Microbiology (2006), **152**, 2137–2147 DOI 10.1099/mic.0.28850-0

Distribution of prophages and SGI-1 antibiotic-resistance genes among different *Salmonella enterica* serovar Typhimurium isolates

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Received 16 January 2006
Revised 17 March 2006
Accepted 30 March 2006

Recently, the authors identified *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) definitive type (DT)104-specific sequences of mainly prophage origin by genomic subtractive hybridization. In the present study, the distribution of the prophages identified, ST104 and ST64B, and the novel prophage remnant designated prophage ST104B, was tested among 23 non-DT104 *S.* Typhimurium isolates of different phage types and 19 isolates of the DT104 subtypes DT104A, DT104B low and DT104L, and the DT104-related type U302. The four *S.* Typhimurium prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 were also included. Analysis of prophage distribution in different *S.* Typhimurium isolates may supply additional information to enable development of a molecular method as an alternative to phage typing. Furthermore, the presence of the common DT104 antibiotic resistance genes for the penta-resistance type ACSSuT, *aadA2*, *floR*, *pse-1*, *sul1* and *tet*(*G*), was also studied because of the authors' focus on this emerging type. Based on differences in prophage presence within their genome, it was possible to divide *S.* Typhimurium isolates into 12 groups. Although no clear relationship was found between different phage type and prophage presence, discrimination could be made between the different DT104 subtypes based on diversity in the presence of prophages ST104, ST104B and ST64B. The novel prophage remnant ST104B, which harbours a homologue of the *Escherichia coli* O157 : H7 HldD LPS assembly-related protein, was identified only in the 14 DT104L isolates and in the DT104-related U302 isolate. In conclusion, the presence of the genes for penta-resistance type ACSSuT, the HldD homologue containing ST104 prophage remnant and phage type DT104L are most likely common features of the emerging subtype of *S.* Typhimurium DT104.

INTRODUCTION

Horizontal gene transfer plays an important role in the evolution of bacteria to adapt to changing niches or to exploit new niches. The acquisition of virulence or antibiotic resistance genes via horizontally transferable genetic elements, such as plasmids (conjugation) or bacteriophages (transduction), has resulted in the emergence of a variety of pathogens (reviewed by Ochman *et al.*, 2000). Also, the foodborne pathogen *Salmonella* has acquired a large number of virulence genes via horizontal gene transfer (Groisman & Ochman, 1997). Within the genomes of different *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) isolates, many virulence factors have been reported to be located on prophages, which are bacteriophages integrated into the bacterial genome (Bakshi *et al.*, 2000; Figueroa-Bossi & Bossi, 1999, 2001; Ho *et al.*, 2002; Miao *et al.*, 1999; Mirold *et al.*, 1999; Stanley *et al.*, 2000). For example, within the genome of *S.* Typhimurium lilleengen type (LT)2, which is an attenuated laboratory strain whose genome has been sequenced (McClelland *et al.*, 2001), prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 all encode one or more genes that are somehow involved in virulence (reviewed by Brussow *et al.*, 2004). These prophages are often identified adjacent to tRNA genes (McClelland *et al.*, 2001), which are hot spots for insertion of transferable elements in general (Cheetham & Katz, 1995; Reiter *et al.*, 1989), because these genes are highly conserved among bacterial genomes (Campbell, 2003; Hou, 1999). Our study focused on the emerging multiple-antibiotic-resistant *S.* Typhimurium definitive type (DT)104. The isolates of *S.* Typhimurium DT104 that have emerged during the past

Abbreviations: DT, definitive type; LT, lilleengen type; RIVM, National Institute of Public Health and the Environment; SGI-1, *Salmonella* genomic island 1.

decades have a core pattern of resistance to the five antibiotics ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, referred to as penta-resistance type ACSSuT (Glynn *et al*, 1998; Humphrey, 2001; Threlfall *et al.*, 1994; Threlfall, 2000). The five genes for this penta-resistance type, *aadA2*, *floR*, *pse-1*, *sul1* and *tet*(*G*), are clustered within the genome on the so-called *Salmonella* genomic island 1 (SGI-1), which also contains horizontally transferable genetic elements such as phage- and plasmid-related genes (Boyd *et al.*, 2001; Briggs & Fratamico, 1999). Based on phage typing for *S.* Typhimurium by the Anderson scheme (Anderson *et al.*, 1977), five subtypes have been described within DT104: DT104A, DT104B, DT04B low, DT104H and DT104L. Phage type U302 was previously referred to as being DT104-related (Briggs & Fratamico, 1999). Interestingly, of 160 DT104 isolates studied, the DT104L subtype isolates harbouring penta-resistance have been revealed to be the most frequently occurring subtype, with 40 % of all isolates (Malorny *et al.*, 2002).

The currently used Anderson phage-typing scheme uses 34 bacteriophages to distinguish 207 DTs (Anderson *et al.*, 1977) and the pattern of sensitivity of an isolate to these bacteriophages results in a phage-type number, such as DT104. However, this typing method has some drawbacks. Several *S.* Typhimurium isolates cannot be classified by this method, and the method itself requires considerable experience for scoring, and to achieve good standardization (Hu *et al*, 2002). Furthermore, Schmieger (1999) noted that, if the original Anderson typing bacteriophage stocks become exhausted, this may be the end of this method. Therefore, molecular methods have been developed and implemented to replace conventional phage typing (Hu *et al.*, 2002; Tucker & Heuzenroeder, 2004). The presence of a prophage in the *S.* Typhimurium genome can affect the susceptibility to a bacteriophage, resulting in a different phage type, referred to as phage-type conversion (Mmolawa *et al.*, 2002; Rabsch *et al*, 2002; Tucker & Heuzenroeder, 2004). Analysis of prophage distribution in different *S.* Typhimurium isolates may supply additional information for such a molecular method for phage typing.

