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ORIGINAL PAPER

Identification of putative ancestors of the multidrug-resistant *Salmonella enterica* serovar typhimurium DT104 clone harboring the *Salmonella* genomic island 1

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Abstract The origin of multidrug-resistant *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) harboring the *Salmonella* Genomic Island 1 (SGI1), which was detected for the first time in the mid-1980s is unknown. In this study, we performed microarray genomotyping of four multidrug-resistant SGI1 positive strains and found that unlike the *S. typhimurium* LT2 strain, the multidrug-resistant strains lacked genes STM0517-0529 allowing the utilization of allantoin as a sole nitrogen source. We extended this observation by PCR screening of additional 120 *S. typhimurium* field strains and found that this locus was absent in all SGI1 positive and also in 24% of SGI1 negative strains, which were proposed to be the original recipients of SGI1. To prove this hypothesis, we compared the STM0517-0529 negative strains (with or without the SGI1) by PFGE and PCR prophage typing and found that 8 out of 11 of the SGI1 negative strains and 17 out of 22 SGI1 positive strains were of identical PFGE pattern and PCR prophage pattern, while this specific pattern was never observed among STM0517-0529 positive strains. We therefore propose that a lineage of the *S. typhimurium* DT104 sensitive strain first lost the ability to metabolize allantoin and then acquired SGI1.

Keywords *Salmonella typhimurium* · DT104 · SGI1 · Microarray genomotyping · Prophage · Allantoin

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Introduction

Salmonella enterica ssp. *enterica* is one of the main causative agents of gastrointestinal and systemic diseases in humans and domestic animals. Although about 2,500 serotypes exist in *S. enterica*, in humans *S. enteritidis* and *S. typhimurium* remain the two most predominant serovars responsible for gastrointestinal disorders. Resistance to antibiotics is increasing in *Salmonella* and many other bacterial species. For unknown reasons, strains of serovar Enteritidis are rarely reported to be antibiotic resistant at present. On the other hand, *S. typhimurium* strains currently circulating in the environment are frequently resistant to one or more antibiotics. Although various genes and genetic elements responsible for antibiotic resistance can be found in *S. typhimurium* (Faldynova et al. 2003; Rychlik et al. 2006), strains with the chromosomally located SGI1 genomic island harboring one or two integrons with antibiotic resistance genes are found most frequently. This genomic island has been

sequenced in *S. typhimurium* DT104 (Boyd et al. 2001), and identical or very similar genomic islands were detected also in other epidemic phage types of *S. typhimurium* such as DT29, 204, 193 and 204c, as well as different *Salmonella* serovars such as Agona (Boyd et al. 2002), Paratyphi B (Meunier et al. 2002) and Albany (Doublet et al. 2003).

Multidrug-resistant *S. typhimurium* DT104 was detected for the first time in cattle in the UK in the mid-1980s (Threlfall et al. 1994) and since that time it has reached a worldwide distribution. The rapid spread of this clone raises the question whether and/or why it is better adapted for infection of humans and/or farm animals. Multidrug-resistant *S. typhimurium* was reported to be capable of biofilm formation (Anriany et al. 2001), several studies also reported increased virulence of multidrug-resistant *S. typhimurium* DT104. However, while a case control study of infection with an epidemic strain of multidrug-resistant *S. typhimurium* DT104 indicated increased virulence of this clone (Wall et al. 1994; Evans and Davies 1996), possibly due to the up-regulation of collagenase (Wu et al. 2002; Carlson et al. 2005), other studies did not find an increase in virulence of DT104 strains (Carlson et al. 2000; Allen et al. 2001). It therefore seems that mere resistance to antibiotics together with increased ability of biofilm formation makes the strains better adapted for survival in the environment.

Since 2001, the complete genomic sequence of *S. typhimurium* strain LT2 has been available in the GenBank (McClelland et al. 2001), which has allowed implementation of functional genomics in the analysis of the *S. typhimurium* genome. Microarray technology may be used for analysis of gene expression using mRNA/cDNA microarray hybridization. In *Salmonella*, microarray genomotyping has also proved to be a useful tool for analysis of genomes of strains belonging to different species, subspecies or serovars (Porwollik et al. 2004a), despite the fact that in closely related strains of the same serotype, e.g., *S. typhimurium*, the genetic variation of strains was essentially limited to their different prophage content (Chan et al. 2003; Porwollik et al. 2004a). Consequently, differentiation of *S. typhimurium* strains by prophage-specific PCR has been recently used in *S. typhimurium* typing (Mikasova et al. 2005; Ross and Heuzenroeder 2005; Hermans et al. 2005).

