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Identification of Novel *Salmonella enterica* Serovar Typhimurium DT104-Specific Prophage and Nonprophage Chromosomal Sequences among Serovar Typhimurium Isolates by Genomic Subtractive Hybridization

Armand P. H. M. Hermans,[1,2]* Tjakko Abee,[2] Marcel H. Zwietering,[2] and Henk J. M. Aarts[1]

RIKILT Institute of Food Safety, 6700 AE Wageningen,[1] and Laboratory of Food Microbiology, Agrotechnology and Food Sciences Group, Wageningen University, 6700 EV Wageningen,[2] The Netherlands

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Genomic subtractive hybridization was performed between *Salmonella enterica* serovar Typhimurium LT2 and DT104 to search for novel *Salmonella* serovar Typhimurium DT104-specific sequences. The subtraction resulted mainly in the isolation of DNA fragments with sequence similarity to phages. Two fragments identified were associated with possible virulence factors. One fragment was identical to *irsA* of *Salmonella* serovar Typhimurium ATCC 14028, which is suggested to be involved in macrophage survival. The other fragment was homologous to HldD, an *Escherichia coli* O157:H7 lipopolysaccharide assembly-related protein. Five selected DNA fragments—*irsA*, the HldD homologue, and three fragments with sequence similarity to prophages—were tested for their presence in 17 *Salmonella* serovar Typhimurium DT104 isolates and 27 non-DT104 isolates by PCR. All five selected DNA fragments were *Salmonella* serovar Typhimurium DT104 specific among the serovar Typhimurium isolates tested. These DNA fragments can be useful for better detection and typing of *Salmonella* serovar Typhimurium DT104.

During the past decades, *Salmonella enterica* subsp. *enterica* serovar Typhimurium infections have increased in many parts of the world. In particular, the multiple-antibiotic-resistant *Salmonella* serovar Typhimurium phage type DT104 has been identified as an emerging pathogen (9, 12, 24). For example, for human isolates in The Netherlands, the percentage of *Salmonella* serovar Typhimurium DT104 increased from 7% of total *Salmonella* serovar Typhimurium isolates in 1990 to 1995 to 29% in 1996 to 2001 (27). *Salmonella* serovar Typhimurium DT104 is multiply antibiotic resistant via a 43-kb *Salmonella* genomic island I (SGI-I), containing phage- and plasmid-related genes, and five antibiotic resistance genes to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (3, 5, 25).

Pathogens can acquire horizontally transferable genetic elements such as plasmids, genomic islands, and prophages, which often contain virulence factors. For example, the acquisition of virulence factors located on prophages can play an important role in the emergence of specific pathogens (4, 11). Various virulence factors located on transferable elements have been described for *Salmonella* serovar Typhimurium; for example, *Salmonella* serovar Typhimurium LT2 contains a *Salmonella* virulence plasmid, *Salmonella* pathogenicity islands, and Gifsy and Fels prophages (15). In addition, strain-specific virulence factors located on prophages have been described for several *Salmonella* serovar Typhimurium strains. Phage *Fels*-1 of *Salmonella* serovar Typhimurium LT2 carries *nanH* and *sodCIII*, phage Gifsy-3 of *Salmonella* serovar Typhimurium ATCC 14028 encodes *pagJ*, and phage SopEΦ of *Salmonella* serovar Typhimurium SL1344 contains *sopE* (8, 16).

Two prophages (PDT17 and ST104) have been identified in *Salmonella* serovar Typhimurium DT104 (22, 23), although no virulence association has been reported. In addition, a *Salmonella* serovar Typhimurium DT104-specific DNA fragment has been identified which is homologous to genes encoded by *Escherichia coli* O157:H7 prophages (14, 20).

