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Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany

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Abstract

Salmonella enterica *serovar Derby (S. Derby) is one of the most prevalent serovars in pigs in Europe and in the U.S. and ranks among the 10 most frequently isolated serovars in humans. Therefore, a set of 82 epidemiologically unrelated S. Derby strains isolated between 2006 and 2008 from pigs, pork and humans in Germany was selected and investigated in respect to the transmission of clonal groups of the serovar along the food chain. Various phenotypic and genotypic methods were applied and the pathogenicity and resistance gene repertoire was determined. Phenotypically 72% of the strains were susceptible to all 17 antimicrobials tested while the others were monoresistant to tetracycline or multi-resistant with different resistance profiles. Four major clonal groups were identified based on PFGE, sequence data of the virulence genes* sopA*,* sopB *and* sopD*, VNTR-locus STTR5 and MLST revealing also the new sequence type ST774. Thirty different PFGE profiles were detected resulting in four clusters representing the four groups. The pathogenicity gene repertoire of 32 representative S. Derby strains analyzed by microarray showed six types with differences in the* Salmonella *pathogenicity islands, pathogenicity genes on smaller islets or prophages and fimbriae coding genes. The pathogenicity gene repertoire of the predominant types PAT DE1 and DE2 were most similar to the ones of S. Paratyphi B (dT+, O5−) and to a minor degree to S. Infantis and S. Virchow PATs. Overall this study showed that in Germany currently one major S. Derby clone is frequently isolated from pigs and humans. Contaminated pork was identified as one vehicle and consequently is a risk for human health. To prevent this serovar from entering the food chain, control measurements should be applied at the farm level.*

1. Introduction

Pork is a major source of human foodborne salmonellosis in the EU and many other countries (Boyen et al., 2008). Therefore all serovars isolated from pig are considered as a possible hazard for public health by the European Food Safety Authority (EFSA) (EFSA, 2006).

In Europe, baseline studies revealed that *Salmonella enterica subsp. enterica* serovar Derby (*S*. Derby) is one of the top 10 *Salmonella* serovars isolated from slaughter pigs (EFSA, 2008) and the most frequently isolated serovar from breeding pigs (EFSA, 2009). This serovar is not exclusively adapted to pigs but most often associated with this source. Nevertheless, *S*. Derby is also isolated from cases of human salmonellosis and from food, mainly pork, and is therefore supposed to spread from pig via pork to human ([Ling et al., 2001], [Valdezate et al., 2005] and [Zhao et al., 2007]).

Between the years 2000 and 2009 the German National Reference Laboratory for *Salmonella* received on routine basis an average of 100 *S*. Derby strains (2.5% of all isolates) per year. Of these 34% were isolated from pigs and 17% from pork. Other sources were meat products of undefined origin and to a smaller degree feeding stuff and environmental sources. *Salmonella* isolates from humans in the same time frame sent to the German National Reference Centre for *Salmonellae* and

other Enterics were serotyped as *S*. Derby at an average of 34 cases per year (0.7% of all isolates). Outbreaks caused by *S*. Derby were sporadically reported worldwide especially from Japan, USA, Australia and Europe ([Ebuchi et al., 2006], [Sanders et al., 1963], [Rubbo, 2010] and [EFSA and ECDC, 2011]) and were traced back to contaminated meat or remained without identification of the source.

The genetic diversity of *S*. Derby strains has rarely been investigated. More than 20 years ago Beltran et al. (1988) described six electrophoretic types within 349 *S*. Derby strains of avian, porcine and human origin. Recently multilocus sequence typing (MLST) became a powerful tool for population studies (Maiden et al., 1998). However, MLST data for *S*. Derby are still limited. The few data available for *S*. Derby (http://mlst.ucc.ie/mlst/mlst/dbs/Senterica/) indicate that the serovar originates from more than one common ancestor which is called polyphyletic. Moreover, some studies used pulsed-field gel electrophoresis to elucidate the spread of *S*. Derby in slaughterhouses (Giovannacci et al., 2001), or to investigate the genetic diversity of the serovar in France ([Kerouanton et al., 2007] and [Kerouanton et al., 2010]), Spain (Valdezate et al., 2005), Hong Kong (Ling et al., 2001), China (Xia et al., 2009) or Brazil (Michael et al., 2006). Clinical, food and animal isolates showed a wide range of PFGE and antimicrobial resistance profiles, often either implicated streptomycin, tetracycline, or sulfonamides ([Valdezate et al., 2005], [Michael et al., 2006], [Gebreyes et al., 2004] and [Clothier et al., 2010]).

This study was conducted to gain a better understanding of the clonality of *S.* Derby and of the subtypes actually transmitted to humans from pigs via pork in Germany. For this purpose three different sequence-based approaches and PFGE were applied to a set of 82 *S*. Derby strains from pigs, pork and humans. Additionally, the genetic relatedness as well as the pathogenicity and antimicrobial resistance gene repertoire of the serovar was compared to six other serovars relevant to human health in Europe by microarray analysis in order to estimate the potential health risk for humans.

2. Materials and methods

2.1. Selection of strains

Altogether 82 *S*. Derby strains were used in this study (Table 1). Of these, 25 *S.* Derby strains were isolated from porcine lymph nodes during an EU-Monitoring study in the years 2006/2007 on the prevalence of *Salmonella* in slaughter pigs (Anonymous, 2006). Another 12 *S.* Derby strains were isolated from pork in the same time frame. These strains received the National Reference Laboratory for *Salmonella* (NRL-BFR), Berlin, Germany on a routine basis for serotyping. The remaining 45 *S*. Derby strains were isolated from epidemiologically unrelated human gastroenteritis cases in 2007 and 2008 selected from the strain collection of the National Reference Centre for *Salmonellae* and other Enterics (NRZ-RKI), Wernigerode, Germany (Table 1). All strains were selected to represent various geographical origins in Germany as well as to cover different seasons. A subset of 32 *S*. Derby strains representing the majority of distinct resistance-, PFGE-, MLST- and sop-types was selected for further microarray analysis (Table 1).