In this study, we have first used microarray genomotyping for comparison of the genomes of four multidrug-resistant *S. typhimurium* DT104 strains amongst themselves and with the reference *S. typhimurium* LT2 strain. Similar to the other reports, the greatest variability was found in the presence or absence of integrated prophages. However, all the multidrug-resistant strains were found to lack genes for anaerobic utilization of allantoin. Similar strains unable to utilize allantoin were then detected also among the antibiotic-sensitive isolates, which we predicted to be the ancestors of the current multidrug-resistant strains that acquired the SGI1 in the mid-1980s. Using PFGE and prophage-specific PCR, we confirmed the high degree of similarity of the allantoin utilization defective strains regardless of the presence or absence of SGI1 in their genome and we propose the recent evolution in *S. typhimurium* resulting in the current distribution of circulating clones.

Materials and methods

Bacterial strains

Five bacterial strains were first subjected to microarray analysis. These included four multidrug-resistant *S. typhimurium* DT104 strains and a control *S. typhimurium* LT2. Antibiotic resistance was tested by disk diffusion method and strains representing the two most frequent antibiotic resistance patterns, ACSSuT (resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, strains 8E1 and 8E2) and SSu (resistance to streptomycin and sulfonamides, strains 8E3 and 8E4) were selected (Faldynova et al. 2003). Within each antibiotic pattern, the two strains differed in the presence (8E1 and 8E3) or absence (8E2 and 8E4) of the retron reverse transcriptase locus (Pilousova et al. 2005). These strains were tested also for growth in M9 minimal medium, PFGE and PCR prophage typing. An additional 120 field strains of *S. typhimurium* were analyzed by PCR for distribution of genes for allantion utilization and growth in glucose M9 minimal medium. Finally, 46 strains of these were subjected also to PFGE and prophage-specific PCRs (see below). Strains selected for the PFGE and prophage-specific PCRs are listed in Table 1. Phage typing has been performed according to Anderson et al. (1977).

Microarray analysis

The microarray chip contained PCR products of 4,466 out of the 4,678 identified ORFs of the *S. typhimurium* LT2 genome (McClelland et al. 2001). Primers designed to amplify the genome of *S. typhimurium* LT2 in a two-round amplification strategy were purchased from Sigma Genosys. The genes were amplified using specific primers with HotStar Taq DNA Polymerase

Table 1 List of strains used in this study

Strain	Phage type	Year	Source	Alla	SGI	PFGE	PCR phage typing					
							sodCI	*bim2*	*gtrA*	*hldD*	*artA*	*sopE*
15B5	U	2005	Human	+	–	G	+	–	–	–	–	–
LT2	ND	–	–	+	–	K	+	–	–	–	–	–
15B4	2	2005	Duck	+	–	A	+	+	–	–	–	–
15B2	2	2005	Pigeon	+	–	E	+	+	–	–	–	–
2452	2	2005	Pigeon	+	–	E	+	+	–	–	–	–
2461	2	2005	Pigeon	+	–	E	+	+	–	–	–	–
2463	2	2005	Pigeon	+	–	E	+	+	–	–	–	–
15B3	DT104	2005	Human	+	–	H	+	+	–	–	–	–
8H2	193	1985	Cattle	+	–	C	+	+	–	–	–	+
9B8	RDNC	1984	Cattle	+	–	F	+	+	–	–	–	+
8H6	RDNC	1985	Cattle	+	–	F	+	+	–	–	–	+
9C4	ND	1984	Cattle	+	–	F	+	+	–	–	–	+
9D10	DT104	1996	Pig	+	–	I	+	+	–	–	–	+
9E7	DT104	1996	Cattle	+	–	I	+	+	–	–	–	+
9A5	114	2000	Human	+	–	J	+	+	–	–	–	+
15A10	1	2005	Poultry	+	–	B	+	+	+	–	–	–
15B1	DT104	2005	Cattle	+	–	B	+	+	+	–	–	–
8H8	14	1985	Cattle	+	–	D	+	+	+	–	–	+
15B6	1	2005	Wild boar	–	–	N	+	–	–	–	–	–
15B7	U	2005	Parrot	–	–	M	+	+	–	–	+	–
9B10	ND	1991	Pig	–	–	A	+	+	+	+	+	–
2002	DT104	2005	Human	–	–	A	+	+	+	+	+	–
2009	DT104	2005	Poultry	–	–	A	+	+	+	+	+	–
2116	DT104	2005	Human	–	–	A	+	+	+	+	+	–
2226	DT104	2005	Pig	–	–	A	+	+	+	+	+	–
9D3	ND	1985	Cattle	–	–	A	+	+	+	+	+	–
9A2	DT104	2000	Human	–	–	A	+	+	+	+	+	–
9A3	DT104	2000	Food	–	–	A	+	+	+	+	+	–
9D7	ND	1997	Cattle	–	–	L	+	+	+	+	+	–
8E2	DT104	2001	Poultry	–	+	A	–	+	+	+	+	+
2025	DT104	2005	Human	–	+	A	+	+	+	+	–	–
15B8	DT104	2005	Human	–	+	A	+	+	+	+	–	–
9B9	DT104	2000	Cat	–	+	A	+	+	+	+	–	–
138/8	DT104	2004	Cattle	–	+	A	+	+	+	+	+	–
145/8	ND	2004	Pig	–	+	A	+	+	+	+	+	–
2003	DT104	2005	Human	–	+	A	+	+	+	+	+	–
2045	DT104	2005	Cattle	–	+	A	+	+	+	+	+	–
2082	DT104	2005	Human	–	+	A	+	+	+	+	+	–
2358	12	2005	Human	–	+	A	+	+	+	+	+	–
2368	120	2005	Cattle	–	+	A	+	+	+	+	+	–
9E1	DT104	1996	Pigeon	–	+	A	+	+	+	+	+	–
9E4	DT104	1996	Cattle	–	+	A	+	+	+	+	+	–
9E2	DT104	1996	Pig	–	+	A	+	+	+	+	+	–
9E5	DT104	1996	Pigeon	–	+	A	+	+	+	+	+	–
9A6	DT104	2000	Poultry	–	+	A	+	+	+	+	+	–
9D1	ND	1998	Pig	–	+	A	+	+	+	+	+	–
15B9	DT104	2005	Pig	–	+	O	+	+	+	+	+	–
8E1	DT104	2001	Pig	–	+	A	+	+	+	+	+	–
8E3	DT104	2000	Human	–	+	P	+	+	+	+	+	–
8E4	DT104	2000	Human	–	+	A	+	+	+	+	+	–
9E6	DT104	1996	Pig	–	+	A	+	+	+	+	+	+