The objective of the present work was to identify and characterize *Salmonella* serovar Typhimurium DT104-specific sequences, which may lead to the identification of novel virulence factors. Therefore, genomic subtractive hybridization (2, 6, 7, 17) was performed between *Salmonella* serovar Typhimurium LT2 and DT104.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1; they included isolates from the strain collections of RIKILT, the Dutch National Institute of Public Health and the Environment (RIVM), the Norwegian Institute of Public Health (isolates 911 and 327), and the American Type Culture Collection (ATCC). All isolates were stored at −80°C in brain heart broth (Merck, Darmstadt, Germany) plus 50% glycerol (Merck). The isolates were grown overnight in brain heart broth (Merck) at 37°C without shaking.

The Dutch phage-typing system for serovar Typhimurium was gauged in 1997 and 1998 against the English phage-typing system and showed no clear one-to-one relationship. The following relationships between the two phage-typing methods were applied to the phage types mentioned in this paper: the Dutch atypically reacting strains (ARS) correspond with ARS in the English system; the nontypeable strains (OS) correspond with OS; PT10 with DT3; PT296 with DT12; PT3 with DT41, DT1, and DT12; PT301 with DT52; PT350 with DT193; PT353 with DT194; PT401 with DT193, DT104, and DT120; PT506 with DT104; PT507 with DT208; and PT510 with DT208 (W. van Pelt, personal communication).

Subtractive hybridization library construction. First, genomic DNA was extracted from *Salmonella* serovar Typhimurium DT104 strain 7945 (tester) and

* Corresponding author. Mailing address: RIKILT Institute of Food Safety, P.O. Box 230, 6700 AE Wageningen, The Netherlands. Phone: (31) 317-475556. Fax: (31) 317-477717. E-mail: armand.hermans@wur.nl.

TABLE 1. *Salmonella* serovar Typhimurium isolates used in this study

Phage type[a]	Strain no.	Isolation source[b]
ARS	435	Turkey
	444	Human
DT104	7945	Pig
	ATCC BAA-188	Human
	so-2945	Human
	so-3633	Cacao
	stm 911	Human
	stm 327	Halva dessert
LT2 (DT4)	ATCC 700720	—
	286 (ATCC 29946)	—
ND[c]	275 (ATCC 13311)	Human feces
	375	—
	389	—
	390	—
OS	254	—
	255	—
	256	—
	257	—
PT3	322	Meat
	419	Chicken
PT10	323	—
PT296	413	Pig
	462	Human
PT301	416	Pig
PT350	412	Pig
	445	Meat
PT353	414	Pig
PT401	408	Pig
	411	Pig
	420	—
	461	Human
PT506[d]	406	Human
	410	Pig
	418	Dairy cow
	427	Human
	433	Human
	436	Pig
	443	Chicken products
	448	Human
	451	Pig
	454	Human
	455	Human
PT507	452	Pig
PT510	415	Pig

[a] PT, phage types according to the Dutch phage-typing system.
[b] —, isolation source unknown.
[c] ND, not determined.
[d] PT506 is typed as DT104 in the English phage-typing system.

strain LT2 (driver) by using a genomic DNA wizard kit (Promega, Madison, Wis.). Subtractive hybridization was carried out using the PCR-Select Bacterial Genome Subtraction kit (BD Clontech, Palo Alto, CA) as recommended by the manufacturer. In addition, glycogen (2 μg/μl; SEQ DTCS kit; Beckman Coulter, Princeton, NJ) was added during the precipitation step after the RsaI digestion to increase the precipitated DNA yield. The PCR products obtained at the end of the subtraction procedure were ligated into the pGEM-T Easy vector (Promega). The subtractive hybridization library was constructed by transforming the ligation mixture to XL2-Blue ultracompetent *E. coli* cells (Stratagene, La Jolla, CA) with ampicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG)–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) selection and screening on Luria-Bertani Lennox agar plates (Difco, Detroit, Mich.) as described by the supplier. Individual colonies (n = 192) were picked and grown overnight at 37°C in Luria-Bertani Lennox broth (Difco) with ampicillin (50 μg/ml) selection. Plasmid DNA was isolated using a miniprep plasmid isolation kit (QIAGEN, Valencia, CA).