2.2. Serotyping

All strains were previously serotyped according to the White–Kauffmann–Le Minor scheme (Grimont and Weill, 2007) by slide agglutination with O- and H-antigen specific sera (Sifin Diagnostics, Berlin, Germany).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility of strains was tested against 17 antimicrobials or antimicrobial combinations by determining the minimum inhibitory concentration (MIC) using the CLSI broth micro dilution method (Clinical and Laboratory Standards Institute, 2006) in combination with the semi-automatic Sensititre system (TREK Diagnostic Systems, Cleveland, Ohio). Breakpoints were applied as previously described (Schroeter et al., 2004). Antimicrobials tested were amoxicillin/clavulanic acid

(AMC), ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), florfenicol (FLO), gentamicin (GEN), kanamycin (KAN), neomycin (NEO), nalidixic acid (NAL), spectinomycin (SPE), streptomycin (STR), sulfamethoxazole (SMX), trimethoprim/sulfamethoxazole (SXT), tetracycline (TET), trimethoprim (TMP) and ceftiofur (XNL).

2.4. Genomic DNA purification

Strains were grown aerobically in Luria–Bertani broth (Merck, Darmstadt, Germany) with shaking at 37 °C for 16–18 h. Genomic DNA isolation was carried out using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol with the addition of 25 µL Proteinase K instead of 20 µL and extended lysis for 3.5 h. The quality and quantity of DNA was measured spectrophotometrically, and a minimum of 4 µg high quality DNA was used for labeling.

2.5. Multilocus sequence typing (MLST)

MLST was carried out as previously described (Kidgell et al., 2002) and the alleles and sequence types were assigned according to the MLST scheme at http://mlst.ucc.ie/mlst/dbs/Senterica. New alleles and sequence types were submitted to the website.

2.6. Variable–number–tandem–repeat and sop gene sequence typing (sop-ST)

Variable–number–tandem–repeat (VNTR) locus STTR5 (Lindstedt et al., 2004) and genes *sopA*, *sopB* and *sopD* were amplified from each strain by polymerase chain reaction (PCR) using the respective primers shown in Table 2. These genes were selected since they were previously reported to vary among different serovars (Prager et al., 2000). A 50-µL PCR contained 0.25 µM of each primer, 25 µL of a 2× HotStarTaq Master Mix (Qiagen) and an aliquot of 5 µL from a colony grown on nutrient agar and suspended in 100 µL of distilled water. All PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA). The cycling conditions were for *sopA* and *sopB* 95 °C, 15 min followed by 25 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 2 min. For *sopD* the annealing temperature was 50 °C and for STTR5 61 °C. Sequencing of PCR products was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the following conditions: 96 °C, 1 min followed by 25 cycles (96 °C, 10 s; 50 °C, 5 s; 60 °C, 4 min). Sequences of all primers used for sequencing the *sop* genes can be obtained on request. The products were analyzed using an ABI capillary electrophoresis equipment (Applied Biosystems) according to the manufacturer's instructions. Nucleotide sequences were investigated using the Lasergene v.8 software package (DNASTAR Inc., Madison, WI). Based on sequence differences, laboratory-internal allele numbers were assigned resulting in a respective *sop*-ST allele pattern (*sopA*–*sopB*–*sopD*) for a given strain. For VNTR locus STTR5 two sequence-types A and B were defined based on the 6 bp repeat unit sequence. For each sequence type alleles were assigned according to the number of repeats.

2.7. Nucleotide sequence accession numbers

The alleles detected by *sop*-ST were entered into GenBank under the accession numbers GU825929 to GU825936 for *sopA*18, 19, 30, 31, 32, 34, 35, 36, respectively, GU825937 to GU825941 for *sopB*18, 19, 20, 21, 22, respectively, and, GU825942 to GU825944 for *sopD*18, 19, 20, respectively.

2.8. Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out after digestion of genomic DNA with the restriction enzyme XbaI according to the Pulse-Net protocol (Ribot et al., 2006). Gel images were analyzed in BioNumerics v.5.1 (Applied Maths, Sint-Martens-Latem, Belgium) and compared by cluster analysis using dice coefficient and unweighted-pair group method with arithmetic averages (UPGMA dendrogram type) with a position

tolerance of 1.5% and optimization of 1.0%. Fragments smaller than 20 kb were not considered for cluster analysis.

2.9. DNA microarray analysis

The DNA microarray used in this study was applied as previously described (Huehn et al., 2009a). A set of 275 gene-specific 57–60mer oligonucleotide probes derived from *Salmonella* sequences deposited in GenBank at NCBI (http://www.ncbi.nlm.nih.gov/) were designed using the program Array Designer 4.1 (Premier Biosoft, Palo Alto, CA). The probes were assigned to seven different marker groups depending on the functionality of the corresponding gene sequence (number of probes): pathogenicity (80), resistance (49), serotyping (33), fimbriae (22), DNA mobility (57), metabolism (21), and prophages (13). In addition, three 57–61mer oligonucleotides derived from the *Arabidopsis thaliana* genes RCA (M86720), RCP1 (NM_12175), and PRKASE (X58149) were designed as negative control probes. Pathogenicity determinants for each strain analyzed were categorized according to their location on the *Salmonella* genome: *Salmonella* pathogenicity islands (SPIs), prophages, plasmid, islets and fimbrial clusters. Analysis of the DNA microarray data was performed as previously described (Huehn et al., 2009a). Normalized data (presence/absence of gene) for every strain were imported in BioNumerics v.5.1 as character values. For comparison of pathogenicity determinants between *S*. Derby, *S*. Typhimurium, *S*. Enteritidis, *S*. Infantis, *S*. Virchow *S*. Hadar (Huehn et al., 2010) and *S*. Paratyphi B (d-tartrate positive, dT+) (Huehn et al., 2009b) a cluster analysis was performed with the simple matching binary coefficient and using the UPGMA dendrogram type. The maximum parsimony cluster analysis was performed with 1000 bootstrap cycles and the exported rendered tree was generated with hidden branches and distance labels shorter than or equal to 1 and rooted tree type.

2.10. Statistical analysis

To assess the discriminatory power of PFGE, *sop*-ST and STTR5 sequence typing Simpson´s index of diversity (ID) and the 95% confidence intervals (CI) were calculated using the Comparing Partitions website (http://darwin.phyloviz.net/ComparingPartitions/index.php?link = Tool).

3. Results

3.1. Antimicrobial resistance

Fifty-nine of the 82 *S*. Derby strains (72%) were susceptible to all 17 antimicrobials tested while three strains were monoresistant to tetracycline, 14 strains multi-resistant to two or three antimicrobials (10 different profiles) and six strains to four or more antimicrobials (5 different profiles) (Table 1). The percentage of resistant strains was slightly higher in those from human origin compared to the other sources but was statistically not significant ($p > 0.05$).