ND not determined, year–year of isolation, *Alla* presence (+) or absence (–) of STM0517–STM0529 genes for allantoin utilization; SGI–PCR amplifying the left junction of SGI1 and flanking genomic DNA. Strains in bold were subjected to microarray genomotyping

(Qiagen) in 25 µl volumes (2.5 ng template DNA, 60 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 5 U HotStar Taq polymerase) with an annealing temperature of 60°C. The product size was determined by gel electrophoresis prior to diluting 25-fold for use in the second round amplification. Aliquots (5 µl) of the diluted products were used in the second reaction using universal primers (TCCTAGGAGCTCTCTTCT as forward primer and TGCCTAGGGCTCTTCG as reverse primer), which annealed to the 19-nucleotide

overhang generated in the first round. Amplification took place in a similar manner in a 100-µl reaction volume. The products were precipitated by ethanol to remove the nucleotides and primers. The dried products were resuspended in 3× SSC containing 1.5 M betaine (Sigma) at a concentration 400 ng/µl and spotted on to in-house-coated poly-L-lysine slides, in duplicates, using a commercially available robotic arrayer (MicroGrid II, BioRobotics) that generates microarrays with a DNA spot size of 150 µm in diameter. The slides were left to rehydrate overnight before fixing by snap drying for 1 min at 90°C on a heating block and UV fixation at 650 mJ. The slides were blocked in dichloroethane (Aldrich), succinic anhydride (Sigma) using 1-methylimidazole (Sigma) as a catalyst.

Genomic DNA was purified from an 18-h-old bacterial culture grown in LB broth at 37°C using the DNeasy Tissue Kit (Qiagen). DNA, 10 µg, was labeled indirectly using random hexamers, 40 U Klenow Fragment DNA Polymerase (Exonuclease minus activity, Epicentre) and 20 mM dNTPs mix and aminoallyl dUTP (the ratio of aa-dUTP to dTTP was 3:2). After clean up (GFX columns, Pharmacia), the samples were labeled for 1 h with a 100 µg aliquot of Cy3 or Cy5 dye (Sigma). After an additional clean up, the probes were resuspended in hybridization buffer containing 40% deionized formamide, 5× SSC, 5× Denhart's solution, 1 mM sodium pyrophosphate, 0.1% sodium dodecyl sulfate and 50 µg yeast tRNA and denatured for 5 min at 95°C. The overnight hybridization at 42°C was followed by two washes in 6× SSC and 0.005% TritonX-102 and additional two washes in 0.1× SSC, 0.005% TritonX-102. Slides dried by spinning at 500 rpm for 5 min at room temperature were subjected to scanning (see below). For each of the strains, data from four independent microarray hybridizations were obtained.

Image acquisition and data analysis

The microarray chips were scanned by ScanArray Express Microarray Scanner (Perkin Elmer). Spots were localized by the adaptive threshold quantification method and spot intensities of each microarray were normalized by the LOWESS algorithm implemented in the ScanArray Express 2.2.0. Normalization between the microarray slides was performed using open source software R project version 2.1.1. (R Development Core Team 2004) and the package SMIDA (Wit and McClure 2004).