DNA sequencing and analysis. DNA sequencing was performed on a capillary sequencer (Beckman Coulter) using the CEQ DTCS kit (Beckman Coulter) according to the supplier's instructions. The sequence reactions were initiated by using forward primer M13. The sequences obtained from the clones were analyzed using BLASTN and BLASTX through the databases mentioned in the next section. The BLASTN or BLASTX hit with the highest similarity was picked and, if possible, linked to functionality. The unique fragments obtained that showed no similarities to the already known *Salmonella* serovar Typhimurium DT104-specific SGI-I (GenBank accession no. AF261825) (3) were additionally sequenced twice in both directions (M13 forward and M13 reverse primer).

Nucleotide databases used. The following databases were used to analyze the sequences of the subtraction library: (i) GenBank at the National Center for Biotechnology Information (NCBI); (ii) the *Salmonella* genomes of the microbial-genome database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) at NCBI (the finished genomes of *Salmonella enterica* serovar Paratyphi A ATCC 9150, *Salmonella enterica* serovar Typhi CT18, *Salmonella* serovar Typhi Ty2, and *Salmonella* serovar Typhimurium LT2 and the unfinished genomes of *Salmonella enterica* serovar Dublin, *Salmonella enterica* serovar Enteritidis PT8 strain LK5, *Salmonella* serovar Typhimurium DT104, *Salmonella* serovar Typhimurium SL1344, *Salmonella* serovar Paratyphi B strain SPB7, and *Salmonella bongori* 12149); (iii) the DNA fragment databases of *Salmonella* serovar Enteritidis PT4, *Salmonella enterica* serovar Gallinarum 287/91, *Salmonella* serovar Typhimurium DT104, *Salmonella* serovar Typhimurium SL1344, and *S. bongori* 12419, the sequence data of which were produced by the *Salmonella* spp. Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella; and (iv) the DNA fragment databases of *Salmonella* serovar Paratyphi A ATCC 9150 and *Salmonella enterica* subsp. *diarizonae* serovar 61:1,v:1,5,(7) ATCC BAA-639, the sequence data of which were produced by the Genome Sequencing Center at the Washington University School of Medicine and can be obtained from http://genome.wustl.edu/blast/client.pl.

Detection of genomic DNA fragments by PCR. Primer sets were designed (Gene Runner, version 3.05) to detect five DNA fragments selected from the fragments obtained from the subtractive hybridization library (see Table 3): two fragments with nonprophage sequence homology (fragments 117 and 144) and three fragments homologous to prophage sequences found only in *Salmonella* serovar Typhimurium DT104 and not in other *Salmonella* genomes (fragments 84, 168, and 180). The two nonprophage fragments (fragments 117 and 144) were named *irsA* and HldD homologue according to their homology to *irsA* of *Salmonella* serovar Typhimurium ATCC 14028 and HldD of *E. coli* O157:H7, respectively. In addition, one control primer set was used to detect a DNA fragment (orf STM1056) in the Gifsy-2 prophage which should be present in all *Salmonella* strains. An overview of the primers used and the expected amplicon sizes is shown in Table 2.

The primers (Isogen, Maarssen, The Netherlands), at a 0.2 nM concentration, were combined with about 1 to 10 ng DNA template and amplified with *Taq* polymerase (Invitrogen, Carlsbad, CA). 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. Samples were fractionated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. All PCRs were performed four times for each *Salmonella* serovar Typhimurium isolate shown in Table 1.

Nucleotide sequence accession numbers. The nucleotide sequences of the 34 *Salmonella* serovar Typhimurium DT104 fragments that are listed in Table 3 have been submitted to GenBank in numerical fragment order with accession numbers AY462969 to AY463002, respectively.

