Analysis of the contribution of bacteriophage ST64B to in vitro virulence traits of *Salmonella enterica* serovar Typhimurium

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Comparison of the publicly available genomes of the virulent *Salmonella enterica* serovar Typhimurium (S. Typhimurium) strains SL1344, 14028s and D23580 to that of the virulence-attenuated isolate LT2 revealed the absence of a full sequence of bacteriophage ST64B in the latter. Four selected ST64B regions of unknown function (sb7–sb11, sb46, sb49–sb50 and sb54) were mapped by PCR in two strain collections: (i) 310 isolates of S. Typhimurium from human blood or stool samples, and from food, animal and environmental reservoirs; and (ii) 90 isolates belonging to other serovars. The region sb49–sb50 was found to be unique to S. Typhimurium and was strongly associated with strains isolated from blood samples (100 and 28.4 % of the blood and non-blood isolates, respectively). The region was cloned into LT2 and knocked out in SL1344, and these strains were compared to wild-type isogenic strains in in vitro assays used to predict virulence association. No difference in invasion of the Int407 human cell line was observed between the wild-type and mutated strains, but the isolate carrying the whole ST64B prophage was found to have a slightly better survival in blood. The study showed a high prevalence and a strong association between the prophage ST64B and isolates of S. Typhimurium collected from blood, and may indicate that such strains constitute a selected subpopulation within this serovar. Further studies are indicated to determine whether the slight increase in blood survival observed in the strain carrying ST64B genes is of paramount importance for systemic infections.

INTRODUCTION

*S. enterica* serovar Typhimurium (S. Typhimurium) is a bacterial pathogen with a broad host range that causes infections ranging from gastroenteritis to systemic disease (Parry et al., 2002; Haraga et al., 2008). After oral ingestion, the bacterium preferentially associates with M cells in the Peyer’s patches of the distal ileum (Jepson & Clark, 2001). It crosses the intestinal epithelium and, through interactions with epithelial and immune cells, it induces inflammation in the intestine leading to diarrhoea (Haraga et al., 2008). In a fraction of cases, the bacterium is transported inside phagocytic cells from the mesenteric lymph nodes to the liver and the spleen (Haraga et al., 2008), where it resides primarily within macrophages and causes substantial inflammation (Richter-Dahlfors et al., 1997; Salcedo et al., 2001). The invasion of eukaryotic cells and replication within phagocytes is mainly mediated by type III secretion systems encoded from chromosomal pathogenicity islands (Schmidt & Hensel, 2004) through the injection of so-called effector proteins into the host cells (Galán & Wolf-Watz, 2006). In addition to genes found in the *Salmonella* chromosomal pathogenicity islands, a great number of virulence-associated genes are carried on mobile elements, such as prophages (Bossi et al., 2003), which are increasingly considered important for both evolution of the bacteria and their contribution to virulence (Brüssow et al., 2004).

Several full genomes of virulent *S. Typhimurium* strains have been published, including those of SL1344 [National Center for Biotechnology Information (NCBI) direct submission, 2010, G. Dougan, P. Barrow, M. Achtman, J. Parkhill & N. R. Thomson – genome sequence of *S. enterica* subsp. *enterica*}
serovar Typhimurium SL1344] and 14028s (Jarvik et al., 2010), both highly virulent and well-characterized isolates that are frequently used as model strains in studies of pathogenicity. The two genomes encode different effector proteins. Thus, S. Typhimurium SL1344 carries the gene sopE (encoding SopE), present in the sopEF prophage, which is absent in S. Typhimurium 14028s. In contrast, S. Typhimurium 14028s harbours the gene ssph1 (encoding SspH1), located within the Gifsy-3 prophage, which is absent in S. Typhimurium SL1344 (Figuerola-Bossi & Bossi, 2004). S. Typhimurium LT2 (annotated reference genome of genus Salmonella) (McClelland et al., 2001) is a highly virulence-attenuated strain in both in vitro and in vivo assays (Swords et al., 1997; Wilmes-Riesenber, et al., 1997). The attenuation is mainly due to suboptimal translation of the RpoS (ΔrpoS) sigma factor (Wilmes-Riesenber, et al., 1997). In a previous work, the S. Typhimurium LT2 and S. Typhimurium SL1344 genomes were compared with the aim of identifying novel virulence effectors (Brown et al., 2011). Nine unique regions, encoding 117 ORFs, were identified in the S. Typhimurium SL1344 chromosome. One identified gene corresponded to ORF sb26 encoded within the prophage ST64B (Mmolawa et al., 2003). This ORF represents the third member of the SskK/NleB family of effectors and was renamed sseK3 (Brown et al., 2011).