For the detection of DNA regions absent in each microarray experiment, a breakpoint detection method based on the adaptive weights smoothing procedure was used. The method is implemented in R package GLAD version 1.0.4. (Hupe et al. 2004). The default setting of the parameters of the basic GLAD function "glad.profileCGH" was used for analysis. For each microarray experiment, GLAD results on both normalized "Log_2 Ratio of Medians" and normalized "Log_2 Ratio of Means" measures were compared for additional quality control. The results of the GLAD analysis were compared also between the dye-swap experiments. Moreover, bacterial strains from the different microarray experiments with the same LT2 reference were also compared. Joined log_2 ratios [log_2 (strain1/strain2)] of these strains were obtained as follows: log_2 (strain1/strain2) = (log_2 (strain1/LT2) − log_2 (strain2/LT2). These joined log_2 ratios were also analyzed using GLAD and compared with log_2 ratios obtained from the corresponding dye swap experiments. Thus, eight GLAD results for each pair of bacterial strains (four for Log_2 Ratio of Means and four for Log_2 Ratio of Medians) were obtained. The region of the DNA was considered as changed when the majority of the eight GLAD results agreed. The genes that were selected as potentially missing in the genome of compared strains were subjected to the PCR verification.

Confirmatory PCRs

PCRs were used for (1) confirmation of data from microarray analysis, (2) classification of strains into SGI1 positive or negative and (3) prophage typing. For all PCRs, the DNA was released from one colony resuspended in 50 µl of sterile distilled water and boiled for 20 min in a dry block incubator. After spinning for 1 min at 13,000*g*, 2 µl of the supernatant was taken as a template for PCR. The PCR reactions were carried out using a PCR Master Mix kit (Qiagen). PCR cycling conditions consisted of 30 cycles of 1 min incubations at 92, 55 and 72°C, followed by a final extension at 72°C for 3 min. The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and analyzed by UV transilluminator. All primers used in this study are listed in Table 2. If necessary, the PCR products were sequenced using ABI Prism 310 Genetic Analyser (Applied Biosystems).

Ability of *S. typhimurium* to utilize allantoin as a sole nitrogen source

Glucose M9 minimal medium supplemented with either 20 mM $(NH_4)_2SO_4$ or 60 mM allantoin as a nitrogen source was used (Cusa et al. 1999). To mimic anaerobic

Table 2 List of primers used in this study

Name	Primer Sequence (5′ to 3′)	Description	Purpose	Reference
STM903 F	TTT GCC TTT GCT GAG TAT TC	Fels-1	MA	This study
STM903 R	GCA CAC CTC TTT CTT TTG TC	Fels-1	MA	This study
STM908 F	AAT TAC CGA CAT GAC GAA GC	Fels-1	MA	This study
STM908 R	TGG CTT CTT CCA TTT TAG TC	Fels-1	MA	This study
STM2702 F	ATC TGG TCG TTC TGG TAG AG	Fels-2	MA	This study
STM2702 R	ATC AAA AAG CAA TCC AAC AC	Fels-2	MA	This study
STM2705 F	CTG GGG TGT TGT TAA TCA TC	Fels-2	MA	This study
STM2705 R	ATC ACT GAT AAT TCC CAA CG	Fels-2	MA	This study
STM1026 F	ACT AAA TTC GTA GGC GAT TC	Gifsy-2	MA	This study
STM1026 R	TAC CTT ATC TCT GGA CCA GG	Gifsy-2	MA	This study
STM1044 F	TAT CGG AGT AAT TGT CAC CG	Gifsy-2, *sodCI*	MA, PT	This study
STM1044 R	ACA ATA TTG TCG CTG GTA GC	Gifsy-2, *sodCI*	MA, PT	This study
STM3844 F	AGC TTT CTC ACT TGC AGA AG	retron, *int2*	MA	This study
STM3844 R	GTG AGT TCT TCG AGT TCC AG	retron, *int2*	MA	This study
STM3846 F	GAA CTA TTG CTC ATC CTT CG	retron, *rrtT*	MA	This study
STM3846 R	GTA ACG TGA CGG TTA TGT CC	retron, *rrtT*	MA	This study
STM519 F	ACC ATC GTC GAT ATG AGT TC	STM0519, *glxR*	MA	This study
STM519 R	TAT GAT TGG CCA TTA ATT CC	STM0519, *glxR*	MA	This study
STM527 F	CGA TAT CGA TGC TGA TAT CC	STM0527, *allC*	MA	This study
STM527 R	ATA GAA GAA ACA TTG CCC TG	STM0527, *allC*	MA	This study
U7-L12[a]	ACA CCT TGA GCA GGG CAA **A**G	SGI1 left junction	S	Boyd et al. (2002)
LJ-R1	AGT TCT AAA GGT TCG TAG TCG	SGI1 left junction	S	Boyd et al. (2002)
STM516 F	AGC AGG CGC GTG AAC AGG GC	STM0517-0529 deletion	S	Garaizar et al. (2002)
STM530 R	TGC GGA CGT GCG CGA CGA ATT T	STM0517-0529 deletion	S	Garaizar et al. (2002)
gtrA F	AGA CCT TTC CGA ATC CGC TG	phage ST64T	PT	Ross and Heuzenroeder (2005)
gtrA R	TAA TTG CCG AGA AAG TGA TAA GGG	phage ST64T	PT	Ross and Heuzenroeder (2005)
BIM2 F	CCA TTA CCG GCG CTT GCA C	phage ST64B	PT	Ross and Heuzenroeder (2005)
BIM2 R	TAA CGT ATA ACC ATG CGA TTT CCG	phage ST64B	PT	Ross and Heuzenroeder (2005)
hldD F	ACA ATG CTT TCG AAC CTG ATG GGC	prophage-like	PT	Hermans et al.(2005)
hldD R	CCA TCG CTT CAA TTG CAA CCA TGC	prophage-like	PT	Hermans et al. (2005)
artA F	ATT ACT ACG TGC ATC ATC CC	Gifsy-1_{DT104}	PT	This study
artA R	TTA GAG GTG ACT CGT GTT CC	Gifsy-1_{DT104}	PT	This study
sopE F	AATTCATCAATCAGATGGAC	phage SopEΦ	PT	This study
sopE R	TCATATTAATCAGGAAGAGG	phage SopEΦ	PT	This study