In a previous genomic subtractive hybridization study, we identified novel *S.* Typhimurium DT104L sequences of mainly prophage origin (Hermans *et al.*, 2005). The sequences obtained were similar to those of bacteriophages ST104 (Tanaka *et al.*, 2004) and ST64B (Mmolawa *et al.*, 2003a). Furthermore, a novel prophage remnant was identified, designated prophage ST104B in the current study, which harbours a homologue of the *Escherichia coli* O157 : H7 HldD LPS assembly-related protein and the *irsA* gene, which is suggested to be involved in macrophage survival (Baumler *et al.*, 1994). The objective of the present study was to investigate the distribution of the prophages ST104, ST104B and ST64B among *S.* Typhimurium isolates of different phage types, with special focus on *S.* Typhimurium DT104 and related types. The four prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 that have been described in the sequenced *S.* Typhimurium LT2 strain were also included. Because of our focus on DT104 and the importance of the multi-antibiotic resistance of this type, all isolates were also tested for the presence of the five SGI-1-located antibiotic-resistance genes. Differences in the presence of prophages can be indicative of the virulence potency of an *S.* Typhimurium strain, since many virulence factors have been reported to be located on prophages. Furthermore, these results will help to increase our knowledge of the relationship between prophage presence and conventional phage typing for *S.* Typhimurium.

METHODS

Bacterial strains, culture conditions and preparation of genomic DNA. The *S.* Typhimurium isolates of different phage types used in this study are listed in Table 1, and were obtained from RIKILT Institute of Food Safety, the Dutch National Institute of Public Health and the Environment (RIVM) and the American Type Culture Collection (ATCC). The isolates were typed at the National *Salmonella* Reference Laboratory of the Federal Institute for Risk Assessment, Germany. All isolates were stored at −80 °C in brain heart broth plus 50 % (v/v) glycerol. The isolates were grown overnight in brain heart broth at 37 °C without shaking. Genomic DNA was extracted from overnight cultures by using a genomic DNA wizard kit (Promega).

Prophage detection. A PCR method was developed for the detection of prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 identified in the attenuated laboratory strain *S.* Typhimurium LT2 (GenBank accession no. NC_003197; McClelland *et al.*, 2001), and prophages ST104 and ST64B and the prophage remnant ST104B, referred to as the non-LT2 prophages, which we could identify in *S.* Typhimurium DT104L. The non-LT2 prophages were identified in *S.* Typhimurium DT104L by using the sequences of: (a) our earlier DT104L genomic subtractive hybridization results (accession nos. AY462969–AY463002; Hermans *et al.*, 2005); (b) bacteriophages ST104 (NC_005841; Tanaka *et al.*, 2004) and ST64B (NC_004313; Mmolawa *et al.*, 2003a); and (c) the DT104 genome (NC_004513). The sequence data of the DT104 genome were produced by the *Salmonella* spp. Sequencing Group at the Sanger Institute, Hinxton, UK, and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella. A comparison between the genome sequences of LT2 and DT104 at the Gifsy and Fels prophage loci, by using the software package Vector NTI Suite version 5.1 (Invitrogen), revealed that the Fels prophages were absent in DT104. This resulted in the identification of the integration positions of the Fels bacteriophages and the design of primers to test the presence or absence of these prophages (Fig. 1). The Gifsy prophages were found in both LT2 and DT104, therefore, primers were designed based only on the annotation of these prophages within the LT2 genome. The genome comparison of both strains also revealed the location of the non-LT2 prophages ST104, ST104B and ST64B within the DT104 genome (Fig. 1). For the seven prophages selected, primer sets were designed on the left (LB-F and LB-R) and right (RB-F and RB-R) borders of the prophages, and an additional primer set on internal DNA fragments for the non-LT2 prophages ST104 (*cI*), ST104B (HldD homologue) and ST64B (non-coding region). Notably, primer sets for internal DNA fragments of the non-LT2 prophages were designed as an additional control because the prophage border primers were based on the bacteriophage sequences while, for the LT2 prophages, the primers were based on the actual prophage sequences. Furthermore, no prophage insertion (prophage absent) could be detected by combining the LB-F and RB-R primers for each prophage. An overview of all PCR primers and amplicons used to detect the seven prophages is shown

in Table 2 and is schematically presented in Fig. 1. The primers (Isogen) at a 0·2 μmol l^{-1} concentration were combined with 0·2 mmol l^{-1} of each dNTP, 3 mmol l^{-1} $MgCl_2$, 1 × PCR reaction buffer and ~1–100 ng DNA template, and were amplified with 1 U

