307	11-25-25	5	4,5,12
11-01505	2010	Human	Susceptible	B	41	43	NT	NT	4,5,12
11-01506	2010	Human	Susceptible	B	35	43	NT	NT	4,5,12
11-01507	2010	Human	AMP SMX	B	34	43	11-25-25	6	4,5,12
11-01508	2010	Human	Susceptible	B	37	43	NT	NT	4,5,12
11-01509	2010	Human	Susceptible	B	56	88	NT	NT	4,5,12
11-01510	2010	Human	STR	B	43	149	NT	NT	4,5,12
11-01511	2010	Human	Susceptible	A	9	88	NT	NT	4,5,12
11-01512	2010	Human	Susceptible	A	4	88	39-28-24	2	4,5,12
11-01513	2010	Human	Susceptible	A	4	88	NT	NT	4,5,12
11-01515	2002	Human	AMP CIP NAL SMX STR TET TMP	C	60	28	NT	NT	4,12
11-01516	2002	Human	Susceptible	B	30	110	12-24-25	8	4,12 (7-bp deletion)
11-01517	2003	Human	CIP NAL SMX STR TMP	C	63	28	NT	NT	4,12
11-01518	2003	Human	AMP CIP NAL SMX STR TMP	C	60	28	NT	NT	4,12
11-01519	2003	Human	STR TMP	C	60	28	NT	NT	4,12
11-01520	2003	Human	AMP CIP NAL SMX STR TMP	C	60	28	NT	NT	4,12
11-01521	2004	Human	AMP FOT STR TAZ TMP	C	60	28	NT	NT	4,12
11-01522	2005	Human	Susceptible	B	48	149	46-34-25	9	4,12 (7-bp deletion)
11-01523	2005	Human	Susceptible	A	21	88	39-28-24	2	4,12 (7-bp deletion)
11-01524	2007	Human	CHL CIP NAL SMX STR TET TMP	C	67	28	NT	NT	4,12
11-01525	2009	Human	AMP CHL CIP COL GEN KAN NAL SMX STR TET TMP	C	72	28	NT	NT	4,12
11-01526	2009	Human	AMP CIP FOT NAL SMX STR TAZ TET TMP	C	64	28	38-27-0	1	4,12
11-01527	2009	Human	Susceptible	B	57	88	41-28-24	2	4,12 (7-bp deletion)
11-01528	2009	Human	Susceptible	B	57	88	NT	NT	4,12 (7-bp deletion)
11-01529	2009	Human	STR	B	58	88	41-28-24	2	4,12 (7-bp deletion)
11-01530	2009	Human	CIP NAL STR TMP	C	61	28	NT	NT	4,12
11-01532	2006	Food, spice	Susceptible	B	53	43	11-25-25	5	4,5,12
11-01533	2008	Sludge	AMP CIP SMX	B	39	43	11-25-25	7	4,5,12
11-01534	2005	Compost	STR TMP	C	60	28	NT	NT	4,12

[a] See Materials and Methods for abbreviations.
[b] NT, not tested.

(23). Alleles and sequence types were assigned according to the MLST scheme (available at http://mlst.ucc.ie/mlst/dbs/Senterica). Unknown alleles were submitted to the website and newly named. The analysis was carried out in BioNumerics, version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). The comparisons were made by advanced cluster analysis using the analysis template maximum spanning tree (MST) for categorical data on merged sequences of the seven genes. The *sop* gene sequence typing (*sop*-ST) was carried out as described previously (18) including the genes *sopA*, *sopB*, and *sopD*. Laboratory-internal allele numbers were assigned to new alleles, resulting in a *sop*-ST allele pattern (*sopA-sopB-sopD*) for a given strain. Based on a multiple alignment of merged *sop* sequences, similarities were analyzed by the unweighted pair group method with arithmetic averages (UPGMA) in BioNumerics, version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium).

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed according to the Pulse-Net protocol (30) using the restriction enzyme XbaI for digestion of genomic DNA. The analyses of the gel images were carried out in BioNumerics, version 6.5. The comparisons were made by cluster analysis using the Dice coefficient and UPGMA with a position tolerance of 1.5% and optimization of 1.0%. Fragments that were smaller than 25 kb were not considered for cluster analysis.

DNA microarray analysis. The DNA microarray used in this study was applied as previously described (20). Altogether, 80 pathogenic markers, 22 fimbrial gene markers, and 49 resistance gene markers were analyzed from 35 strains representing the diversity of PFGE profiles. Analysis of raw data was performed as previously described (20). After normalization (presence/absence of gene), the data for each strain were imported in BioNumerics, version 6.5, as character values. For comparison, a cluster analysis with the simple matching binary coefficient, using the UPGMA dendrogram type, was applied on the basis of the 80 pathogenicity and 22 fimbriae markers.