Purpose purpose of use of primer pair, *MA* confirmation of data from microarray analysis, *S* classification of strains into SGI1 and STM0517-0529 positive or negative, PT–PCR prophage typing

[a] The sequence of this primer differs from the original one reported by Boyd et al. (2002) in that there is an extra A at the 3′ end of the primer (marked in bold), according to the complete sequences of both LT2 and DT104 strains

conditions, 15 ml close-capped tubes were filled with 14 ml of the medium. The strains were inoculated into the medium at an initial concentration of 10^6 CFU/ml and incubated without opening at 37°C. The terminal OD of the culture was determined 72 h after the inoculation using a tube-adapted densitometer (Ultrospec 10 Cell Density Meter, Amersham Biosciences).

Pulsed-field gel electrophoresis

DNA was purified from strains selected to represent the original antibiotic-sensitive population ($n = 18$), the SGI1 negative population with the absence of STM0517-0529 ($n = 11$, all available strains of this type), and the SGI1 positive population ($n = 22$). DNA purification for macrorestriction analysis, restriction enzyme digestion and pulsed-field gel electrophoresis was performed essentially as described elsewhere (Hunter et al. 2005). Pulsed-field gel electrophoresis of *Xba*I digested DNA was performed using the CHEF-DRIII system (Bio-Rad) in 0.5× TBE. After the electrophoresis, the gels were stained with ethidium bromide and the DNA was visualized under UV light. *S*. Braenderup H9812 digested with *Xba*I was used as a molecular weight standard (Hunter et al. 2005). The dendrogram was generated by the GelCompar software (Applied Maths, Belgium) using Dice coefficient and UPGMA algorithms.

Results

Microarray genomotyping

Microarray analysis indicated six genomic regions of potential variation among analyzed strains. These

included STM0517-0529, STM0893-0932, STM1005-1057, STM2694-2741 STM3844-3846 and STM4090-STM4120 (Fig. 1). The presence or absence of these regions was confirmed by PCR, which confirmed the results of the microarray analysis in all the cases except for the STM4090-4120 genomic region. This region was apparently absent in the multidrug-resistant strain; however, all the CPR resulted in positive amplifications. This region was recently reported as of variable fluorescence due to genomic rearrangements (Porwolik et al. 2004b).

Open reading frames STM3844-3846 represent the retron reverse transcriptase locus. This locus was missing in two multidrug-resistant strains, 8E2 and 8E4, a fact that we knew prior to the microarray analysis. The correct detection of the presence or absence of this locus in the strains analyzed confirmed the specificity of the microarray genomotyping.

Open reading frames STM0893-0932, STM1005-1057 and STM2694-2741 represent Fels-1, Gifsy-2 and Fels-2 phages, respectively. Fels-1 and Fels-2 prophages were present only in the genome of strain LT2 and were absent in the genome of all the multidrug-resistant strains. Gifsy-2 prophage was present in all the analyzed strains except for a single multidrug-resistant strain, 8E2.

The last genomic cluster, which showed variation among the strains, was a block of genes STM0517-0529. The corresponding part of the *E. coli* genome encodes its ability to utilize allantoin as the only nitrogen source under anaerobic conditions via the allantoin–glyoxylate metabolic pathway (Cusa et al. 1999). These genes were present in strain LT2 but were absent in all four multidrug-resistant strains (Fig. 2).