Table 1. *S.* Typhimurium isolates used in this study

Strain no.	Phage type*	Isolation source†	Resistance pattern‡	Prophage profile§
DT104 and DT104-related				
406	U302	Human	ASu	1
408	DT104B low	Pig		4
410	DT104L	Pig	ACSSuT	1
417	DT104L	Dairy cow	ASu	1
418	DT104L	Dairy cow	ACSSuT	1
420	DT104B low	−		4
427	DT104L	Human	ACSSuT	1
433	DT104L	Human	ACSSuT	10
436	DT104L	Pig	ACSSuT	1
443	DT104L	Chicken products	ACSSuT	1
448	DT104L	Human	ACSSuT	1
451	DT104L	Pig	ACSSuT	1
454	DT104L	Human	ACSSuT	1
455	DT104L	Human	ACSSuT	10
461	DT104B low	Human		4
462	DT104A	Human		2
2945	DT104L	Human	ACSSuT	1
3633	DT104L	Cocoa	ACSSuT	1
7945	DT104L	Pig	ACSSuT	1
Non-DT104				
254	DT194	−		7
255	OS	−		7
256	OS	−		7
257	OS	−		7
275 (ATCC 13311)	DT120	Human faeces		5
286 (ATCC 29946)	DT4 (LT2)‖	−		9
322	ARS	Meat		6
323	ARS	−		3
375	DT41	−		5
389	DT193	−		8
390	DT193	−		8
411	DT193	Pig		4
412	DT208	Pig	A	7
413	DT208	Pig		5
414	ARS	Pig		2
415	ARS	Pig		5
416	ARS	Pig		7
419	DT1	Chicken		11
435	ARS	Turkey		2
444	DT193	Human		2
445	ARS	Meat		12
452	DT193	Pig		4
ATCC 700720	DT4 (LT2)‖	−		9

*ARS, atypically reacting strain; OS, nontypable strain.

†Isolation source unknown.

‡Presence of the following SGI-1 antibiotic resistance genes (resistance to): A, *aadA2* (ampicillin); C, *floR* (chloramphenicol); S, *pse-1* (streptomycin); Su, *sul1* (sulfonamides); and T, *tet*(*G*) tetracycline. Presence determined as described by van Hoek *et al* (2005).

§Prophage profile, as identified in Table 4.

‖LT2 is typed as DT4 in the English phage-typing system and is the only attenuated laboratory strain listed in Table 1.