***oafA* and D-tartrate PCRs.** Presence of the *oafA* gene responsible for O:5 antigen expression in *Salmonella* was performed according to Hauser et al. (19) using primers P-439 and P-440 amplifying a 433-bp PCR product. To detect a 7-bp deletion within the open reading frame, primers P-439 and P-1072 were used, resulting in a PCR product of 170 bp. In case of the 7-bp deletion, no PCR product was obtained. The ability of *S. enterica* serovar Paratyphi B (dT+) strains to ferment D-tartrate was analyzed according to the PCR protocol described by Malorny et al. (26).

Statistical methods. To assess the discrimination index of PFGE, Simpson's index of diversity (ID) and 95% confidence intervals (CI) were calculated using software at the Comparing Partitions website(http://darwin.phyloviz.net/ComparingPartitions/index.php?link = Tool)

Nucleotide sequence accession numbers. New alleles detected by *sop*-ST were deposited in GenBank under the following accession numbers: JQ045575 to JQ045580 for *sopA11*, *sopA12*, *sopA38*, *sopA39*, *sopA41*, and *sopA46*; JQ067612 to JQ067615 for *sopB28*, *sopB27*, *sopB25*, and *sopB24*; JQ067610 for *sopB34*; JQ067622 for *sopD24*; and JQ067623 for *sopD25*.

RESULTS

O:5 antigen distribution in *S. enterica* serovar Paratyphi B (dT+). A minority of *S. enterica* serovar Paratyphi B (dT+) strains collected between 2002 and 2010 at the National Reference Laboratory for Salmonella (NRL-BfR) (33 strains, 4.4%) expressed the O:5 antigen in addition to the 4,12 antigen (O:4,5,12). These were isolated mostly from reptiles (27 strains, 82%) and only sporadically from food (5 strains, 15%) and the environment (1 strain, 3%) (Table 1). In contrast, O:5-antigen-negative strains were isolated mainly from poultry and products thereof (569 strains, 79%) and sporadically from pigs, cattle, and diverse food products but not from reptiles. The National Reference Centre for Salmonella and other Enterics (NRZ-RKI), Wernigerode, Germany, received between 2002 and 2010 a total of 211 *S. enterica* serovar Paratyphi B (dT+) strains isolated from humans in Germany. Most of them (195 strains, 92%) expressed the O:5 antigen. It is obvious that data show a contrast with respect to expression of the O:5 antigen between strains isolated from humans and those isolated from livestock, especially poultry.

For the investigation of clonality and genetic relatedness, 108 epidemiologically unrelated *S. enterica* serovar Paratyphi B (dT+) strains from the collection of the NRL-BfR and NRZ-RKI were selected (Table 2) (for selection criteria, see Materials and Methods).

Antimicrobial resistance. Fifty-nine of the 108 *S.* Paratyphi B (dT+) strains (55%) tested were susceptible to all 14 antimicrobials. Three strains were monoresistant to tetracycline, one strain was monoresistant to streptomycin, and one was monoresistant to colistin. Forty-two strains (39%) were multidrug resistant to two or more antimicrobials. All strains isolated from livestock or poultry meat were multidrug resistant to up to 10 antimicrobials (Table 2) and did not express the O:5 antigen. Strains isolated from humans and lacking the O:5 antigen were mainly multidrug resistant, but five strains were susceptible. A PCR screening of the presence of the *oafA* gene encoding the O:5 antigen revealed the absence of the gene in multidrug-resistant strains. Susceptible O:5-antigen-negative strains harbored the *oafA* gene, but a 7-bp deletion within the open reading frame obviously caused a frameshift leading to the loss of O:5 antigen expression, as previously described by Hauser et al. (19) for *S. enterica* serovar Typhimurium. Human strains expressing factor 5 were mainly susceptible. Three strains exhibited multidrug resistance to AMP, CHL, FFN, SMX, STR, and TET, indicating the existence of a *Salmonella* genomic island 1 (SGI1), a cluster of genes encoding multidrug resistance (35). Responsible genes for antimicrobial resistance were determined by DNA microarray. Poultry-associated multidrug-resistant strains harbored a class 2 integrase gene. In combination with *dfrA1*, *sat1* (Tn*7*), and *aadA1*, this indicated that a class 2 integron was carried by these strains, as previously described by Miko et al. (28). Additionally, six strains had a class 1 integrase gene in combination with *sul1*. Multidrug-resistant strains expressing the O:5 antigen showed the typical resistance genes usually found in SGI1, namely, *floR*, *aadA2*, bla_{PSE1}, *tet*(G), and *sul1*. Two strains isolated from humans and one strain isolated from chicken meat were resistant to extended-spectrum β-lactams TAZ and FOT. Responsible antimicrobial resistance gene families could be assigned by DNA microarray to bla_{CMY-2}-like (09-04217, chicken), bla_{CTX-M2}-like (11-01526, human), and bla_{TEM1}-like (11-01521, human) genes.