RESULTS

DNA sequencing and analysis. The sequence reactions performed on 192 picked colonies of the subtractive hybridization library resulted in 126 different DNA fragments, of which 57 fragments were not found in the *Salmonella* serovar Typhimurium LT2 genome by using BLASTN analysis. After BLASTN and BLASTX analysis through GenBank, these 57 *Salmonella* serovar Typhimurium DT104 fragments were divided into four groups showing sequence similarity to either SGI-I (group A; n = 23), phage sequences (group B; n = 30), nonphage sequences (group C; n = 2), or nonsignificant sequences (group D; n = 2). Table 3 shows the BLAST search results for all fragments of groups B to D. The fragments of group A with sequence similarities to SGI-I were not further

TABLE 2. Overview of the PCR primers used for the PCR detection of genomic fragments

PCR fragment	Primer name	Sequence (5′–3′)[a]	Amplicon size (bp)
HldD homologue	hldDF	ACAATGCTTTCGAACCTGATGGGC	510[b]
	hldDR	CCATCGCTTCAATTGCAACCATGC	
irsA	irsAF	ATTCAGGCTGCGCTCCGTCTTTAC	416[b]
	irsAR	CTTAATGAGGCGGCGGAACAGTAC	
Fragment 84	frag-84F	ATGGCACAACCACTCAGTAATCCG	372[b]
	frag-84R	AGATGGCATCGGCGTAGTTATGAG	
Fragment 168	frag-168F	CATCCATTACCGGGTTGTCCATCC	492[b]
	frag-168R	CGCGTTTAACGGTACAGATGGTGG	
Fragment 180	frag-180F	CCATAATAGGAAGCATTGCGTGAG	476[b]
	frag-180R	ATGCCATCTAGCTATCTCTGCGAC	
Gifsy-2 control	gifsy-2 RB-F	GGTGGCTAAATGTAAATGACGTGG	488[c]
	gifsy-2 RB-R	TGAGCGAGATCGAGATGAAGCTTG	

[a] All primers were designed and used first in this study.
[b] Amplicon size based on *Salmonella* serovar Typhimurium DT104.
[c] Amplicon size based on *Salmonella* serovar Typhimurium LT2.

analyzed, because this *Salmonella* serovar Typhimurium DT104 island had already been sequenced (GenBank accession no. AF261825) (3). The fragments of group B were additionally divided into four phage subgroups showing sequence similarity to either (i) the *Salmonella* serovar Typhimurium DT64 bacteriophage ST64B, (ii) the *Salmonella* serovar Typhimurium DT104 bacteriophage ST104, (iii) the *Escherichia coli* (STEC) bacteriophage P27, or (iv) other prophages present in the genome of *Salmonella* serovar Typhi CT18, *Salmonella* serovar Typhimurium, *E. coli* K-12, *E. coli* O157:H7, or *Shigella flexneri* 2a.

Although fragments 22, 66, 75, and 84 were not similar to prophage sequences, these fragments were placed in the group of phages (see Table 3) because the adjacent genome regions of the matching BLAST hits were similar to prophage sequences (data not shown). Fragment 117, fragment 158, and all fragments of phage subgroups i and ii matched to sequences of *Salmonella* serovar Typhimurium origin. In addition, fragment 158 was the only fragment matching to a sequence of phage type DT104 origin that was not located on SGI-I or prophage ST104. All other fragments had not been associated with *Salmonella* serovar Typhimurium before. Notably, the fragments with DNA sequence similarities (phage subgroup i to iii fragments) could be clustered into a subgroup of similar origin, such as bacteriophage ST104, while the fragments with amino acid sequence similarity (phage subgroup iv and groups C and D) could not be clustered into subgroups of similar origin.

The presence of the 34 *Salmonella* serovar Typhimurium DT104 fragments of groups B to D was found, using BLASTN analysis, to be different in available finished and unfinished *Salmonella* genomes (Table 3). Ten fragments were found only in the *Salmonella* serovar Typhimurium DT104 genome, while the other fragments were randomly found in the other *Salmonella* genomes. Among all fragments, three fragments (62, 66, and 144) were found in a strain of *S. bongori*.

Identification of possible virulence factor candidates. Based on sequence homology, three *Salmonella* serovar Typhimurium DT104 DNA fragments obtained could be associated with possible virulence factors: fragments 66, 117, and 144.