3.2. Typing by pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis revealed 30 different XbaI profiles within the 82 strains analyzed (ID, 84.6, [95% CI, 77.2 to 92.0]) (Fig. 1). The main restriction pattern was X02, found in 31 strains (38%) isolated from pigs, pork and humans with no significant assignment ($p > 0.05$). Three other profiles (X03, X23 and X29) were associated with two of the three sources. The remaining 26 profiles were associated with pig, pork or human, often represented by one single strain (37 strains). Four different clusters (clusters A, B, C and D) were assigned after generating an UPGMA tree using the dice similarity coefficient. Cluster A harbored 52 of the 82 strains (63.5%) including the most prominent restriction profile X02. Cluster B was represented by 23 strains (28.0%), cluster C by five strains (6.0%) and cluster D by two strains (2.5%) with identical profile (Table 1, Fig. 1).

3.3. Multilocus sequence typing (MLST) of S. Derby

All strains were further typed by MLST including seven housekeeping genes *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA* (Kidgell et al., 2002). Five different sequence types (STs) were found. The most prominent sequence type found was ST39 (48 strains) followed by ST40 (25 strains), ST682 (5 strains), ST71 (2 strains) and ST774 (2 strains). Thereof four were previously reported (ST39, ST40, ST71, ST682) and one was newly assigned (ST774) (Table 1, Fig. 2). ST774 (strains 08-03157 and 08-03159) differed from ST39 by one nucleotide in *aroC* only and ST40 from ST 39 by 8 nucleotides in *sucA*.

3.4. VNTR and sop sequence typing (sop-ST)

VNTR sequence typing applied to locus STTR5 (ID, 65.3 [95% CI, 58.2 to 72.5]) revealed two different repeat units which differed in one nucleotide (type A: ACCATG; type B: ACCACG). Type A occurred in 75 strains (91%) and type B in seven strains (9%) of those five were isolated from humans. Most of the *S*. Derby strains (49%) had 9 tandem repeats in STTR5 equally distributed among strains from pig, pork and human. Other strains showed 7 repeats (1 strain), 10 repeats (29 strains), 11 repeats (9 strains) or 12 repeats (3 strains). The type-B-sequence repeat unit was associated with either 10 or 11 tandem repeats (Table 1).

Sequencing the virulence genes *sopA*, *sopB* and *sopD* of the 82 selected *S*. Derby strains identified eight different *sopA* alleles with the most prominent allele *sopA*30 (53 strains), six different *sopB* alleles with mainly allele *sopB*18 (50 strains) and three different *sopD* alleles with most prevalent allele *sopD*18 (75 strains). Altogether 12 different combinations of *sopA*, *sopB* and *sopD* were found. The *sop*-ST 30–18–18 (*sopA*–*sopB*–*sopD*) was identified in 49 out of 82 strains (60%) (ID, 62.1 [95% CI, 50.7 to 73.5]) and was equally distributed among all three sources (Fig. 2).

3.5. Microarray analysis of pathogenicity genes in S. Derby

Thirty-two strains were selected for microarray analysis for identification of 102 pathogenicity associated genes. The selection represented diversity in resistance-, PFGE-, MLST- and sop-types. Six different pathogenicity array types (PATs) could be assigned. PAT DE1 was defined by the presence of genes located in the *Salmonella* pathogenicity islands (SPIs) 1, 2, 4, 5 but truncated in SPI-3 lacking *rhuM* and *sugR* encoding a putative cytoplasmic protein and a putative ATP binding protein, respectively. Pathogenicity genes found in SPI-7 and the prophages Gifsy-1, Gifsy-2, Gifsy-3, Fels-1 and other prophages as well as genes located on the *Salmonella* virulence plasmid were absent. Some other genes located on genomic islets (*pagK* and *trhH*) were also absent. PAT DE2 differed by PAT DE1 only in the absence of *stcC* (encoding a putative outer membrane fimbrial protein) (Fig. 3). Thirteen strains were assigned to PAT DE1 and ten strains to PAT DE2. In PAT DE3 (1 strain, no. 07-01111) two genes of SPI-2 (*sseC* encoding a translocation machinery component required for systemic infection and *sseF* gene for secretion system effector) as well as *pipA*, *pipD* and *sopB* as representatives of SPI-5 were absent. Four strains grouped together in PAT DE4 harboring a complete SPI-3 and, in addition, *sspH1* usually located in Gifsy-3 encoding the leucine-rich repeat protein SspH1. Furthermore, PAT DE4 was positive for the outer membrane receptor gene *fhu*A_STM instead of *fhuA*_Spa but no signal was obtained for the islet genes *htrE* (probable porin/fimbrial assembly protein), *sopD2* (secreted effector protein), *srfJ* (putative virulence factor, activated by transcription factor SsrB) and *sseK2* (translocated effector protein SSEK2 by type III secretion system) while *pagK* (PhoPQ-activated protein) was present only in this array type. Also positive were the fimbrial genes *lpfD* and *stkC* but STM4595 was solely negative in PAT DE4. PAT DE5 was defined by the presence of *sopE1* (translocated effector protein encoded by P2-like cryptic bacteriophage) and the *trhH* gene for a putative pilus assembly protein usually present in the *Salmonella* genomic island 1 (SGI1). This combination was found in two strains. PAT DE6 was represented by two strains again. This type harbored *sugR* but not *rhuM* in SPI-3. Furthermore, the prophage located genes *hldD*_DT104 and *irsA* initially found in *S*. Typhimurium phagetype DT104 strains were detected and *fhuA*_STM instead of *fhuA*_Spa showed a positive probe signal as in PAT DE4. The *sseK2* gene was absent and the only two strains with positive signal for *stjB* (encoding a putative fimbrial usher protein)

were associated with this PAT. The most prominent PATs DE1 and DE2 were detected in strains from all three sources while other PATs were associated with one or only two sources (Table 1).