PCR screening of STM0517-0529 deletion and SGI1 in *S. typhimurium*

While phages are well-established sources of genomic variation and the absence of the retron locus in some of the strains was known before the microarray genomotyping was started, the potential inability of the multidrug-resistant strains to utilize allantoin as a nitrogen source has not been described so far. To characterize the extent and significance of genes STM0517-0529 among the current *S. typhimurium* field strains, we performed a PCR screening for this genomic region in 120 current field strains. In parallel, PCR of the left junction of SGI1 island was used as a marker for the presence of SGI1 encoded multidrug resistance. These PCR results enabled us to classify the field strains into three groups (including the five genomotyped strains). The first group was formed by STM0517-0529 locus-positive and SGI1 negative strains ($n = 34$), the second group consisted of STM0517-0529 locus-negative and SGI1 negative strains ($n = 11$) and the last group comprised STM0517-0529 locus-negative and SGI1 positive strains ($n = 80$). Strains of the latter two groups predominantly belonged to the phage type DT104. Sequencing of the PCR products of SGI1 left junction and the STM0517-0529 deletion in ten selected SGI1 positive and 2 SGI1 negative strains resulted in an identical sequence further confirming similarity of these strains. While the number of SGI1 positive

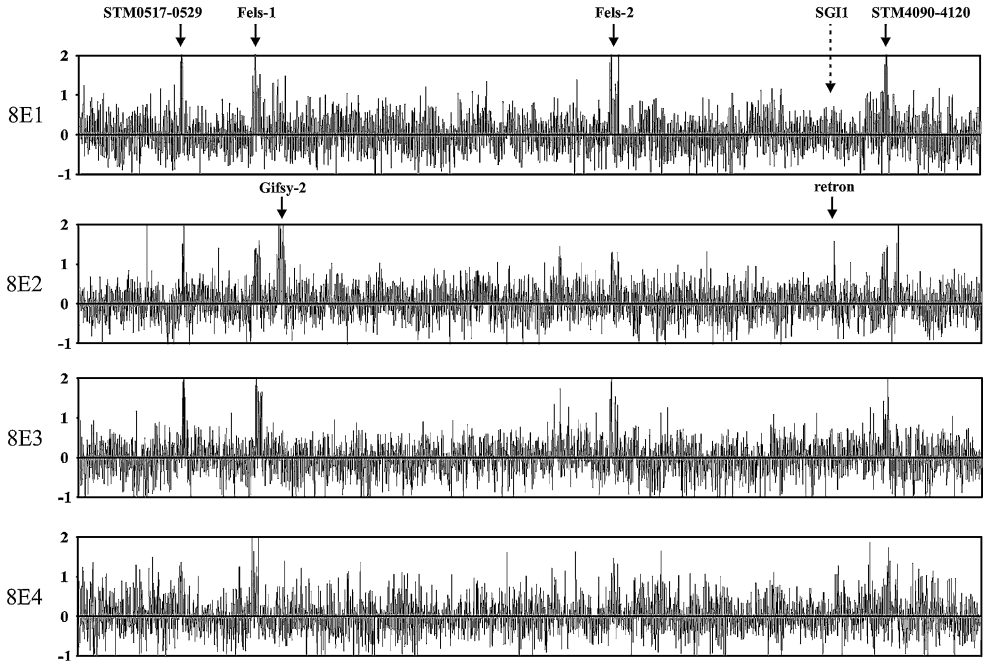

Fig. 1 Microarray genomotyping of four multidrug-resistant strains of *S. typhimurium* expressed as $\log_2$ ratios of fluorescence signal intensities to the LT2 reference strain. Genomic regions of potential variations between the strains are indicated. Site of SGI1 insertion is also indicated by *dashed arrow*, although this was not identified by the microarray analysis since the chip contained only *S. typhimurium* LT2 genes and ORFs

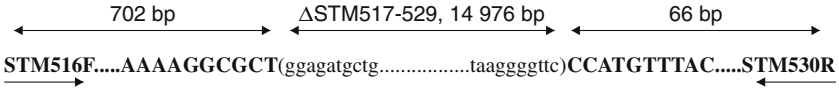

Fig. 2 Map of the STM0517-0529 genomic region, which was subjected to deletion in all SGI1 positive multidrug-resistant *S. typhimurium* strains and also in a small proportion of antibiotic-sensitive strains. The deletion is defined by the *upper* and *lower* case letters. The *lower* case letter sequence, altogether 14,976 bp, is present only in the *S. typhimurium* LT2 strain but absent from the genome of SGI1 positive strains. Primers STM0516F and STM0530R used for the amplification over the deletion are shown

strains (group 3) was deliberately increased in this study, the ratio of strains in group 1 and 2 allowed a realistic estimation of the proportion of allantoin gene-defective strains in the antibiotic-sensitive *S. typhimurium* population to 24%.

Ability of *S. typhimurium* to utilize allantoin as a sole nitrogen source

Although the same deletion has already been described in other *Salmonella* serotypes (Porwollik et al. 2002; Garaizar et al. 2002; Reen et al. 2005), the biological consequences of the deletion were not investigated in those studies at all. Because the STM0517-0529 locus should encode the ability of *S. typhimurium* to utilize allantoin as a sole nitrogen source under anaerobic conditions, we tested experimentally the consequence of this deletion for the growth of *S. typhimurium*. The growth of STM0517-0529 locus-positive and SGI1 negative strains (group 1) was quite variable in glucose minimal medium. Strains of this group were capable of growth in minimal medium supplemented with $(NH_4)_2SO_4$ (average OD among all the strains 1.07 ± 0.32) and, except for three strains, all the strains grew also when allantoin was used as a nitrogen source (OD 0.75 ± 0.28).