Recently, another S. Typhimurium genome (strain D23580) has been sequenced, annotated and deposited (Kingsley et al., 2009). This isolate originated from a human blood sample collected in Malawi and was assigned to the sequence type 313 (ST313). This new multidrug-resistant pathogenic clade has emerged in sub-Saharan Africa causing epidemics in different African countries, and it may have adapted to cause invasive disease in humans (Kingsley et al., 2009; Feasey et al., 2012).

Data mining of available genomes is a promising approach for the identification of putative candidate genes for development of vaccines and novel drug targets, and for identification of genes associated with virulence (Pizza et al., 2000; Andreatta et al., 2010; Katara et al., 2011; Vejborg et al., 2011). Genomic information in itself, however, is not sufficient. Confirmation of the importance of the genes and, if possible, their precise function, is essential. The aims of the present work were as follows. (a) The first aim was to compare the three sequenced and annotated S. Typhimurium genomes of the virulent strains SL1344, 14028s and D23580 to that of the attenuated strain LT2 in an attempt to identify new determinants putatively involved in virulence. We identified a region corresponding to the ST64B prophage that was rudimentary in LT2. ST64B was first described in S. Typhimurium DTF64 and shows a mosaic structure (Mmolawa et al., 2003). (b) The second aim was to examine the presence of selected genes harboured by the prophage in two large Salmonella strain collections of global origin. (c) The third aim was to assess the role of the prophage ST64B in the invasion of human epithelial cell lines and human bloodstream survival of S. Typhimurium.

METHODS

Ethical Statement. The study was performed in accordance with principles for good scientific and ethical practice and approved by the director of the National Food Institute, Technical University of Denmark (DTU-Food). All experiments were conducted following the principles of the Declaration of Helsinki. All volunteer blood donors provided written informed consent.

Bioinformatic analyses. Four S. Typhimurium sequenced genomes, SL1344 (FQ312003), 14028s (CP001363), D23580 (FN424405) and LT2 (AE006468), were compared. The sequences were analysed using BLAST alignment (Altschul et al., 1990), phylogeny tools available at http://www.ncbi.nlm.nih.gov/BLAST atlas and BLAST matrix software (Jacobsen et al., 2011). Information available at NCBI and the Uniprot databases (http://www.uniprot.org) was used for the prediction of the ST64B ORFs and determination of the putative role of the encoded proteins, respectively (Fig. 1b).

Bacterial strains and growth conditions. A list of the isolates used in the present work is given in Table 1. The strains were grown in LB broth (Oxoid) at 37 °C with vigorous shaking or on LB agar plates. For the experiments under anaerobic conditions the strains were incubated in plastic jars using the AnaeroGen (Oxoid) atmosphere generation system.

Determination of the prevalence of the identified regions in two global strain collections of Salmonella. In all the isolates, the presence of the prophage ST64B-associated regions, sb5–sb11, sb46, sb49–50 and sb54, was assessed by PCR as previously described (Hasman et al., 2005). Initially, a screening was carried out in a collection of 310 S. Typhimurium strains (99 isolates from human blood samples and 211 isolates from other sources such as stools and food) (Table 1). Secondly, a collection of 90 Salmonella isolates of serovars other than Typhimurium (Table 1) was tested for the presence of the regions. Specific primers (TAG Copenhagen) were designed for each of the regions (Table 2). S. Typhimurium LT2 and the mouse-virulent S. Typhimurium SL1344 strains were used as negative and positive controls, respectively, for all the genes tested.