3.6. Determination of resistance gene repertoire

Of the 32 strains investigated 24 were susceptible with none of the analyzed resistance genes present. Two strains (07-01111 and 08-03154) were phenotypically resistant to tetracycline showing a positive signal for *tet*(B) encoding an efflux pump. Furthermore there was one strain (08-03159) resistant to AMP and STR attributed to genes bla_{TEM1_like} (encoding ß-lactamase) and *strA-strB* (encoding aminoglycoside-phosphotransferase). Another strain (07-00923) with resistance to SMX and TMP was positive for *sul3* (dihydropteroate synthase), *dfrA1-like* (dihydrofolate reductase), *aadA1a* (aminoglycoside-adenyltransferase) and *int1* (DNA integrase 1). One strain (07-02316), phenotypically resistant to SMX, SPE, STR and TET, revealed the associated genes *sul1* (dihydropteroate synthase), *aadA2–3–8* (aminoglycoside-adenyltransferase), *int1*, *qacEΔ* (multidrug exporter) and *tet*(A) (efflux pump). Another strain (08-03163) with resistance profile AMP, SMX, STR, TET, TMP was positive for the genes $bla_{TEM1\_like,}$*sul1, sul2, aadA1a*, *aadA2–3–8, tet*(A), *int1*, *qacEΔ and dfrA1-like.* Two further multi-resistant human strains (08-03151 and 08-03152) were found to possess the SGI1 associated genes bla_{PSE1}, *floR*, *sul1*, *tet*(G), *aadA2-3–8*, *qacEΔ*, *intSGI1* and *int1* (data not shown), exhibiting resistances to AMP, CHL, FFN, SMX, TET and TMP. Both strains were assigned to PAT DE6.

3.7. Characterization of clonal groups in S. Derby

Four clonal groups were defined based on the sequence and PFGE typing results (Fig. 2). The most prominent group comprised 48 strains characterized by MLST-ST39, and in addition two strains harboring the closely related sequence type ST774. All strains belonged to PFGE cluster A, showed STTR5 sequence type A with 9, 10 or 11 tandem repeats, respectively, and the *sop*-ST 30–18–18 except for one strain with *sop*-ST 19–18–18. Thirty-one of these strains belonged to PFGE-profile X02. All strains with *sop*-ST 30–18–18 belonged to PAT DE1.

A second, highly similar group is based on the MLST sequence type ST40. Strains of this group (25 out of 82 strains) belonged to PFGE cluster B except two strains assigned to cluster A. Allele *sopB*21 was specifically associated with this group (Fig. 2). The six different *sopA* alleles observed varied by very few nucleotides. The STTR5 locus was highly diverse in terms of the number of tandem repeats identified. All 13 strains except one tested belonged either to PAT DE2 (10 strains) or to PAT DE5 (2 strains). The exceptional strain (PAT DE3) lacked the *sopB* locus and therefore was not further considered in the UPGMA dendrogram (Fig. 2). Both groups included strains from pig, pork and human.

The third group comprised two strains isolated from humans. Both strains were identical with respect to its genotypic properties. They were assigned to PFGE cluster D, showed the ST71 MLST sequence type in combination with *sop*-ST 34–19–19, and PAT DE6. The STTR5 locus had 10 tandem repeats of sequence type B. Both strains were multi-resistant and resistance genes present indicated that they possessed a variant of SGI1.

The remaining five strains isolated from pigs or humans formed a fourth group with a uniform genotype pattern including ST682, *sop*-ST 31–22–20, 11 tandem repeats in STTR5 (sequence type B) and PFGE cluster C. Four strains were analyzed by microarray revealing PAT DE4.

3.8. Comparison of pathogenicity gene repertoire to other *Salmonella* serovars

Comparing the pathogenicity gene repertoire of *S*. Derby strains from this study with other epidemiological important *Salmonella* serovars in humans and animals namely *S*. Typhimurium, *S*. Enteritidis, *S*. Virchow, *S*. Infantis, *S*. Hadar, and *S*. Paratyphi B (dT+) previously investigated with the same DNA-microarray ([Huehn et al., 2009b] and [Huehn et al., 2010]) indicated that most prominent PATs of *S*. Derby (PAT DE1 and DE2) were most similar to the pathogenicity gene repertoire from *S*. Paratyphi B (dT+) O5-antigen negative (O5−) strains and had the most distantly repertoire with *S*.

Typhimurium. A rendered maximum parsimony tree based on the presence or absence of the 102 pathogenicity determinants (pathogenicity and fimbrial markers) clustered all *S*. Derby strains tested in one clade (Fig. 4). Clonal groups could be differentiated by the six PATs identified. PAT DE1 is associated with group1, PAT DE2, DE3 and DE5 with group 2, PAT DE6 with group 3 and PAT DE4 with group 4. A unique gene present in all *S*. Derby strains except in four strains belonging to PAT DE4 was *htrE* (encoding putative porin/fimbrial assembly protein). Since those strains lacked *sugR* and *rhuM*, they possessed a previously described variant of SPI-3 (Amavisit et al., 2003).

4. Discussion

S. Derby is frequently isolated in breeding and slaughter pigs in Europe and in the U.S. ([Arguello et al., 2011], [De Busser et al., 2011], [EFSA, 2009], [EFSA, 2008] and [Foley et al., 2008];) and ranks within the top 10 serovars reported from human salmonellosis cases in Europe in 2009 (EFSA and ECDC, 2011). Therefore, this serovar plays a major role in public health concern. This study provides an extensive characterization of *S*. Derby isolated from pigs, pork and humans with the aim to understand the transmission of *S*. Derby from animal to human through pork and to determine the clonal structure as well as pathogenicity gene repertoire compared to other serovars on molecular level. Therefore different methods like PFGE, microarray, MLST and partial sequencing of the virulence genes *sopA*, *sopB* and *sopD* (*sop*-ST) as well as analysis of the variable tandem repeat locus STTR5 were applied. To our knowledge, no other study has investigated *S*. Derby so comprehensively on a molecular level.

Phenotypically 72% of the strains were susceptible to all 17 antimicrobials tested while the others were mono-resistant to tetracycline or multi-resistant with different resistance profiles. Data from other countries reported fewer susceptible strains but a higher proportion of resistance to tetracycline. A reason for these differences might be varying antimicrobial usage regimes in pig production ([Ling et al., 2001], [Valdezate et al., 2005], [Zhao et al., 2007], [Huang et al., 2009] and [Xia et al., 2009]). An increased antimicrobial resistance to sulfadimethoxine and spectinomycin has been also reported in *S*. Derby isolated from U.S. livestock between 2003 and 2008 (Clothier et al., 2010). Genotyping of antimicrobial resistance genes in strains from Southern Brazil and America ([Michael et al., 2006], [Zhao et al., 2007] and [Anjum et al., 2011]) revealed *sul1*, *sul2*, *aadA2*, *tet*(A), *tet*(B), bla_{TEM} and *dfrA14*. Except for *dfrA14* antimicrobial resistance was encoded by the same genes among *S*. Derby strains investigated in this study. Furthermore, two strains from humans harbored resistance to additional genes generally associated with SGI1. A number of different SGI1 variants were described previously but it remains to be elucidated which variant occurred in the two strains described here ([Akiba et al., 2006] and [Amar et al., 2008]).