STM0517-0529 and SGI1 locus-negative strains (group 2) grew to high cell densities in $(NH_4)_2SO_4$ minimal medium (OD 1.34 ± 0.09) but did not grow when $(NH_4)_2SO_4$ was replaced with allantoin, consistent with the absence of the STM0517-0529 locus. Similar growth characteristics were observed also in STM0517-0529 negative and SGI1 positive strains (group 3). These strains grew to high cell densities in $(NH_4)_2SO_4$ minimal medium with low variability (OD 1.39 ± 0.18). However, none of these strains grew when $(NH_4)_2SO_4$ was replaced with allantoin.

Pulsed-field gel electrophoresis

The results presented above indicated that the strains in group 2 might be the recipients of SGI1. To prove this hypothesis, similar numbers of strains representing each of the three groups were characterized by pulsed-field gel electrophoresis and PCR prophage typing. If strains in group 2 were the ancestors of SGI1 positive *S. typhimurium* (group 3), these should be of identical or highly similar genotypes.

Altogether, 16 different PFGE profiles were identified among the 51 tested strains (Table 1). Of the 18 selected STM0517-0529 locus-positive and SGI1 negative strains (group 1), 11 profiles were observed, confirming a considerable variability among these strains (Fig. 3). The distribution of profiles was relatively random and no dominant profile was observed. The PFGE profile designated as "E" was found in four strains from pigeons, profile "F" was identified in three strains and the remaining profiles were identified in one or two strains only. The profile designated as "A", found to be dominant in strains with deletion of STM0517-0529 (see below), was found in one strain of this group.

Among the 11 strains negative both in STM0517-0529 and SGI1 (group 2), four different profiles were recorded. The distribution of different profiles was not as random as in the STM0517-0529 positive strains. Three profiles were recorded in three individual strains, while profile "A" was recorded in eight strains, being clearly the dominant one in this group.

In the last group of 22 strains positive in SGI1 (group 3), three profiles were identified. In 20 strains, profile "A" was observed and in only individual strains, profiles designated as "O" and "P" were determined.

PCR detection of phage loci in *S. typhimurium* field strains

To further confirm the high degree of similarity of strains of group 2 and 3, PCR prophage typing was performed. Phages are known sources of genomic variability and PCRs specific for phage sequences have been successfully used for the differentiation of closely related strains of *S. typhimurium* (Ross and Heuzenroeder 2005). As a target for PCR in this study, we focused on phages that were described in the genome of *S. typhimurium* DT104. This is the reason that the Fels-1 and Fels-2 prophages, which are relatively

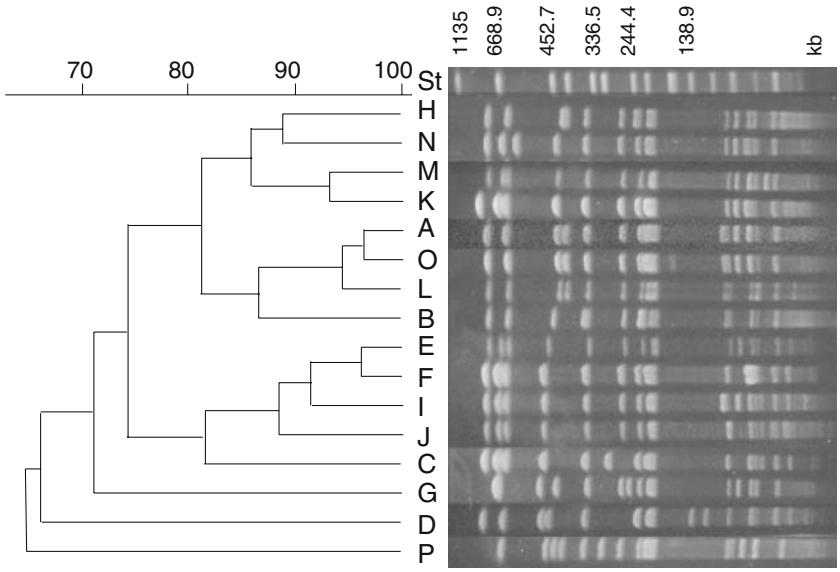

Fig. 3 *Xba*I-digested DNA of representatives of various clones found among the strains of *S. typhimurium* analyzed in this study. *Lane* St—reference *Salmonella* serovar Braenderup H9812 DNA digested with *Xba*I

specific for LT2 strain (Porwollik and McClelland 2003), were not detected by PCR in field strains despite the fact that using microarray analysis for these phages differentiated the LT2 strain from all the DT104 isolates.