Fragment 66 was similar (91%) to a *Salmonella* serovar Typhi CT18 gene (orf STY1362). This *Salmonella* serovar Typhi CT18 gene is described as being homologous to a putative toxin subunit 1 gene of *Bordetella pertussis* based on the amino acid sequence. However, this gene represents a pseudogene due to at least one frameshift (19). Therefore, it is unlikely that fragment 66 encodes a virulence factor. In addition, four genes encoding the other subunits necessary to form the active *B. pertussis* toxin (18) were not found in the *Salmonella* serovar Typhimurium DT104 genome (data not shown).

Fragment 117 was highly similar (99%) to a part of the *irsA* gene of *Salmonella* serovar Typhimurium ATCC 14028. The *irsA* locus in *Salmonella* serovar Typhimurium ATCC 14028 is described as being involved in macrophage survival (1). Finally, fragment 144 was homologous (75%) to the lipopolysaccharide (LPS) assembly-related protein HldD (formerly named WaaD) of *E. coli* O157:H7, based on the amino acid sequence.

Detection of genomic DNA fragments by PCR. The presence of five selected DNA fragments—fragment 117 (*irsA*), fragment 144 (HldD homologue), and three fragments homologous to prophage sequences (fragments 84, 168, and 180)—and a Gifsy-2 prophage control fragment was tested among 44 *Salmonella* serovar Typhimurium isolates by PCR (Table 4). The five selected fragments appeared to be present in all 17 *Salmonella* serovar Typhimurium DT104 isolates and absent in all 27 non-DT104 phage type isolates. In addition, the Gifsy-2 prophage control fragment was indeed present in all *Salmonella* serovar Typhimurium DT104 and non-DT104 isolates.

DISCUSSION

The objective of the present work was to identify and characterize *Salmonella* serovar Typhimurium DT104-specific sequences, which may lead to the identification of novel virulence factors. Therefore, genomic subtractive hybridization was performed between *Salmonella* serovar Typhimurium LT2 and DT104, which resulted in novel DNA fragments not found in *Salmonella* serovar Typhimurium DT104 before. Notably, a large number of fragments were homologous to prophage sequences.

Based on sequence homology, three *Salmonella* serovar Typhimurium DT104 DNA fragments identified were associated with possible virulence factors: fragments 66, 117, and 144. Fragment 66 was homologous to the putative toxin subunit 1 gene of *B. pertussis* found in *Salmonella* serovar Typhi CT18.

TABLE 3. BLAST search results for the *Salmonella* serovar Typhimurium DT104 fragments generated by genomic subtraction between the *Salmonella* serovar Typhimurium DT104 and LT2 strains excluding the fragments similar to SGI-I