Thirty different PFGE profiles were detected clustering in four clades. Therefore PFGE showed the highest index of diversity followed by VNTR sequence typing and *sop*-ST. The main PFGE profile was X02. This profile was also reported from France in two cases, one from pork and one from human (Kerouanton et al., 2010) and from slaughtered pigs in Spain (Valdezate et al., 2005). Generally, there was a high diversity of PFGE profiles observed in this and other studies but the most prominent profile detected in this study was only rarely found in the other studies ([Kerouanton et al., 2007], [Michael et al., 2006], [Valdezate et al., 2005] and [Xia et al., 2009]). Profiles similar to the ones presented in cluster C and D were also observed in *S*. Derby isolated outside of Germany.

Within the last decade, multilocus sequence-based typing methods were established for the determination of bacterial population structure, especially based on a combination of seven housekeeping genes (Maiden, 2006). Sequencing of virulence genes might discover subgroups of clonal groups reported by MLST, since these genes are assumed to evolve faster than housekeeping genes due to the selective pressure exhibited by the contact of their gene products with varying environmental and host defense factors during adaptation in different hosts. In this study, sequence typing of the three virulence genes *sopA*, *sopB*, *sopD* encoding translocated effector proteins (*sop*-ST) was applied in addition to MLST in order to describe clonal groups and subgroups among serovar *S*. Derby. Previously some studies used virulence genes in combination with housekeeping genes in their MLST schemes but *sop* sequences were not considered ([Sukhnanand et al., 2005] and [Tankouo-Sandjong et al., 2007]). The *sop* virulence genes were selected since they are present in all *Salmonella* strains but DNA sequences (available at GenBank http://www.ncbi.nlm.nih.gov) vary among different serovars (Prager et al., 2000). The four major clonal groups identified by MLST were

in agreement with the sequence data obtained for the three *Salmonella sop* genes. Both typing methods resulted in a similar tree structure representing the four main groups. Whereas five MLST types were detected within the set of *S*. Derby strains tested, *sop* gene sequencing revealed 12 *sop*-STs splitting two of the four groups in further branches (Fig. 2). This indicates that the combination of *sopA–sopB–sopD* sequences has the potential to determine the clonal structure of a serovar. Nevertheless, this needs to be elucidated for other serovars as well as the role of recombination in these *sop* genes needs to be addressed. If sequence variations within the effector proteins may alter the host cellular response remained unclear. VNTR sequence typing of locus STTR5 was not appropriate for population studies since tandem repeat numbers seem to be rather randomly distributed in strains tested unless differences in the VNTR sequence unit.

Among the five MLST types identified in this study a new one (ST774) has been recognized which differed only in one nucleotide to ST39. Five additional STs were previously reported for *S*. Derby to the MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica) not detected in this set of strains. Some STs differed only marginally in their nucleotide sequence, e.g. ST40 and ST678 (five nucleotides different in *purE*) or ST71 and ST683 (10 nucleotides different in *aroC*, *dnaN*, *sucA*) (data not shown).

Recently the pathogenicity gene repertoire of several *Salmonella* serovars isolated in Denmark was compared to MLST (Litrup et al., 2010). *S*. Derby strains investigated had a truncation in SPI-3 and were negative for *msgA* (encoding macrophage survival protein) and most often for *lpfD* (encoding long polar fimbrial protein). This was also found for most of the strains analyzed here with the exception of the pathogenicity array type PAT DE4 harboring the complete SPI-3 and possessing *lpfD*. Whether this contributes to the hazard of these strains is not clear.

A comparison of the pathogenicity gene repertoire of *S*. Derby with other epidemiologically important serovars grouped all *S*. Derby PATs together although PATs differed in up to 19 of the 102 pathogenicity genes investigated. The pathogenicity gene repertoire of the predominant types PAT DE1 and DE2 grouped nearest to those of *S*. Paratyphi B (dT+, O5−) and most distantly related to the ones of *S*. Typhimurium (Fig. 4). This was surprising since *S*. Paratyphi B (dT+, O5−) was previously reported as a serovar partly adapted to poultry (Huehn et al., 2009b) with low pathogenicity potential for humans compared to *S*. Typhimurium (Huehn et al., 2010). However, ascribing a low pathogenicity potential to *S*. Derby would be inappropriate since other types (PAT DE4 and PAT DE6) have more consensus in their pathogenicity gene repertoire with *S*. Infantis and *S*. Virchow. Nevertheless, it might be possible that some genes or genome islands important for *S*. Derby virulence might not be detected by the microarray.

Interestingly there was one strain isolated from pork missing genes in SPI-2 and all targets for SPI-5 including *sopB*. Previous studies investigated *sopB* mutants for their virulence phenotype and found for example reduced fluid accumulation in bovine ligated ileal loops or decreased invasiveness in epithelial cells ([Zhang et al., 2002] and [Raffatellu et al., 2005]). Because of the absence of *sopB* this strain was not included in the *sop*-based dendrogram (Fig. 2) but according to MLST typing it belonged to clonal group 2.

The vast majority of strains tested were assigned to clonal groups 1 (61%) and 2 (30%). They originated from pigs, pork and humans and were approximately equally distributed among these sources. These data clearly show that these groups are prominent within the food chain in Germany and are transmitted from pig to human through contaminated pork. The other rather distantly related two groups 3 and 4 were mainly linked to human strains and none of these strains originated from pork. Since only few strains were assigned to groups 3 and 4 it is unclear whether their transmission to human can be caused by pork. It might be possible that these humans were infected by *S*. Derby from another source like birds. Based on multilocus enzyme electrophoresis typing two divergent clone clusters of *S*. Derby were previously observed. Both differed in their host distribution between birds and mammals including swine (Beltran et al., 1988). However, the frequencies of human infections caused by strains from either division were found approximately equal, suggesting both, mammals (swine) as well as birds as a reservoir for distinct but pathogenic *S*. Derby.