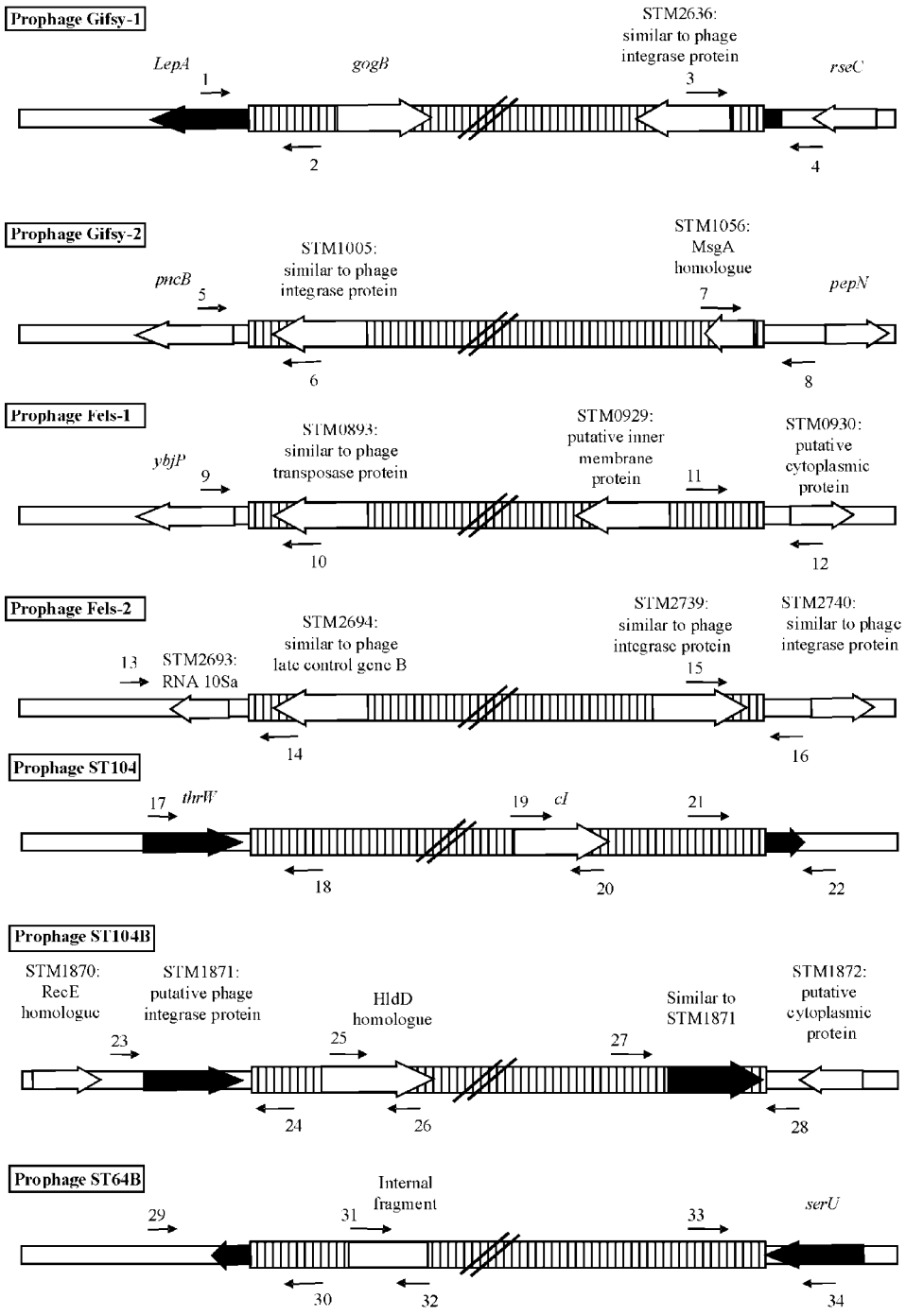

Fig. 1. Overview of the genome loci, characteristics and amplicons for the four *S.* Typhimurium LT2 prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2, and the three non-LT2 prophages ST104, ST104B and ST64B (not to scale), based on, or derived from, the GenBank sequences AF001386, AY462969–AY463002, NC_003197, NC_004313, NC_004513 and NC_005841. The prophages are depicted as larger vertically-hatched regions and the adjacent *S.* Typhimurium LT2 genome segments are depicted as white regions. Arrow boxes denote the direction of transcription of the genes indicated. For prophage Gifsy-1, the black arrow box represents the *lepA* gene, which is interrupted due to Gifsy-1 integration. The other black arrow boxes represent genes which are partly duplicated in the *S.* Typhimurium DT104 genome due to prophage integration. The position on the genome of the PCR primers developed is represented by small arrows and the numbers refer to the primer numbers listed in Table 2.

Table 2. Overview of the PCR primers used for prophage detection

Prophage detection	Primer name*	No.†	Sequence (5′→3′)	Amplicon size (bp)
Prophage present				
Gifsy-1	gifsy-1 LB-F	1	GCTAACGAACGGGAAACTTCATAG	506‡
	gifsy-1 LB-R	2	CTGCTGTTTAGTCAGGACTGACTG	
	gifsy-1 RB-F	3	GCCCGAGGCACGACTTTAGATAAC	359‡
	gifsy-1 RB-R	4	AACAGACGTTAAGCTCAGAACAGC	
Gifsy-2	gifsy-2 LB-F	5	GCAACGAGTGCAGAACAGGAGAAG	322‡
	gifsy-2 LB-R	6	AGAGAAGAGCGCAGAACAGGTTTC	
	gifsy-2 RB-F	7	GGTGGCTAAATGTAAATGACGTGG	488‡
	gifsy-2 RB-R	8	TGAGCGAGATCGAGATGAAGCTTG	
Fels-1	fels-1 LB-F	9	AAGCGCGGCGATATCATTGCTGTG	504‡
	fels-1 LB-R	10	CCAGTCCCGACATACCTATGCATG	
	fels-1 RB-F	11	GGAGGCTAATCGTGTTTGAGTTTG	407‡
	fels-1 RB-R	12	ACCACACCGCAATACTCCACGATG	
Fels-2	fels-2 LB-F	13	TAACCTGCTTAGAGCCCTCTCTCC	361‡
	fels-2 LB-R	14	CTGAACAGAACCCGCTTTAATGGC	
	fels-2 RB-F	15	AACGGCGGAAACATACTGGTACTG	318‡
	fels-2 RB-R	16	TGAATGAATGTTTGGTGGAGCTGG	
ST104	phageST104 LB-F	17	ATTCCGCTGCGGTTTATGTCAACG	484§
	phageST104 LB-R	18	CAAATCACCTGACTGAACATGCTC	
	phageST104 *cI*-F	19	TGGAACTGGCTGGTATGTCTCAAG	576§
	phageST104 *cI*-R	20	CTCTTTCAATTGGGTCCCAAGCTG	
	phageST104 RB-F	21	GTTCCCATGAATCCCACATACATC	810§
	phageST104 RB-R	22	ATTACGCGGGTAGGATCAGAGTAC	
ST104B	phageST104B LB-F	23	GACAGGAAATTACAACGGACGGTG	1299§
	phageST104B LB-R	24	ACTCATGCAATCAGGAGAGCTAAC	
	phageST104B hldD-F	25	ACAATGCTTTCGAACCTGATGGGC	510§
	phageST104B hldD-R	26	CCATCGCTTCAATTGCAACCATGC	
	phageST104B RB-F	27	AGCCGAATAAAGTGGGACTTGTGC	1425§
	phageST104B RB-R	28	CATCTATTCTTAAAGGGCAAGGCG	
ST64B	phageST64B LB-F	29	GCGTTTCCCTCACAGCAATTAATC	673§
	phageST64B LB-R	30	AAAAGCATGAGGGAAGGTTGTGGC	
	phageST64B int-F	31	CACAACGTAATGATGCTCGCTGGC	527§
	phageST64B int-R	32	GGACACTCCGCCAGTAGCTTATTG	
	phageST64B RB-F	33	CTCTTGACTGCACTTTCCACGATC	489§
	phageST64B RB-R	34	GGGTTATTTCTTGTGCTTTCCAGG	
Prophage absent				
Gifsy-1	gifsy-1 LB-F	1	GCTAACGAACGGGAAACTTCATAG	+/−500‖
	gifsy-1 RB-R	4	AACAGACGTTAAGCTCAGAACAGC	
Gifsy-2	gifsy-2 LB-F	5	GCAACGAGTGCAGAACAGGAGAAG	+/−500‖
	gifsy-2 RB-R	8	TGAGCGAGATCGAGATGAAGCTTG	
Fels-1	fels-1 LB-F	9	AAGCGCGGCGATATCATTGCTGTG	378§
	fels-1 RB-R	12	ACCACACCGCAATACTCCACGATG	
Fels-2	fels-2 LB-F	13	TAACCTGCTTAGAGCCCTCTCTCC	252§
	fels-2 RB-R	16	TGAATGAATGTTTGGTGGAGCTGG	
ST104	phageST104 LB-F	17	ATTCCGCTGCGGTTTATGTCAACG	675‡
	phageST104 RB-R	22	ATTACGCGGGTAGGATCAGAGTAC	
ST104B	phageST104B LB-F	23	GACAGGAAATTACAACGGACGGTG	1207‡
	phageST104B RB-R	28	CATCTATTCTTAAAGGGCAAGGCG	
ST64B	phageST64B LB-F	29	GCGTTTCCCTCACAGCAATTAATC	558‡
	phageST64B RB-R	34	GGGTTATTTCTTGTGCTTTCCAGG	