Group	Fragment no.	Sequence similarity to:	Accession no.	Similarity region[a]	% Similarity[b]	Present in[c]:
(B) Phage						
(i) ST64B	4	Serovar Typhimurium bacteriophage ST64B genome: putative DNA methyltransferase	AY055382.1	32607–32895	93	Serovar Typhimurium SL1344
	5	Serovar Typhimurium bacteriophage ST64B genome: putative transcriptional activator	AY055382.1	31896–32507	98	Serovar Typhimurium SL1344
	59	Serovar Typhimurium bacteriophage ST64B genome: hypothetical protein Sb40	AY055382.1	29290–29704	100	Serovar Dublin, serovar Enteritidis PT4, serovar Typhimurium SL1344
	95	Serovar Typhimurium bacteriophage ST64B genome: putative tail proteins Sb22 and Sb23	AY055382.1	16126–16645	97	Serovar Dublin, serovar Enteritidis PT4, serovar Typhimurium SL1344
	149	Serovar Typhimurium bacteriophage ST64B genome: putative DNA methyltransferase Sb44	AY055382.1	32897–33312	100	Serovar Typhimurium SL1344
	155	Serovar Typhimurium bacteriophage ST64B genome: hypothetical protein Sb46	AY055382.1	33908–34104	98	Serovar Enteritidis PT4, serovar Typhimurium SL1344
	170	Serovar Typhimurium bacteriophage ST64B genome: integrase protein Sb28	AY055382.1	21702–22241	99	Serovar Dublin, serovar Enteritidis PT4, serovar Typhimurium SL1344
	178	Serovar Typhimurium bacteriophage ST64B genome: putative tail protein Sb22	AY055382.1	15708–16007	97	Serovar Dublin, serovar Enteritidis PT4, serovar Typhimurium SL 1344
(ii) ST104	2	Serovar Typhimurium bacteriophage ST104 genome: tailspike protein	AB102868.1	40450–40738	100	Serovar Dublin, serovar Paratyphi A, serovar Paratyphi B
	17	Serovar Typhimurium bacteriophage ST104 genome: NinZ	AB102868.1	19809–20268	99	*S. enterica* subsp. *diarizonae*, serovar Dublin
	36	Serovar Typhimurium bacteriophage ST104 genome: ORF15 (endopeptidase) and ORF19 (lysozyme)	AB102868.1	21707–22377	99	*S. enterica* subsp. *diarizonae*, serovar Paratyphi A, serovar Paratyphi B
	50	Serovar Typhimurium bacteriophage ST104: tail spike protein	AB102868.1	41078–41391	100	Serovar Dublin, serovar Paratyphi A, serovar Paratyphi B
	54	Serovar Typhimurium bacteriophage ST104 genome: Mnt and tail spike protein	AB102868.1	38879–39670	100	Serovar Dublin, serovar Paratyphi A, serovar Paratyphi B
	78	Serovar Typhimurium bacteriophage ST104 genome: ORF46, ORF47, and ORF48	AB102868.1	23183–24148	100	*S. enterica* subsp. *diarizonae*, serovar Dublin, serovar Paratyphi A, serovar Paratyphi B
	98	Serovar Typhimurium bacteriophage ST104 genome: ORF19	AB102868.1	9548–9791	100	
	101	Serovar Typhimurium bacteriophage ST104 genome: ORF23 (antitermination protein Q homologue)	AB102868.1	20269–21264	99	*S. enterica* subsp. *diarizonae*, serovar Paratyphi A, serovar Paratyphi B
	110	Serovar Typhimurium bacteriophage ST104 genome: NinG	AB102868.1	18853–19808	99	*S. enterica* subsp. *diarizonae*
	122	Serovar Typhimurium bacteriophage ST104 genome: SieB	AB102868.1	10671–10897	99	*S. enterica* subsp. *diarizonae*, serovar Paratyphi A, serovar Paratyphi B
(iii) P27	8	Serovar Typhi CT18 genome segment 6: putative holin of bacteriophage P27	AL627270.1	15663–15915	94	Serovar Paratyphi A, serovar Paratyphi B, serovar Typhi Ty2, serovar Typhi CT18, serovar Typhimurium SL1344
	62	Serovar Typhi CT18 genome segment 4: putative holin of bacteriophage P27	AL627268.1	266762–267028	97	*S. bongori*, serovar Enteritidis PT4, serovar Typhi Ty2, serovar Typhi CT18
	66	Serovar Typhi CT18 genome segment 6: putative toxin subunit 1 of *Bordetella pertussis*	AL627270.1	14526–15084	91	*S. bongori*, serovar Paratyphi A, serovar Paratyphi B, serovar Typhi Ty2, serovar Typhi CT18
(iv) Others	16	Putative prophage terminase large subunit of a prophage in serovar Typhi	CAD05440.1	aa 291–453	82 (I), 89 (P)	
	22	Unnamed protein product in *Photorhabdus luminescens* subsp. *laumondii* TTO1	CAE15767.1	aa 1–132	63 (I), 81 (P)	
	56	Unknown protein of prophage CP-933U in *E. coli* O157:H7	AAG57029.1	aa 23–99	61 (I), 79 (P)	