In conclusion this study showed that in Germany one major *S*. Derby clone is currently predominant in pigs and humans. Contaminated pork was identified as one vehicle and consequently presents a risk for human health. To prevent this serovar from entering the food chain, control measurements should be applied at the pig farm level.

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Tables and Figures

Table 1. *S*. Derby strains used for phenotypic and molecular analysis in this study.

Strain no.	Year of isolation	Origin	Resistance[a]	PFGE cluster	PFGE profile no.	STTR5 locus[b]	*sop*-ST	MLST (ST)	Microarray (PAT DE)
06-04282	2006	Pork	Susceptible	A	2	A9	30–18–18	39	1
06-04691	2006	Pork	Susceptible	B	24	A10	30–21–18	40	2
06-04789	2006	Pork	Susceptible	B	23	A9	19–21–18	40	2
06-05059	2006	Pork	Susceptible	A	2	A9	30–18–18	39	n.t.[c]
07-00011	2006	Pork	Susceptible	B	23	A10	19–21–18	40	2
07-00832	2007	Pork	Susceptible	A	2	A10	30–18–18	39	1
07-01111	2007	Pork	TET	A	13	A10	30–00–18	40	3
07-01947	2007	Pork	Susceptible	A	3	A10	30–18–18	39	1
07-02313	2007	Pork	AMP GEN	A	2	A9	30–18–18	39	n.t.
07-02316	2007	Pork	SMX SPE STR TET	B	26	A9	32–21–18	40	5
07-04034	2007	Pork	Susceptible	A	2	A9	30–18–18	39	1
07-04781	2007	Pork	Susceptible	B	23	A10	19–21–18	40	n.t.
06-04420	2006	Pig	Susceptible	B	25	A10	32–21–18	40	2
06-04671	2006	Pig	SMX SXT TMP	A	5	A10	30–18–18	39	n.t.
06-04887	2006	Pig	AMP TET	A	2	A9	30–18–18	39	n.t.
07-00245	2007	Pig	SMX SXT TMP	A	1	A10	30–18–18	39	n.t.
07-00725	2007	Pig	Susceptible	B	23	A9	35–21–18	40	2
07-00730	2007	Pig	Susceptible	A	1	A9	30–18–18	39	1
07-00923	2007	Pig	SMX TMP	B	21	A10	19–21–18	40	2
07-01009	2007	Pig	Susceptible	A	2	A9	30–18–18	39	n.t.
07-01016	2007	Pig	Susceptible	B	23	A9	19–21–18	40	n.t.
07-01174	2007	Pig	Susceptible	A	2	A9	30–18–18	39	n.t.
07-01244	2007	Pig	AMP STR	A	2	A10	30–18–18	39	n.t.
07-01731	2007	Pig	Susceptible	A	2	A10	19–18–18	39	1
07-01839	2007	Pig	Susceptible	A	2	A9	30–18–18	39	n.t.
07-02007	2007	Pig	Susceptible	B	27	A11	18–21–18	40	2
07-02050	2007	Pig	Susceptible	B	23	A7	35–21–18	40	n.t.
07-02201	2007	Pig	Susceptible	A	2	A9	30–18–18	39	n.t.
07-02272	2007	Pig	Susceptible	A	8	A10	30–18–18	39	1
07-02434	2007	Pig	SMX SPE STR	B	23	A10	19–21–18	40	n.t.
07-02436	2007	Pig	Susceptible	B	22	A10	36–21–18	40	2
07-02686	2007	Pig	Susceptible	C	28	B11	31–22–20	682	4
07-02790	2007	Pig	Susceptible	C	29	B11	31–22–20	682	4
07-02791	2007	Pig	Susceptible	A	4	A10	30–18–18	39	1
07-02808	2007	Pig	Susceptible	A	2	A9	30–18–18	39	n.t.
07-02856	2007	Pig	Susceptible	A	2	A9	30–18–18	39	n.t.
07-03879	2007	Pig	Susceptible	B	17	A9	30–21–18	40	n.t.
08-03147	2007	Human	Susceptible	A	2	A9	30–18–18	39	1
08-03148	2007	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03149	2007	Human	Susceptible	A	7	A9	30–18–18	39	1
08-03150	2007	Human	AMP SMX STR TMP	A	2	A9	30–18–18	39	n.t.
08-03151	2007	Human	AMP CHL FFN SMX TET TMP	D	30	B10	34–19–19	71	6
08-03152	2007	Human	AMP CHL FFN SMX TET TMP	D	30	B10	34–19–19	71	6
08-03153	2007	Human	Susceptible	A	3	A9	30–18–18	39	n.t.
08-03154	2007	Human	TET	A	9	A9	30–20–18	40	2
08-03155	2007	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03156	2007	Human	Susceptible	A	6	A10	30–18–18	39	n.t.
08-03157	2007	Human	Susceptible	A	11	A10	30–18–18	774	1
08-03158	2007	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03159	2007	Human	AMP STR	A	11	A10	30–18–18	774	1
08-03160	2007	Human	Susceptible	A	6	A10	30–18–18	39	n.t.
08-03161	2007	Human	Susceptible	A	6	A10	30–18–18	39	n.t.
08-03162	2007	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03163	2007	Human	AMP SMX STR TET TMP	B	14	A12	32–21–18	40	5
09-04665	2007	Human	Susceptible	A	11	A10	30–18–18	39	1
08-03164	2007	Human	Susceptible	A	3	A10	30–18–18	39	n.t.
08-03165	2007	Human	Susceptible	B	18	A10	19–21–18	40	2
08-03166	2007	Human	AMP STR TET	A	2	A9	30–18–18	39	n.t.
08-03167	2007	Human	TET	B	20	A11	32–21–18	40	n.t.
08-03168	2007	Human	Susceptible	A	10	A10	30–18–18	39	n.t.
08-03169	2007	Human	AMP SMX STR TMP	A	2	A9	30–18–18	39	n.t.
08-03170	2007	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03171	2007	Human	SMX STR	B	19	A9	19–21–18	40	n.t.
08-03172	2007	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03173	2007	Human	Susceptible	A	12	A11	30–18–18	39	n.t.
08-03174	2007	Human	Susceptible	A	12	A11	30–18–18	39	n.t.
08-03175	2007	Human	SMX STR	B	16	A12	32–21–18	40	n.t.
08-03176	2007	Human	SMX TMP	B	19	A9	19–21–18	40	n.t.
09-04666	2007	Human	SMX STR TET	B	15	A12	32–21–18	40	n.t.
08-03177	2007	Human	SMX TMP	B	19	A9	19–21–18	40	n.t.
08-03178	2008	Human	Susceptible	B	19	A9	19–21–18	40	n.t.
08-03179	2008	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03180	2008	Human	Susceptible	A	10	A10	30–18–18	39	n.t.
08-03181	2008	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
09-04664	2008	Human	Susceptible	A	2	A9	30–18–18	39	n.t.