Construction of plasmids, cloning and mutagenesis. PCR amplicons corresponding to the regions sb45, sb50 and sb49–sb50 obtained from S. Typhimurium SL1344 strain were independently cloned into the HindIII–XbaI site of the vector pUK-1921 (Herrero et al., 2008), giving rise to the plasmids pAHF1, pAHF2 and pAHF3, respectively. The presence of the regions was confirmed by PCR and restriction endonuclease assays. LT2 competent cells were prepared and electroporated using standard conditions (O’Callaghan & Charbit, 1990) with the recombinant vectors mentioned above. Selection of transformants was performed on LB agar plates containing kanamycin (50 μg ml⁻¹). The resulting strains were named LT2 + sb45, LT2 + sb50 and LT2 + sb49+sb50. Mutagenesis of S. Typhimurium SL1344 for the regions sb45, sb50 and sb49–sb50 was performed using the one-step inactivation protocol (Datiko & Wanner, 2000) leading to the isogenic strains ASL1344sb49, ASL1344sb50 and ASL1344sb49–sb50. The mutations were verified by PCR. The primers used for the cloning and mutagenesis of sb45, sb50 and sb49–sb50 are listed in Table 2.

Bacterial infection assays. Invasion assays were performed in triplicate using published protocols for gentamicin protection assays (Watson et al., 1995). Briefly, Int407 human epithelial cell lines were seeded in 12-well plates to reach a density of 5 × 10⁵ cells per well at the time of infection. Bacteria were grown overnight for 18 h without shaking, followed by subculturing 1/100 (~10⁶ cfu ml⁻¹) into fresh LB and were then regrown to exponential phase (OD₆00 0.4 ± 0.01). Bacteria were added to INT+407 cells at a m.o.i. of 100:1 (bacteria to eukaryotic cell) and incubated for another hour. Following this,
monolayers were washed twice with PBS and incubated for 2 h in Eagle’s minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (Gibco) containing gentamicin (100 μg ml⁻¹).

Finally, the culture cells were washed twice with PBS and then lysed in a PBS solution containing Triton X-100 (0.1% w/v, Sigma). Serial dilutions of the lysates were spread on LB agar plates to determine the

Fig. 1. (a) BLAST atlas of the genomes of the S. Typhimurium strains LT2, 14028s, D23580 and SL1344. The latter represents the reference genome. The location of prophage ST64B is indicated with a purple arrowhead. (b) Map of the ST64B prophage (Mmolawa et al., 2003; modified, shown with a scale in kb). The prophage harbours a total of 56 ORFs. Of these, 48 are present in the genomes of the strains SL1344, 14028s and D23580, and absent in the attenuated isolate LT2. Of the 48 genes shared by the virulent strains, 29 encode prophage-related or other known functions, while for the other 19 the roles of the encoded proteins have not been determined. The latter are indicated in bold. The regions under study in the present work are indicated with an asterisk. Sequencing data have been extracted from the sequenced genome of the prophage ST64B (accession number AY055382).
Table 1. *Salmonella* isolates used in this work and the presence of ST64B-associated genes

All the strains were collected between the years 2000 and 2011. Isolates were serotyped in Denmark or Thailand as previously described (Bhatta et al., 2007; Fashae et al., 2010; Vandenberg et al., 2010). The strain collection is conserved at the National Food Institute, Technical University of Denmark (DTU-Food). *n*, Number of isolates.