Typing by PFGE. Seventy-three different XbaI profiles could be distinguished among the 108 strains analyzed (Fig. 1) (ID, 0.962 [95% CI, 0.937 to 0.988]). They were classified into three clusters (A, B, and C). Cluster A contained 32 strains of the 108 strains (30%), cluster B contained 43 strains (40%), and cluster C contained 33 strains (31%). XbaI profile number 60 harbored 20 strains of the 33 strains (61%) in cluster C and was previously described as an X8 profile (21, 28). These strains were isolated from poultry/poultry meat, other livestock, and humans but not from reptiles. All strains in cluster C lacked the *oafA* gene completely and therefore did not express the O:5 antigen. Strains isolated from reptiles clustered preferentially in cluster A. Human strains were distributed over all three clusters. The similarity coefficient (*F* value) ranged for cluster C from 0.6 to 0.95 and was slightly lower for clusters A (0.54 to 0.93) and B (0.56 to 0.93). The

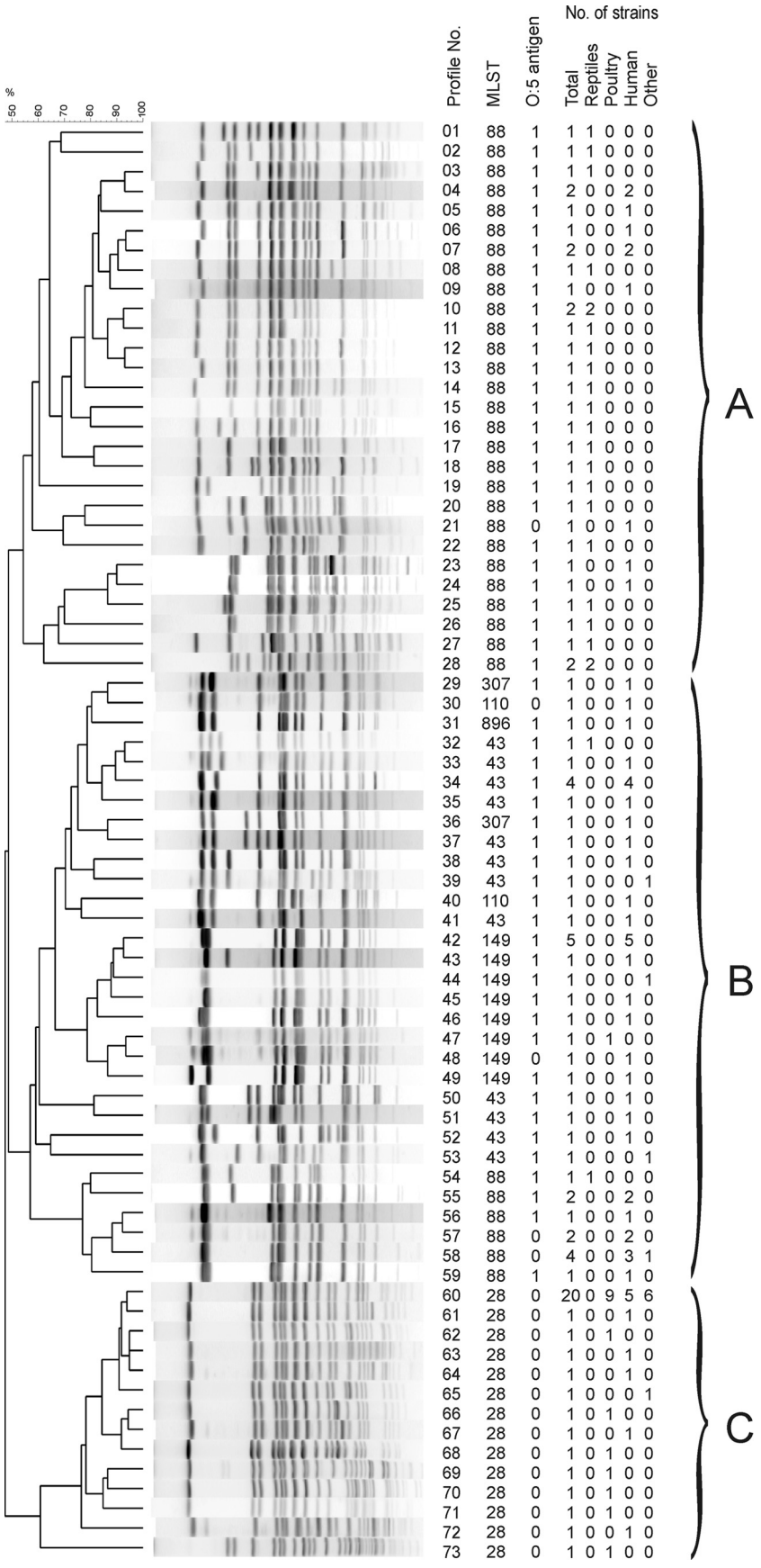

FIG 1 UPGMA dendrogram of PFGE profiles identified in 108 *S. enterica* serovar Paratyphi B (dT+) strains after digestion with XbaI. Profiles were numbered serially from 1 to 73. The number of strains belonging to each source (total, reptile, poultry, human, and other), corresponding MLSTs, and present (1) or absent (0) O:5 antigen are shown on the right side. Assigned clusters A to C are indicated by curly brackets.