	75	Unnamed protein product in *Photorhabdus luminescens* subsp. *laumondii* TTO1	CAE15775.1	aa 142–233	65 (I), 75 (P)	
	84[d]	Unnamed protein product in *Photorhabdus luminescens* subsp. *laumondii* TTO1	CAE15762.1	aa 1–113	59 (I), 78 (P)	
	100	Exodeoxyribonuclease of *Gifsy* prophages in serovar Typhimurium LT2	AAL19943.1	aa 58–355	46 (I), 58 (P)	Serovar Gallinarum, serovar Paratyphi A, serovar Paratyphi B, serovar Typhimurium SL1344
	158[e]	DNA fragment of serovar Typhimurium DT104: hypothetical protein YdaU of Rac prophage in *E. coli* K-12	AF275268.1	229–516	100	Serovar Dublin, serovar Gallinarum
	168[d]	*S. flexneri* 2a genome: putative bacteriophage protein	AE005674.1	2693454–2693818	93	
	180[d]	Hypothetical protein YmfD of prophage e14 in *E. coli* K-12	F64858	aa 102–216	69 (I), 82 (P)	
(C) Nonphage	117[d]	*irsA* gene in serovar Typhimurium ATCC 14028	AY328029.1	1627–1948	99	Serovar Dublin, serovar Enteritidis PT4, serovar Enteritidis PT8, serovar Gallinarum
	144[d]	LPS biosynthesis enzyme HldD (WaaD) of *E. coli* O157:H7	AAC69662.1	aa 29–111	75 (I), 87 (P)	*S. bongori*
(D) Not significant	47	Hypothetical protein in *Caenorhabditis elegans*	CAA88607.1	aa 64–216	23 (I), 41 (P)	
	64	Hypothetical protein in *Anaeromyxobacter dehalogenans* 2CP-C	EAL78506.1	aa 38–155	34 (I), 55 (P)	

[a] Region of similarity in base pairs or amino acids (aa) based on accession number.
[b] Similarities are based on nucleotide or amino acid sequences. When identities (I) and positives (P) are given, values for positives (similarity based on similar amino acid properties) of ≤55% are not significant.
[c] Present or partly present, based on >80% similarity by BLASTN analysis, in the genomes listed. All fragments are present in *Salmonella* serovar Typhimurium DT104.
[d] DNA fragment used for PCR detection of genomic fragments.
[e] DNA fragment described in the literature as *Salmonella* serovar Typhimurium DT104 specific (20).

TABLE 4. PCR results for the detection of six genomic fragments in different *Salmonella* serovar Typhimurium isolates

Phage type[a] (no. of isolates)	*irsA*	Presence or absence[b] of:				
		HldD homologue	Fragment 84	Fragment 168	Fragment 180	Gifsy-2 control
DT104						
DT104 (6)	+	+	+	+	+	+
PT506 (11)[c]	+	+	+	+	+	+
Non-DT104						
ARS (2)	−	−	−	−	−	+
LT2 (2)	−	−	−	−	−	+
ND (4)	−	−	−	−	−	+
OS (4)	−	−	−	−	−	+
PT3 (2)	−	−	−	−	−	+
PT10	−	−	−	−	−	+
PT296 (2)	−	−	−	−	−	+
PT301	−	−	−	−	−	+
PT350 (2)	−	−	−	−	−	+
PT353	−	−	−	−	−	+
PT401 (4)	−	−	−	−	−	+
PT507	−	−	−	−	−	+
PT510	−	−	−	−	−	+

[a] PT, phage types according to the Dutch phage-typing system. ND, not determined.
[b] +, PCR product present; −, PCR product absent.
[c] PT506 is typed as phage type DT104 in the English phage-typing system.

As mentioned earlier, it is unlikely that fragment 66 encodes a virulence factor because of its similarity to a pseudogene and the lack of other genes in the *Salmonella* serovar Typhimurium DT104 genome necessary to form the *B. pertussis* toxin.

Fragment 117 was highly similar to a part of the *irsA* gene of *Salmonella* serovar Typhimurium ATCC 14028. The *irsA* locus in *Salmonella* serovar Typhimurium ATCC 14028 is described as being involved in macrophage survival (1). In contrast, the *irsA* amino acid sequence is 91% homologous to a CP933R prophage protein of *E. coli* O157:H7 with unknown function (GenBank accession no. AAG56427.1) and 73% homologous to Gifsy prophage proteins (GenBank accession no. AAL19954.1 and AAL21514.1). Due to unknown functionality and homology to common prophage sequences, the virulence association of *irsA* remains to be elucidated.