*F, forward; R, reverse; LB, left prophage border; RB, right prophage border. *cI*, hldD and int are internal prophage fragments.

†Numbers refer to primer numbers in Fig. 1.

‡Amplicon size in *S.* Typhimurium LT2 (NC_003197).

§Amplicon size in *S.* Typhimurium DT104 (NC_004513).

‖No amplicon size could be calculated when a Gifsy phage was absent; amplicon size was revealed to be ~500 bp.

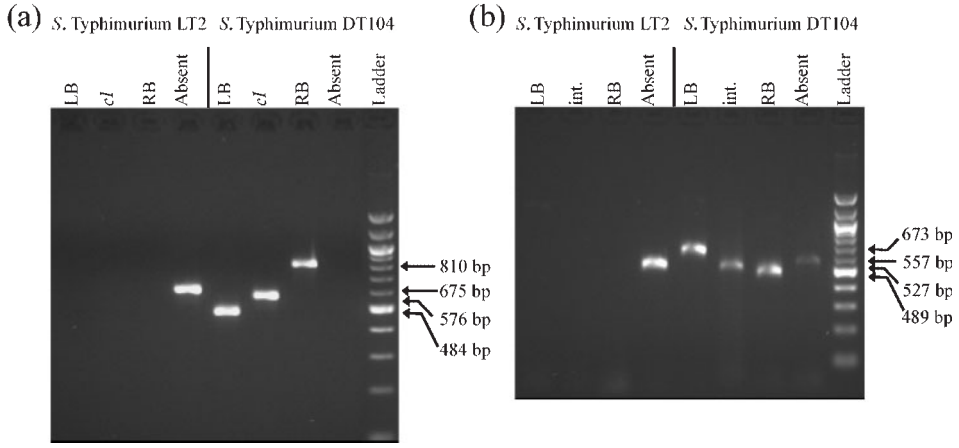

Fig. 2. Results of agarose gel electrophoresis of the PCR products of prophages ST104 (a) and ST64B (b) for *S.* Typhimurium LT2 ATCC 700720 and *S.* Typhimurium DT104L isolate 7945, where left prophage border (LB), right prophage border (RB), internal prophage fragment (*cI* or int.), and outer-left and right prophage border (prophage absent) PCR fragments are shown. A 100 bp ladder was used as a size marker and the PCR fragment lengths are depicted on the right.

Taq polymerase (all Invitrogen) in a total volume of 50 µl. After an initial denaturation at 95 °C for 3 min, the samples were subjected to 30 cycles of 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 45 s, followed by a final 7 min incubation at 72 °C. The elongation step of 60 °C was performed for 90 s for the phage ST104B border primer combination. Samples were fractionated by 2 % (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining. All PCRs were performed at least three times for each isolate. For several isolates, a weak prophage absent band was observed, although the prophages were detected as present (Fig. 2b, Table 3). Therefore, to exclude for these isolates any DNA contamination by other isolates of the PCRs performed, all PCR materials were tested for contamination, DNA was isolated from single colonies, and colony PCRs were performed directly from 50 single colonies. The PCR materials were all negative, and for the PCRs of the single colonies, again, a weak prophage absent band was observed, although the prophages were detected as present. A possible explanation for observing weak prophage absent bands while the prophages were detected as present, will be mentioned in the discussion.

RESULTS

S. Typhimurium LT2 and non-LT2 prophages

An overview of the genome loci and characteristics of the four *S.* Typhimurium LT2 prophages Gifsy-1, Gifsy-2, Fels-1, and Fels-2, and the three non-LT2 prophages ST104, ST104B and ST64B, is depicted in Fig. 1. The loci of the LT2 prophages were derived from GenBank accession no. NC_003197 (McClelland *et al.*, 2001). Bacteriophage Gifsy-1 was integrated into the GTP-binding elongation factor encoding the *lepA* gene of *S.* Typhimurium LT2 (GenBank accession no. AF001386), resulting in prophage Gifsy-1 as indicated, and prophage Gifsy-2 was located between the nicotinate phosphoribosyltransferase-encoding *pncB* and aminopeptidase N-encoding *pepN* genes. Prophage Fels-1 was located between the ORFs STM0892 (*ybjP*, putative lipoprotein) and STM0930 (putative cytoplasmic protein), and prophage Fels-2 between STM2693 (regulatory RNA 10Sa) and STM2740 (phage integrase protein).