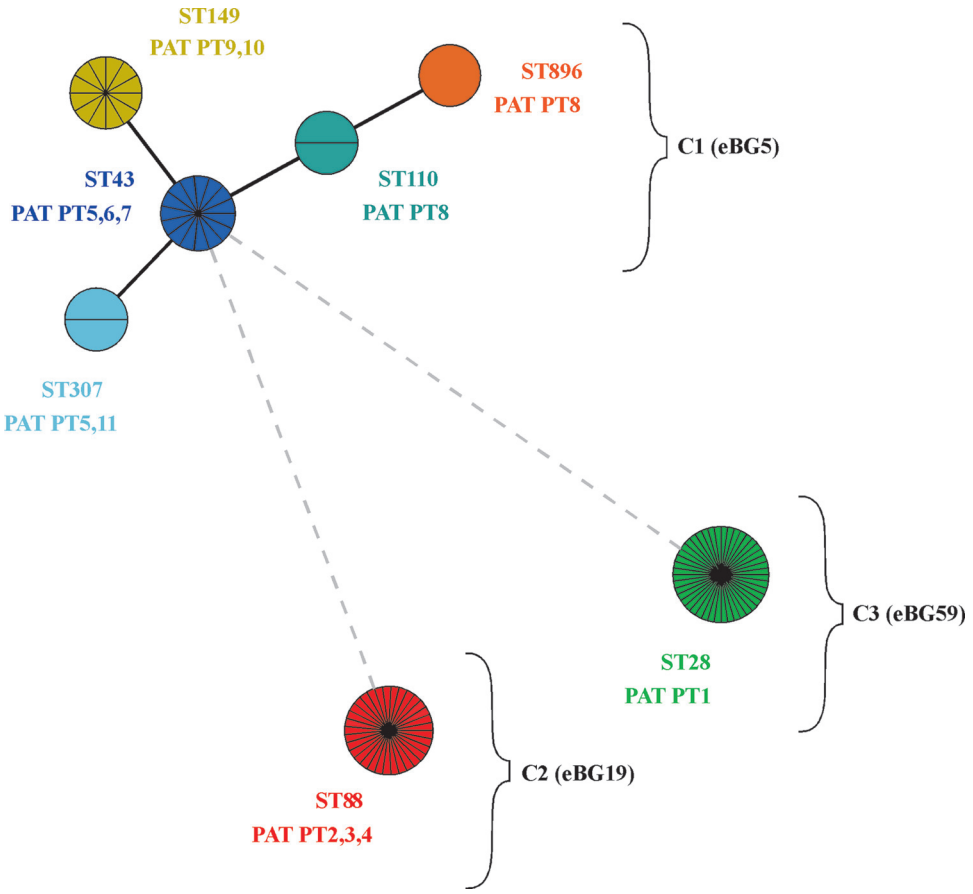

FIG 2 Minimal spanning tree of MLST data on 108 *S. enterica* serovar Paratyphi B (dT+) isolates. Each circle refers to one ST subdivided into one pie slice per strain. STs that share six identical gene alleles are linked by a black line, and STs sharing only one or no common gene allele are linked by a gray dotted line. Based on their similarity, STs were grouped in three complexes (C1, C2, and C3). According to the nomenclature of Achtman et al. (1), C1 is designated eBG5, C2 is eBG19, and C3 is eBG59. Pathogenicity array types (PATs) found in each ST are shown below ST designations.

Simpson's index of discrimination (ID) was considerably higher for clusters A (ID, 0.992 [95% CI, 0.982 to 1.000]) and B (ID, 0.973 [95% CI, 0.952 to 0.995]) than for cluster C (ID, 0.640 [95% CI, 0.444 to 0.836]), indicating lower diversity within the poultry/poultry meat strains.

MLST. Seven different sequence types (STs) were identified, namely, ST28, -43, -88, -110, -149, -307, and -896 (ID, 0.722 [95% CI, 0.675 to 0.769]). Forty-three strains (39.8%) of the 108 strains investigated belonged to ST88, 33 strains (30.5%) belonged to ST28, 12 strains (11.1%) belonged to ST149, 15 strains (13.8%) belonged to ST43, and 2 strains (1.8%) belonged to ST307 and ST110 each. One single strain was assigned to ST896. STs were categorized by BioNumerics in three clonal complexes (Fig. 2). The founder of the largest complex (C1) was identified to be ST43.

ST43 differed from ST149 in one nucleotide in the *purE* allele and from ST307 in one nucleotide in the *thrA* allele. ST110 had 5 nucleotides different from ST43 in the *dnaN* allele. ST896 differed in two alleles from ST43 (*dnaN*, five nucleotides, and *aroC*, one nucleotide). The other two clonal complexes were represented by only one ST each, ST88 (complex C2) and ST28 (complex C3), with six and seven different alleles compared to ST43, respectively.