First, we aligned the genome of *S. typhimurium* LT2 with a raw sequence of *S. typhimurium* DT104 (available at http://www.sanger.ac.uk/Projects/*Salmonella*/) and we identified prophages ST64B and ST64T, and a prophage-like sequence containing the *hldD* gene in the genome of DT104. In addition, we found that genes STM2610-STM2629, a part of the Gifsy-1_{LT2} prophage, were replaced with a similarly sized sequence in the genome of DT104 including the *artA* gene. Since ST64B (*bim2*), ST64T (*gtrA*) and *hldD* specific PCRs were already described (Ross and Heuzenroeder 2005; Hermans et al. 2005) we designed only primer pairs specific for *artA* of Gifsy-1_{DT104}, *sodCI* of Gifsy-2 prophage and *sopE* encoded by the Fels-like SopE prophage. Altogether 10 different PCR prophage profiles were described among 51 tested strains. The vast majority of strains of group 2 and group 3 were positive in all prophage-based PCRs (which should be specific for *S. typhimurium* DT104) except for the *sopE*, while none such strain was found in group 1 (Table 1).

Discussion

In this study, using microarray genomotyping, we first compared the genomes of four multidrug-resistant *S. typhimurium* strains of phage type DT104 with the antibiotic sensitive strain LT2. Five variable genomic regions were found. Consistent with previous reports, we found that the genomic variation was associated with three different prophage sequences (Porwollik et al. 2004a; Thomson et al. 2004; Mikasova et al. 2005; Garaizar et al. 2002) and STM3844-3846, which encodes the retron reverse transcriptase and two additional ORFs (Matiasovicova et al. 2003; Pilousova et al. 2005). The last variable genomic region, the STM0517-0529 locus, was absent in all four multidrug-resistant DT104 strains but present in strain LT2. It is distinct from the previous genomic variations since no obvious mobile sequences in its surroundings could be identified, although this region was proposed to be acquired by *Salmonella* as a result of lateral gene transfer (Porwollik et al. 2002). This genomic variability has already been described in six *S. typhimurium* DT104 isolates from different animal sources in Ireland (Reen et al. 2005) and in a *Salmonella* clone defective in flagella biosynthesis, which was otherwise similar to serovar *S. typhimurium* (Garaizar et al. 2002). The STM0517-0529 deletion can also be found in the raw sequence of the multidrug-resistant DT104 strain currently being sequenced at the Sanger Institute, UK (http://www.sanger.ac.uk/Projects/*Salmonella*/). However, it has never been associated with the multidrug-resistant SGI1 positive strains and their inability to utilize allantoin as a sole nitrogen source. The deletion of STM0517-0529 was physically separated with the insertion of SGI1, which occurred immediately downstream the STM3843 gene (Fig. 1). During the PCR screening of *S. typhimurium* field strains, we found that the deletion was present in all SGI1 positive strains and also in 24% of SGI1 negative strains indicating that a strain of this group could have been the original recipient of SGI1. If this hypothesis is correct, the strains of group 2 and group 3

still should be genetically highly related. In agreement with the assumption, PFGE profile "A" was observed in 28 out of 33 strains of groups 2 and 3, while profile "A" was found in only a single strain of group 1. Furthermore, 26 out of 33 strains of both of these groups were characterized by the presence of the same prophages in their genomes. We therefore propose that strains of group 2 are the ancestors of currently SGI1 positive stains, i.e., that a strain of group 2 must have acquired the SGI1 in the mid-1980s when the SGI1 positive strains of *S. typhimurium* were first recorded in cattle in Great Britain (Threlfall et al. 1994).

When the results of this study are combined with other reports, a recent evolution of multidrug-resistant *S. typhimurium* can be proposed. First, the original allantoin utilization-positive and SGI1 negative strain lost the STM0517-STM0529 locus. Subsequently, a strain of this lineage acquired SGI1, a fact that is supported by the observation of the mobility of SGI1 (Doublet et al. 2005). It is quite difficult to imagine that the evolution went the other way, i.e., that the insertion of SGI1 resulted in the loss of STM0517-0529 and that in some of these clones the SGI1 was subsequently lost. Finally, due to the activity of IS6100, a part of SGI1, more recent genome modification of this clone continues, as can be documented, either by the formation of a chloramphenicol-sensitive SGI1 E variant (Boyd et al. 2002) or loss of the retron reverse transcriptase locus (Pilousova et al. 2005).

Identification of SGI1 recipients will allow much more detailed comparison of the characteristics of ancestors and SGI1 positive strains and may help to elucidate the reasons for their efficient spread in the environment. For example, it has been shown that multidrug-resistant *S. typhimurium* are capable of biofilm formation (Anriany et al. 2001) and we observe that this is true also for the antibiotic-sensitive STM0517-0529 negative strains (unpublished observations). It would be interesting if other features could be identified, which may explain the spread of the SGI1-dependent multidrug-resistant strains of *S. typhimurium*.

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