A comparison of the *S.* Typhimurium LT2 (NC_003197) and DT104 (NC_004513) genome sequences, by using the previously identified DT104 sequences derived from bacteriophages ST104 (NC_005841) and ST64B (NC_004313), and subtractive hybridization results (AY462969–AY463002), resulted in the identification of the genome loci and characteristics of the non-LT2 prophages ST104, ST104B and ST64B. Prophage ST104 was found in *S.* Typhimurium DT104, when compared to the LT2 genome, adjacent to the threonine tRNA gene *thrW*. The integration of bacteriophage ST104 into the DT104 genome resulted in a duplication of the 3′ part of *thrW*. Prophage remnant ST104B, which contains an ORF homologous to HldD of *E. coli* O157 : H7 involved in LPS assembly, was found adjacent to ORF STM1871 (a putative phage integrase), which is part of the prophage remnant containing the *sopE2*, *pagO* and *pagK* genes. An identical copy of ORF STM1871 was identified on prophage remnant ST104B. Prophage ST64B was located adjacent to the serine tRNA gene *serU*. The genomic integration of the ST64B bacteriophage resulted in duplication of the 3′ part of the tRNA *serU*.

Prophage detection

The PCR primers designed for the detection of the seven prophages (as presented in Table 2 and indicated with arrows in Fig. 1) were initially tested by using genomic DNA isolated from *S.* Typhimurium LT2 strain ATCC 700720 and *S.* Typhimurium DT104L strain 7945. Primer sets were designed to detect the left and right borders for each prophage and to detect internal prophage fragments for the non-LT2 prophages. Furthermore, if the combination of the outer-left and right border resulted in a product, we concluded that the prophage was not inserted into the genome

Table 3. Results of PCR for the detection of the seven *S.* Typhimurium LT2 and non-LT2 prophages

+, Present; −, absent; LB, left prophage border; RB, right prophage border; Absent, prophage absent and for prophages ST104, ST104B and ST64B an internal prophage fragment (*cI*, HldD hom. or int.) present.

Amplicon		PCR result	
		Phage type DT104L, strain 7945	Phage type LT2, strain ATTC 700720
Prophage Gifsy-1	LB	+	+
	RB	+	+
	Absent	+*	+*
Prophage Gifsy-2	LB	+	+
	RB	+	+
	Absent	−	−
Prophage Fels-1	LB	−	+
	RB	−	+
	Absent	+	−
Prophage Fels-2	LB	−	+
	RB	−	+
	Absent	+	+*
Prophage ST104	LB	+	−
	cI	+	−
	RB	+	−
	Absent	−	+
Prophage ST104B	LB	+	−
	HldD hom.	+	−
	RB	+	−
	Absent	−	+
Prophage ST64B	LB	+	−
	int.	+	−
	RB	+	−
	Absent	+*	+

*Observed as a weak band on agarose gel (see text for explanation).

(prophage absent). For example, the results of agarose gel electrophoresis of the PCR products of prophages ST104 (Fig. 2a) and ST64B (Fig. 2b) for the LT2 and DT104L strains are shown, for which left prophage border (LB), right prophage border (RB), internal prophage fragment (*cI* or int.), and outer-left and right prophage border (prophage absent) PCRs were performed. Both prophages were absent from the LT2 strain and present in the DT104L strain, based on the finding that, for LT2, only the prophage absent band was visible on the gels, while, for DT104L, three dominant bands were obtained, corresponding to the LB, RB and internal prophage fragments, respectively. Notably, an additional weak prophage absent band was observed for prophage ST64B in this DT104L strain (Fig. 2b). The PCR results obtained for these two strains for the seven prophages are presented in Table 3. The Gifsy prophages were detected in both strains, the LT2 Fels prophages only in LT2, and the non-LT2 prophages (ST104, ST104B and ST64B) were detected only in the DT104 isolate. Finally, prophages Fels-2 Gifsy-1 and ST64B showed an additional weak prophage absent band, although the prophages were detected as present, which was found not to be caused by any contamination (see Methods).

Prophage distribution among different *S.* Typhimurium isolates

All prophage detection PCRs, as mentioned above, were performed for the *S.* Typhimurium isolates listed in Table 1. For each isolate, the PCR results were scored as prophage present (+), prophage absent (−) or prophage borders present and internal fragment absent (o), as depicted in Table 4. Prophage Gifsy-1 was present in all isolates, except for the two DT104L isolates 433 and 455, and isolates 419 and 445. The Gifsy-2 prophage was present in all isolates. Prophage Fels-1 was only found in four isolates: the two LT2 isolates ATTC 700720 and 286, and the two DT193 isolates 389 and 390. Prophage Fels-2 was found in the two LT2 isolates and nine other isolates of different phage types. Prophages ST104 and ST104B were present in all 14 phage