It was obvious that strains isolated from poultry/poultry meat were, with one exception, always associated with ST28. Of the 16 strains isolated from humans between 2002 and 2010 and not expressing the O:5 antigen, 10 strains were assigned to ST28, 4 were assigned to ST88, and 1 was assigned to ST110 and ST149 each. All human O:5-antigen-negative strains distinct from ST28 harbored a nonfunctional *oafA* gene due to the 7-bp deletion within the ORF, whereas the *oafA* gene in ST28 strains was completely absent (Fig. 1). ST88 was identified with one exception in strains isolated from reptiles (53%) and humans (44%). The exceptional strain was isolated from fish. Strains assigned to the first clonal complex originated mainly from humans (84%), and single strains were from reptile, sludge, chicken, fertilizer, and spice (Table 2).

***sop*-ST.** For sequencing of the virulence genes *sopA*, *sopB*, and *sopD*, 35 strains were chosen representing the diversity of XbaI PFGE profiles. Among them, six different *sopA* alleles, five different *sopB* alleles, and two different *sopD* alleles resulting in seven different combinations of *sopA*, *sopB*, and *sopD* (*sop*-STs) were found (Fig. 3). Strains of the first clonal complex with the founder ST43 comprised four highly similar *sop*-STs. Strains with ST88 (second clonal complex) showed two different *sop*-STs (39-28-24 and 41-28-24) where the *sopA* allele 41 differed from allele 39 only in a 1-bp deletion at position 615 leading to a frameshift. All strains with *sopA* allele 41 clustered in one branch of the PFGE tree in cluster B. All ST28 strains (third clonal complex) were *sopD* negative and had identical *sopA* and *sopB* alleles (38-27-0). The

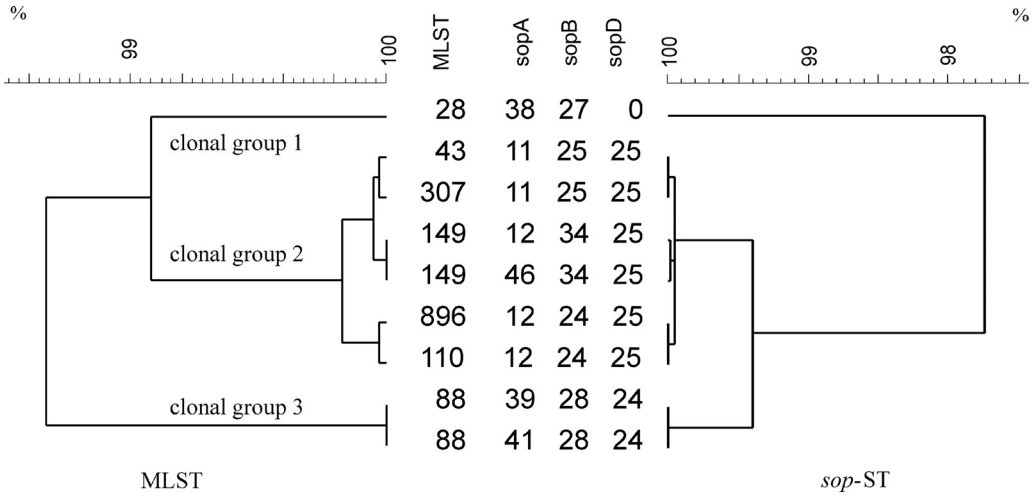

FIG 3 UPGMA dendrograms of 35 *S. enterica* serovar Paratyphi B (DT+) strains based on the merged DNA sequences. On the left side, the MLST dendrogram with corresponding sequence types (STs) is shown, and on the right side the *sop* dendrogram with corresponding *sop*-ST pattern types (*sopA-sopB-sopD*) is shown. The scale indicates the similarity of merged DNA sequences.

sopA allele 11 had a major deletion of 47 base pairs, indicating a recombination event. However, the three major clonal complexes identified by MLST were in agreement with the clustering of *sop*-ST-based single nucleotide polymorphisms (SNPs) within all three genes (Fig. 3).