Finally, the possible virulence factor association of fragment 144, which resulted in homology to HldD of *E. coli* O157:H7, is further analyzed. Recent insight into *E. coli* O157:H7 LPS assembly showed that HldD, in addition to HldE (formerly named WaaE or RfaE), is involved in the nucleotide-activated *glycero-manno*-heptose biosynthesis for inner core oligosaccharide assembly (13, 26). The HldD homologue found in *Salmonella* serovar Typhimurium DT104 may also be involved in the *glycero-manno*-heptose biosynthesis pathway. Notably, all known *Salmonella* serovar Typhimurium LT2 *waa* genes were also found in *Salmonella* serovar Typhimurium DT104 by using BLASTX analysis (data not shown). Therefore, the HldD homologue will most likely be an additional protein in *Salmonella* serovar Typhimurium DT104. The HldD homologue, as an additional protein for inner core oligosaccharide assembly, may lead to a different inner core structure of the LPS. A different inner core structure can result in a more stable outer membrane or in altered host recognition, leading to an altered immune response (reviewed in reference 21), resulting in increased survival and/or virulence. However, more research is

needed to assess this role of the HldD homologue in *Salmonella* serovar Typhimurium DT104 virulence.

The five DNA fragments selected from the subtractive hybridization library, fragment 117 (*irsA*), fragment 144 (HldD homologue), and three fragments homologous to prophage sequences (fragments 84, 168, and 180), were *Salmonella* serovar Typhimurium DT104 specific among the tested serovar Typhimurium isolates (Table 4). Notably, in our PCR results, the *irsA* fragment appeared to be *Salmonella* serovar Typhimurium DT104 specific; however, this fragment is also present in the non-DT104 strain *Salmonella* serovar Typhimurium ATCC 14028 (1). Additional BLAST searches revealed that the upstream DNA regions of *irsA* in *Salmonella* serovar Typhimurium ATCC 14028 and DT104 differ (data not shown). Therefore, the tested *irsA* fragment is not *Salmonella* serovar Typhimurium DT104 specific, but the genome locus may be DT104 specific.

Many DNA fragments obtained in our study were grouped into larger genome fragments, such as SGI-I and the ST64B and ST104 prophages (Table 3). In this and earlier subtractive hybridization studies, almost all differences between closely-related strains were found to be located on large transferable elements such as prophages, plasmids, or fimbrial operons (6, 7, 17). In addition, our PCR results revealed *Salmonella* serovar Typhimurium DT104-specific prophage DNA fragments (Table 4), similar to a previously described *Salmonella* serovar Typhimurium DT104-specific DNA fragment that encodes *E. coli* O157:H7 prophage homologues (14, 20). These findings lead us to the assumption that several DNA fragments obtained from our subtractive hybridization are probably located on a novel *Salmonella* serovar Typhimurium DT104-specific prophage. Matching the fragments obtained to the *Salmonella* serovar Typhimurium DT104 unfinished genome revealed that all fragments of prophage subgroup iv and groups C and D (see Table 3), including *irsA* and the HldD homologue, are clustered (data not shown). This specific prophage may have contributed to the successful clonal expansion of *Salmonella* serovar Typhimurium DT104, as with *Salmonella* serovar Typhimurium DT49 and DT204, which contain phage SopEΦ and emerged in the 1970s and 1980s (8, 10, 16).

In summary, genomic subtraction is a useful tool for finding strain-specific genes, including possible virulence factor candidates. In addition, the PCR method developed revealed that the *irsA* and HldD homologue fragments and the three prophage fragments 84, 168, and 180 were *Salmonella* serovar Typhimurium DT104 specific among the tested serovar Typhimurium isolates and can be useful for better detection and typing of *Salmonella* serovar Typhimurium DT104.

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