Table 4. Prophage distribution among different *S.* Typhimurium isolates

Prophage profile	Phage type*	Strain no.	Presence or absence of prophage† for:						
			Gifsy-1	Gifsy-2	Fels-1	Fels-2	ST104	ST104B	ST64B
1	DT104L (12)	410, 417, 418, 427, 436, 443, 448, 451, 454, 2945, 3633, 7945	+	+	−	−	+	+	+
	U302	406	+	+	−	−	+	+	+
2	ARS (2)	414, 435	+	+	−	−	o	−	+
	DT104A	462	+	+	−	−	o	−	+
	DT193	444	+	+	−	−	o	−	+
	DT208	413	+	+	−	−	o	−	+
3	ARS	323	+	+	−	−	o	−	o
4	DT193 (2)	411, 452	+	+	−	−	o	−	−
	DT104B low (3)	408, 420, 461	+	+	−	−	o	−	−
5	ARS	415	+	+	−	−	−	−	+
	DT41	375	+	+	−	−	−	−	+
	DT120	275	+	+	−	−	−	−	+
6	ARS	322	+	+	−	−	−	−	o
7	OS (3)	255, 256, 257	+	+	−	+	o	−	−
	DT194	254	+	+	−	+	o	−	−
	ARS	416	+	+	−	+	o	−	−
	DT208	412	+	+	−	+	o	−	−
8	DT193 (2)	389, 390	+	+	+	+	o	−	−
9	DT4 (=LT2) (2)	286, 700720	+	+	+	+	−	−	−
10	DT104L (2)	433, 455	−	+	−	−	+	+	+
11	DT1	419	−	+	−	−	−	−	+
12	ARS	445	−	+	−	+	o	−	−

*Number of isolates given in parentheses.

†Symbols: +, present; −, absent; o, borders present and internal fragment absent.

type DT104L isolates and the U302 isolate. All isolates of prophage profiles 1 and 10 that contained the ST104 and ST104B prophages, except the DT104L isolate 417 and the U302 isolate, were of penta-resistance type ACSSuT (depicted in Table 1). In addition, the prophage borders of ST104, without detecting the internal *cI* fragment, were also detected in 20 other isolates belonging to different phage types, including DT104A and DT104B low. Prophage ST64B was present in all 14 phage type DT104L isolates, the U302 isolate and nine other isolates. Also, for prophage ST64B, the borders were detected in two additional isolates (322 and 323), although the internal fragment was not detected.

For each isolate, the PCR results for the detection of the seven different prophages were converted into prophage profiles, as indicated in Table 4. In total, 12 different prophage profiles were identified. Based on the phage-typing numbers, several isolates of the same phage type resulted in the same profile, such as the two LT2 isolates resulting in profile 9, the three DT104B low isolates resulting in profile 4, and the 12 DT104L isolates, except for isolates 433 and 455, which lacked prophage Gifsy-1, resulting in profile 1. In contrast, analysis of the five DT193 isolates resulted in more, different profiles (2, 4 and 8), and the two DT208 isolates resulted in profiles 2 and 7.

DISCUSSION

By combining and analysing *S.* Typhimurium LT2 and DT104 sequences, we were able to identify prophages ST104 and ST64B and a prophage remnant ST104B in the genome of *S.* Typhimurium DT104. Both prophages, ST104 and ST64B, were found adjacent to tRNA genes, which have been commonly observed to be sites for the insertion of transferable elements (Cheetham & Katz, 1995; Reiter *et al.*, 1989). The HldD homologue, which has been described as a putative virulence factor candidate (Hermans *et al.*, 2005), was located on a prophage-like, horizontally transferable genetic element designated prophage ST104B. The presence of virulence factors on such elements has also been described for other *S.* Typhimurium isolates (Bakshi *et al.*, 2000; Figueroa-Bossi & Bossi, 1999; Figueroa-Bossi *et al.*, 2001; Ho *et al.*, 2002; Miao *et al.*, 1999; Mirold *et al.*, 1999; Stanley *et al.*, 2000). If prophages Gifsy-1, Fels-2 and ST64B were

detected in an isolate, shown by strong prophage present bands on the agarose gel, an additional weak prophage absent PCR band was observed. The latter observation may be explained by spontaneous prophage induction, leading to excision of the prophage from the genome, in a small fraction of the stationary-phase cells lacking the prophage in their genome (Bossi *et al.*, 2003; Figueroa-Bossi & Bossi, 1999; Weinbauer, 2004). In our study, DNA was isolated from overnight-grown cultures (stationary growth phase), similar to the overnight cultures used for conventional phage typing, and the PCR results may have been obtained from cells with and without the prophages in their genome. For the other prophages, no double PCR results were obtained, indicating that these prophages were not spontaneously induced or induced at lower, non-detectable levels (Bossi *et al.*, 2003).