Determination of pathogenicity genes. Eleven different pathogenicity array types (PATs) were identified among the same 35 strains tested as for *sop*-ST (Fig. 4). PATs differed in up to 21 targets from 102 markers tested. Almost all PATs corresponded to specific MLST types (Fig. 2). Only two PATs (PT5 and PT8) were found in two STs belonging to clonal complex 1. Markers targeting within the *Salmonella* pathogenicity islands (SPIs) 1 to 5 were partially absent in SPI-1 and SPI-3. PAT PT1 (exclusively associated with ST28 strains) lacked the *sugR* gene, and PAT PT2 to PT4 (ST88 strains) lacked the *rhuM* and *sugR* genes, both located in SPI-3. All strains belonging to clonal complex 1 (PAT PT5 to PT11) lacked the *avrA* gene present in SPI-1 (encoding a protein inhibiting the key proinflammatory immune response). In addition, markers for *msgA*, *pagK*, *slrP*, *sopD*, and *stcC* were specifically absent in PAT PT1. Three PATs (PT2 to PT4) were linked to ST88 strains which differed only in up to two targets (*irsA* and *stiC*). Strains tested belonging to clonal complex 1 showed seven different PATs (PT5 to PT9). These differed in up to nine pathogenicity gene markers. Strains with PAT PT6 and PAT PT7 probably contained a variant of the *Salmonella* genomic island 1 (SGI1) since their resistance genes, bla_{PSE1}, *floR*, *sul1*, *tet*(G), *aadA2-3-8*, and *qacE*Δ were detected in combination with the presence of markers *trhH* and *intSG1* (data not shown). Markers indicating the presence of a virulence plasmid (pSLT) were negative in all strains tested.

DISCUSSION

Population structure of *S. enterica* serovar Paratyphi B (DT+) in Germany. The application of genotypic methods to 108 *S. enterica* serovar Paratyphi B (DT+) strains isolated from diverse sources between 2002 and 2010 in Germany revealed that the serovar is polyphyletic. Polyphyletic serovars originate from more than one common ancestor, and they consist of several distantly related STs within one serovar. Phylogenetic trees based on data from PFGE, MLST, *sop*-ST, and the pathogenicity gene repertoire resulted, independently of the method, in three divergent lineages (referred to also as clonal complexes). The first clonal complex is composed of five closely related STs (ST43, ST110, ST149, ST307, and ST896) which correlated to PFGE cluster B (Fig. 1) and four closely related *sop*-STs. The other two clonal complexes were each represented by one single ST (ST88 and ST28) associated with PFGE clusters A and C. An exceptional set of 10 ST88 strains were grouped in cluster B instead of cluster A. Three of these strains had a unique *sop*-ST41-28-24 instead of *sop*-ST39-28-24. *sopA* allele 41 differed from *sopA* allele 39 by a deletion of one nucleotide, leading to a frameshift mutation. This shows that PFGE is not a reliable mirroring of the phylogeny of *S. enterica* serovar Paratyphi B (DT+) but is a highly discriminative method for short-term evolutionary studies since the Simpson index for discrimination was highest for PFGE in the strain set tested.

The three clonal complexes identified and characterized here were also recently detected within an international set of *S. enterica* serovar Paratyphi B strains based on MLST analysis (1, 31). Achtman et al. (1) have grouped STs into clonal complexes on the basis of an eBurst analysis. According to this nomenclature, clonal complex 1 was designated eBG5, clonal complex 2 was named eBG19, and clonal complex 3 was named eBG59. Specific information on the sources of strains and their origins or the differences in O:5 antigen expression for *S. enterica* serovar Paratyphi B (DT+) were not considered by Achtman et al. (1). However, the study contributed to resolve the evolutionary relationship between the D-tartrate fermenting (DT+) and nonfermenting (DT−) variants of *S. enterica* serovar Paratyphi B. The DT− variant is regarded as a cause of typhoid-like illness in humans, whereas the DT+ variant is associated with gastroenteritis in animals and humans (2, 22). Interestingly, DT− strains belonged to one of the same complex, eBG5, as the DT+ variant, but they are represented by other sequence types.

Recent studies indicated that genetic diversity within *S. enterica* is derived also from homologous recombination in addition to mutational changes (9, 11, 14). However, in this study it was ob-