Table 1 (continued)

Strain no.	Year of isolation	Origin	Resistance[a]	PFGE cluster	PFGE profile no.	STTR5 locus[b]	*sop*-ST	MLST (ST)	Microarray (PAT DE)
08-03182	2008	Human	Susceptible	C	29	B11	31–22–20	682	4
08-03183	2008	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03184	2008	Human	Susceptible	C	29	B11	31–22–20	682	4
08-03185	2008	Human	Susceptible	C	29	B11	31–22–20	682	n.t.
08-03186	2008	Human	Susceptible	A	2	A9	30–18–18	39	n.t.
08-03187	2008	Human	Susceptible	A	2	A10	30–18–18	39	n.t.
08-03188	2008	Human	Susceptible	A	2	A9	30–18–18	39	n.t.

a See Materials and methods for abbreviations.
b Sequence type (A or B) and number of tandem repeats in STTR5.
c n.t., not tested.

Table 2. PCR primers for amplification of *sopA*, *sopB*, *sopD* and STTR5.

Target	Primer designation	Primer sequences	Fragment size in bp	Reference
sopA	sopA-Pf sopA-Pr	5′-TTAATTTTTCTACTATTCCTAACCAGG-3′ 5′-GATGGATGACAGAACACTCG-3′	2306	This study
sopB	sopB-Pf sopB-Pr	5′-ATGCAAATACAGAGCTTCTATC-3′ 5′-AGATGTGATTAATGAAGAAATGC-3′	1683	This study
sopD	sopD-Pf sopD-Pr	5′-ATGCCAGTCACTTTAAGCTT-3′ 5′-AGTAATATATTACGACTGCAC-3′	947	This study
STTR5	STTR5-F STTR5-R	5′-ATGGCGAGGCGAGCAGCAGT-3′ 5′-GGTCAGGCCGAATAGCAGGAT-3′	223–253	(Lindstedt et al., 2004)

Figure 1. UPGMA dendrogramof PFGE profiles identified in 82 S. Derby strains after digestion with XbaI. Profiles were designated X01 to X30. The number of strains belonging to each source (total, pig, pork and human) is shown on the right side. Assigned clusters A to D are indicated by curly brackets. A rectangle highlights themost prominent PFGE profile, X02.

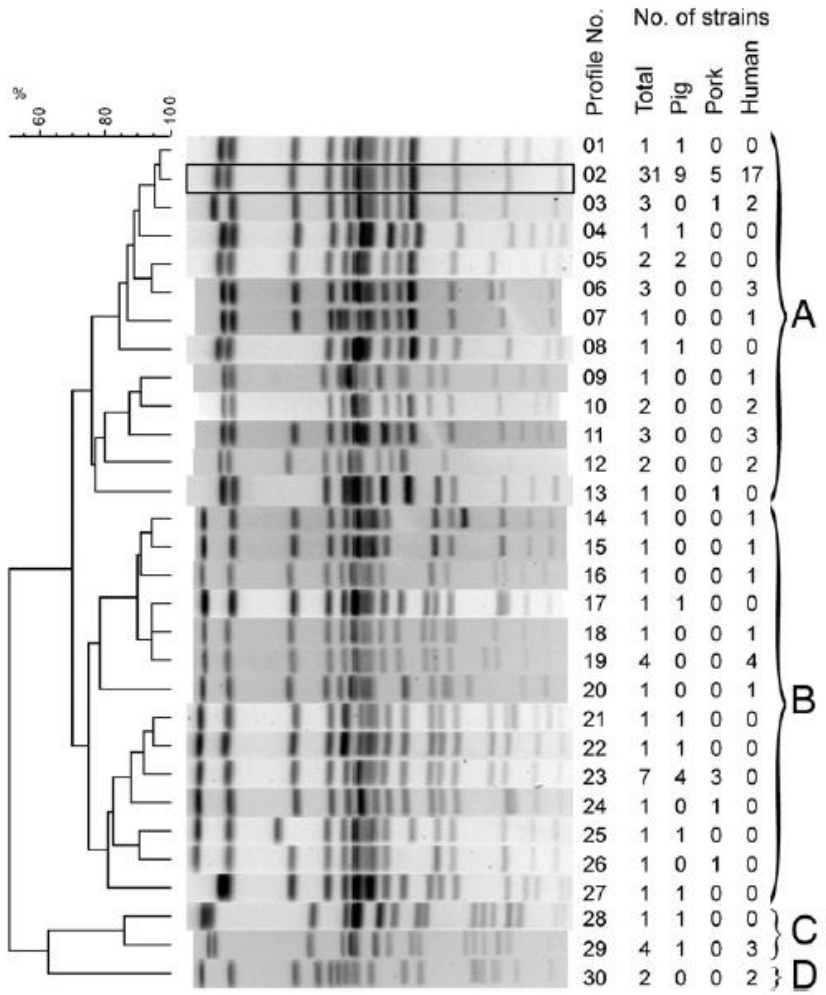

Figure 2. UPGMA dendrogram of 82 S. Derby strains generated by a “composite data set” using Bionumerics v5.1. On the left side the MLST dendrogram with corresponding sequence types (ST). On the right side the sop-dendrogram with corresponding sop-ST pattern types (sopA–B–D). Clonal groups (1 to 4) are indicated at MLST branches. The scale indicated the similarity of DNA sequence of the “composite data set”.