The prophage borders of ST104 and ST64B were detected in many isolates, although for some of these isolates, the internal fragments of the prophages were not found. In these cases, the internal DNA fragment(s) may indeed be absent, resulting in a so-called prophage remnant, but alternatively, the internal sequence at this position may be different, which could indicate the presence of a different prophage, due to recombinations, rearrangements or deletions, which are common features of prophages (Casjens, 2005; Casjens *et al.*, 2005; Mmolawa *et al.*, 2003a, b; Pedulla *et al*, 2003; Yamamoto, 1969). Genetic diversity has previously been reported for prophage ST64B. This prophage has been found to be defective in different phage types of *S.* Typhimurium, and several strains appear to contain different sizes of internal prophage fragments (Tucker & Heuzenroeder, 2004). In *S.* Typhimurium DT104, active ST64B (Figueroa-Bossi & Bossi, 2004) and ST104 (Tanaka *et al.*, 2004) prophages have been reported. The induced ST104 prophage has been isolated only from DT104 isolates. In our study, the internal fragment of prophage ST104, representing *cI*, was detected only in the *S.* Typhimurium DT104L isolates and the U302 isolate. Most likely, the other isolates contained a remnant or recombination of ST104, because the internal fragment was not detected.

Our study revealed 12 different prophage profiles for the 23 non-DT104 and 19 DT104 *S.* Typhimurium isolates. When the isolates resulting in similar prophage profiles were gauged against their phage-type numbers, no clear relationships were found with specific phage types. In some cases, the prophage typing distinguished more groups than conventional phage typing (DT104L or DT193), while in other cases, different phage types had a similar prophage profile (profile 2 or 5). Notably, discrimination between DT104A, DT104B low and DT104L isolates could be made based on the presence of prophages ST104, ST104B and ST64B. In earlier studies, the Fels prophages could not be detected, whereas the Gifsy prophages could be detected in the genomes of non-LT2 *S.* Typhimurium isolates (Porwollik *et al.*, 2004; Reen *et al.*, 2005). In our study, more variation in the presence of these LT2 prophages was identified among different non-LT2 isolates. Prophage Gifsy-1 was absent in four isolates, and prophage Fels-1 was detected in two and Fels-2 in nine non-LT2 isolates. Although prophage Gifsy-1 was absent in four isolates, both Gifsy prophages can be seen as common *S.* Typhimurium prophages. The prophage remnant ST104B, which harboured the putative virulence factor HldD homologue, was detected mainly in phage type DT104L isolates with prophage profile 1 and penta-resistance type ACSSuT (depicted in Table 1). Notably, the antibiotic-resistance genes for penta-resistance were detected only in DT104 isolates. The HldD homologue may be involved in LPS assembly in *S.* Typhimurium DT104, resulting in modification of the LPS structure and/or architecture (Hermans *et al.*, 2005). This observation may offer an explanation for the phenotypic gel-based differences described elsewhere in LPS with various DT104 types (Lawson *et al.*, 2002).

This study indicates the potential for molecular typing of *S.* Typhimurium based on prophage sequences. Previously, this potential was also described by using an amplified fragment length polymorphism (Hu *et al.*, 2002), multilocus sequence typing (Ross & Heuzenroeder, 2005) or microarray-based approach (Pelludat *et al.*, 2005). It is well established that phage-type conversion can occur when lambdoid bacteriophages integrate into the genome of *S.* Typhimurium (Mmolawa *et al.*, 2002; Rabsch *et al.*, 2002; Tucker & Heuzenroeder, 2004). In addition, the detection of prophages can also give information about the virulence potency of an *S.* Typhimurium strain, since many virulence factors have been reported to be located on prophages (Bakshi *et al.*, 2000; Figueroa-Bossi & Bossi, 1999; Figueroa-Bossi *et al.*, 2001; Ho *et al.*, 2002; Miao *et al.*, 1999; Mirold *et al*, 1999; Stanley *et al.*, 2000), and can therefore be useful for risk assessment. The different isolates used in our study may contain as yet unidentified additional prophages, or recombinations of known prophages, called mosaics (Casjens, 2005; Casjens *et al.*, 2005; Mmolawa *et al.*, 2003a, b; Pedulla *et al.*, 2003; Yamamoto, 1969). Our study is believed to be the first to reveal a relationship between prophage presence and phage type that may be useful for the development of a molecular method to replace conventional phage typing. However, more genome sequences of other *S.* Typhimurium strains or bacteriophages, combining earlier work (Hu *et al.*, 2002; Pelludat *et al.*, 2005; Ross & Heuzenroeder, 2005) and screening of the genomic regions adjacent to tRNAs of new emerging salmonellae as hot spots for bacteriophage integration into the genome (Campbell, 2003; Cheetham & Katz, 1995; Hou, 1999; Reiter *et al.*, 1989), will help to gain more insight into the relationship between the presence of prophages and conventional phage typing, and their impact on virulence. Finally, our results suggest that the presence of the genes for penta-resistance type ACSSuT, the HldD homologue containing ST104 prophage remnant and phage type DT104L are most likely common features of the emerging subtype of *S.* Typhimurium DT104.

ACKNOWLEDGEMENTS

This study was supported by The Netherlands Organization for Health Research and Development, 'Nutrition: Health, Safety and Sustainability' programme. The authors would like to thank Ingrid Scholtens, Emmy van de Ven and Sükrü Yigit for technical assistance. We are grateful for the policy of the Sanger Centre to make sequences available before completion. The *S.* Typhimurium isolates were kindly provided by Wim Wannet, RIVM, and Dik Mevius, CIDC-Lelystad, The Netherlands. Phage typing was performed at the National *Salmonella* Reference Laboratory of the Federal Institute for Risk Assessment (BfR), Germany. For advice and help about phage typing, thanks are due to Andreas Schroeter and Burkhard Malorny from BfR.

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