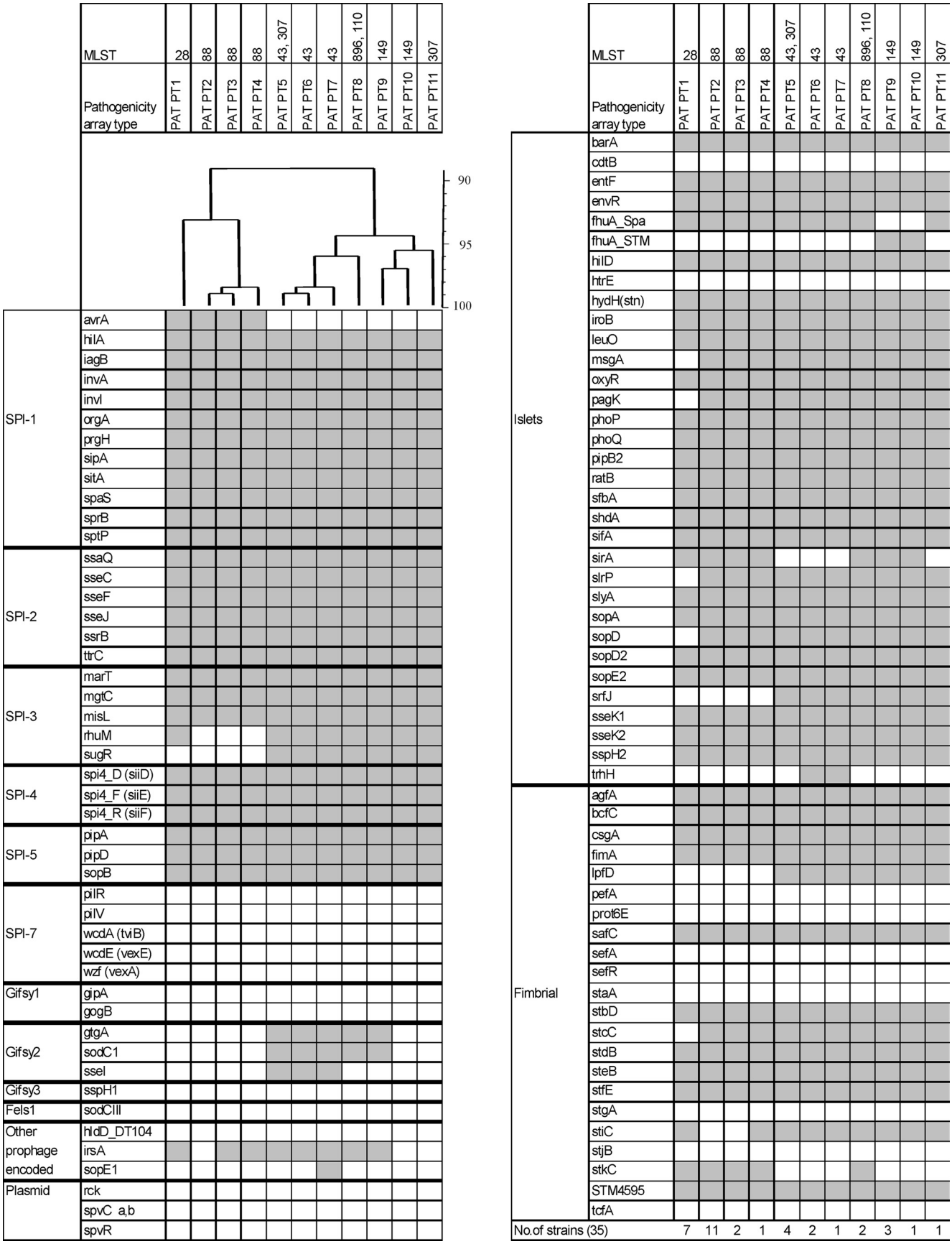

FIG 4 Virulence determinants of 35 *S. enterica* serovar Paratyphi B (dT+) strains analyzed. On the left side the analyzed genes are indicated and grouped according to their particular genomic location (SPI-1 to SPI-7; prophages Gifsy-1, Gifsy-2, Gifsy-3 and Fels-1; plasmids; and islets) or function (fimbrial). At the top, assigned PATs and corresponding STs are indicated. Immediately below this, an UPGMA dendrogram shows the similarity of PATs in percentages. The hybridization result of each PAT is shown by column. A white box indicates the absence, and a gray box indicates the presence of the target sequence.

vious that the tree structures (genetic relatedness) resulting from independent genotypic typing methods (MLST, *sop*-ST, and DNA microarray) were highly congruent (Fig. 3 and 4). The conclusion is that the three complexes evolved independently within specific niches or hosts with a small opportunity for recombination between them. We speculate that humans, as the only common host for all three clonal complexes, might have formerly played a role in recombination leading to the same serovar expression (O:4,[5],12:b:1,2) shared by all three complexes.

Association of lineages with origin of strains. The three divergent lineages could clearly be associated with the origin of the strains. Strains isolated from humans were found in all three lineages, with preference in clonal complex 1 (ST43 founder). Strains which were isolated from reptiles belonged to clonal complex 2 (ST88), and strains which were isolated mainly from poultry and occasionally from other livestock (pig and cattle) belonged to clonal complex 3 (ST28). However, the selection of the 108 epidemiologically unrelated strains did not reflect the frequency of isolation within a specific source. Despite the high number of *S. enterica* serovar Paratyphi B (dT+) strains received at the NRL-BfR isolated from poultry/poultry meat and the potentially high exposure of poultry meat to humans, NRZ-RKI obtained only 10 human strains which could be associated with the typical poultry-adapted ST28 (O:5-antigen-negative) complex within the last 9 years (Table 2). This indicates that apparently ST28 strains are not as pathogenic for humans as the other clonal complexes (represented by ST88 and ST43 as founders) which were frequently isolated from humans. A reason might be that ST28 strains lacked a number of unique virulence genes (*msgA*, *pagK*, *slrP*, *sopD*, and *stcC*) compared to the other STs (Fig. 4). In contrast, the other clonal complexes (C2 and C3) possess virulence determinants that possibly enable them to interact with a broad range of hosts and the environment.