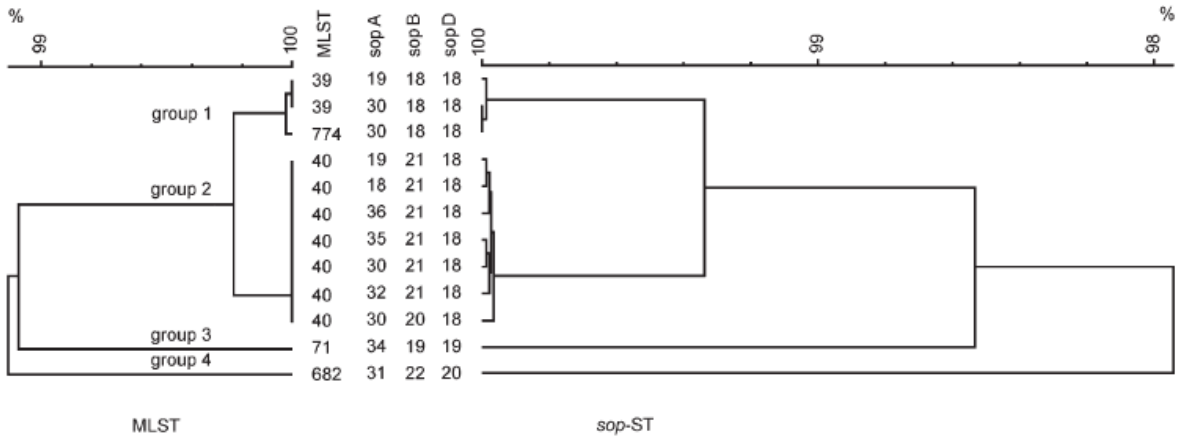

Figure 3. Pathogenicity array types observed in 32 S. Derby strains tested. On the left side the genes tested are indicated and grouped according to their particular genomic location (SPI-1 to SPI-7; Prophages Gifsy-1, Gifsy-2, Gifsy-3 and Fels-1; plasmids and islets) or function (fimbrial). At the top assigned pathogenicity array types (PATs) are indicated. The hybridization result of each type is shown by row. A white box indicates the absence and a gray box indicates the presence of the target sequence. SPI, Salmonella pathogenicity island.

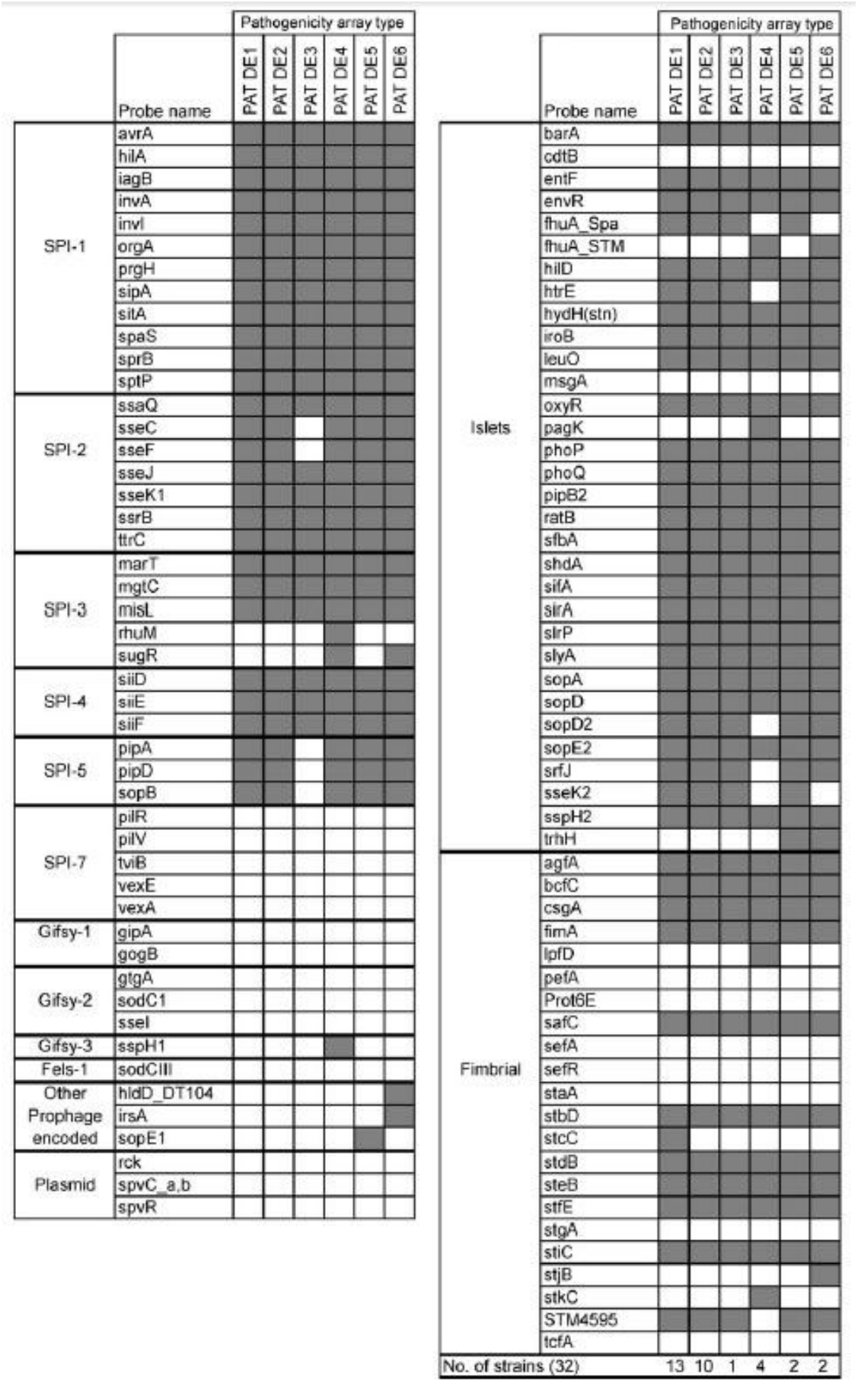

Figure 4. Rendered maximum parsimony tree. The tree shows the differences of Salmonella strains of serovar S. Derby (n=32), S. Typhimurium (n=21), S. Enteritidis (n=19), S. Virchow (n=11), S. Infantis (n=11), S. Hadar (n=14), and S. Paratyphi B (dT+) (n=36) based upon the presence/absence of 102 pathogenicity determinants. The count displays the number of “character state changes” for each branch throughout the dendrogram. Ellipses covered over the branches assigned strains belonging to the same serovar. Pathogenicity array types (PAT DE) assigned to S. Derby are indicated at branches.