Reptiles could play a major role as a potential source for human salmonellosis caused by *S. enterica* serovar Paratyphi B because ST88 isolates were frequently found in both sources. This sequence type was continuously and specifically isolated in Germany from reptiles within the last 9 years. Only one of the 24 reptile strains analyzed was assigned to another type, namely, ST43. Unfortunately, information provided with strains for typing at the NRL-BfR was incomplete and did not allow investigation of whether they originated from reptiles living in a zoological garden or in a private terrarium. However, there are many studies worldwide reporting reptile-associated salmonellosis and sepsis (4, 7, 29). Because reptiles are known to shed *Salmonella* frequently and because they have become more and more popular in households and petting zoos, they are recognized as an emerging public health problem (4).

A potential source for human infection caused by the first clonal complex (ST43 as founder) could not be identified. It was only sporadically isolated from various sources within the last 9 years. Possibly, human-to-human transmission might play a role for dissemination or other unidentified sources such as vegetables (32), fish aquariums (25), or goat's milk cheese (10).

Despite the strong association of the clonal complexes with the origin, there was no exclusive host adaptation observed. Host-adapted variants typically causes systemic disease in a limited number of related species, as thought for the D-tartrate-nonfermenting variant of *S. enterica* serovar Paratyphi B. In contrast, host-associated or -restricted variants are primarily associated with one or two closely related host species and are able to persist in the population but may also infrequently cause illness in other hosts (34). Host adaptation can be triggered by the specific organization of the immune system in birds, mammals, or cold-blooded vertebrates, leading to an adapted pathogenicity gene repertoire of the serovar or variant (3, 24).

Antimicrobial resistance in *S. enterica* serovar Paratyphi B (dT+). A multidrug-resistant subgroup of *S. enterica* serovar Paratyphi B (dT+) has become relatively widely distributed throughout the world. Strains encode the *Salmonella* genomic island 1 (SGI1) originally described in *S. enterica* serovar Typhimurium DT104 (5). They are usually resistant to ampicillin, chloramphenicol, streptomycin, spectinomycin, sulfonamides, and tetracyclines and have not yet been found in poultry but in tropical fish aquaria (25) and cattle (13). In France, the SGI1-containing clone was frequently found in clinical isolates of *S. enterica* serovar Paratyphi B (dT+), but no potential sources of infection could be identified (35). In this study, strains with SGI1 were rarely identified. Only three strains isolated from humans harbored typical SGI1-associated resistance and other genes. All of them belonged to ST43. Unfortunately, a comparison of the antimicrobial resistance patterns gave no hints on any infection source from food or animals.

Another multidrug-resistant clone has been genotypically described in this and previous studies (21, 27). Strains belonged invariably to ST28 (clonal complex 3) and are mostly isolated from poultry/poultry meat. They possess a chromosomally located Tn*7*-like class 2 integron carrying a *dfrA1-sat1-aadA1* gene cassette. Since the manifestation of this clone in poultry in the mid-1990s in several European countries, strains have apparently in addition accumulated quinolone resistance and extended-spectrum β-lactam resistance (35) (B. Guerra, personal communication). Three strains investigated (one chicken and two human strains) harbored bla_{CMY-2}-like, bla_{CTX-M2}-like, and bla_{TEM1}-like genes, indicating that variety of genes lead to β-lactam resistance within this complex.

In conclusion, *S. enterica* serovar Paratyphi B (dT+) is an example of a serovar that can consist of groups with divergent evolutionary paths. Here, data indicated that three clonal complexes evolved independently from each other in different niches. By recombination events in recent time, we speculate that complex 2 and 3, represented by only one ST, acquired structural surface antigens (e.g., O, H1, and H2 antigens) leading to *S. enterica* serovar Paratyphi B (dT+) expression and, consequently, to disruption of the phylogeny within this serovar. Serotyping is therefore for polyphyletic serovars of limited value for the study of population structure and the description of risk characters. Furthermore, we hypothesize that clonal complex 1 is much older than complex 2 or 3 because of its observed genetic diversity. Sequencing of the core genome could clarify this hypothesis. This study showed also that the different complexes were associated with unique sources. Each complex had a specific pathogenicity gene repertoire which might contribute to the frequency of isolation of the complexes from humans and animals. Preferentially by contact with reptiles and to a less extent by exposure to poultry or poultry meat, *S. enterica* serovar Paratyphi B (dT+) poses a health risk for humans. However, other nonidentified sources might play a role for human salmonellosis caused by *S. enterica* serovar Paratyphi B (dT+).

ACKNOWLEDGMENTS

This work was funded by the Bundesministerium für Bildung und Forschung (BMBF), FBI-Zoo 2 (01KI1012I and 01KI1012F).

We thank Gabriele Berendonk, Manuela Jaber, Marta Brohm, and Katharina Thomas for technical assistance. We thank Andreas Schroeter and Beatriz Guerra for scientific discussion about antimicrobial resistance.

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