<table>
<thead>
<tr>
<th>Salmonella serovar (n)</th>
<th>Origin /specimen (n)*</th>
<th>Country of origin (n)†</th>
<th>sb7–sb11 (n)</th>
<th>sb46 (n)</th>
<th>sb49–sb50 (n)</th>
<th>sb54 (n)</th>
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<tr>
<td>Typhimurium (310)</td>
<td>Blood (99)</td>
<td>Congo (87), Mauritius (6), Nigeria (6)</td>
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<td>99</td>
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<td>99</td>
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<td></td>
<td>Stools (118)</td>
<td>Belarus (35), Mauritius (26), Nigeria (1), China (56)</td>
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<td>101</td>
<td>32</td>
<td>114</td>
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<td></td>
<td>Animal (73)</td>
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<td>Food (6)</td>
<td>Mauritius (5), Thailand (1)</td>
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<td>Others (14)</td>
<td>Nepal (9), Thailand (4), Congo (1)</td>
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<td>3</td>
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<td>4</td>
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<td>1</td>
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<td>6</td>
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<td>2</td>
<td>6</td>
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<td>3</td>
</tr>
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</table>

*The animal strains were collected from poultry (*n*=50), pig (*n*=22) and shrimp (*n*=1).

†A total of 29 out of the 84 isolates from the Congo have been previously described (Vandenberg et al., 2010). A total of 16 strains from Mauritius (1 from blood, 2 from food and 13 from stools) were associated with the same outbreak (Issack et al., 2009). The isolates from Nepal (all of them isolated from water), Belarus, China and Nigeria also have been published elsewhere (Bhatta et al., 2007; Tapalski et al., 2007; Xia et al., 2009; Fashae et al., 2010).
number of invaded bacteria as c.f.u. ml\(^{-1}\). In parallel, bacterial enumerations of the inoculum were determined to ensure equal starting numbers. The strain S. Typhimurium 4/74 invH201::TphopA (Watson et al., 1995) was used as a negative control for the infection assays.

**Growth assays in LB, human plasma and blood.** The S. Typhimurium SL1344 wild-type and the mutant strain Δ[ST64B] (isogenic strain cured for prophage ST64B) (Alonso et al., 2005) were tested in triplicate for growth in human blood and plasma. Four healthy volunteers donated blood, which was used immediately after collection in order to avoid blood cell lysis. Plasma was extracted from the fresh blood after storage at room temperature for 45 min. The blood was then centrifuged for 15 min at 2500–3000 r.p.m (Eppendorf 5810R, F-34-6-38 rotor). The supernatant corresponding to the plasma fraction was recovered in a sterilized tube. Fresh human blood, plasma and LB (used as control to monitor the experiments) were independently inoculated with ~10\(^{9}\) c.f.u. ml\(^{-1}\) of each isolate (overnight cultures) and further incubated at 37 °C with shaking for 48 h. Growth of the strains was monitored by determining the number of c.f.u. at different times. Growth curves were obtained and growth was compared between both strains in each of the media. In addition, blood that had been incubated for 8 h at 37 °C was inoculated with both strains and the growth was analysed as mentioned above.

In order to assess the role of complement in the growth of the isolates, plasma was heat treated for 35 min at 56 °C in order to cause complement degradation, cooled down and inoculated with both strains. Growth was studied for 8 h as described before.

**Growth studies under direct competition in LB and human blood and iron-uptake assays.** The S. Typhimurium SL1344 wild-type and the isogenic strain Δ[ST64B] were co-cultured in LB, fresh blood and fresh blood supplemented with 10 μM FeCl\(_3\). The isolate Δ[ST64B] was resistant to kanamycin allowing the differentiation between the strains. Competitions were performed over a period of 48 h. Initially, the bacteria were inoculated at a final concentration of ~10\(^{9}\) c.f.u. ml\(^{-1}\) (ratio 1 : 1) and incubated at 37 °C with shaking for 24 h. After the first incubation, the cultures were diluted 100-fold and reincubated for a further 24 h. The subculturing routine was repeated up to two times every 24 h (T0, T24 and T48). Samples were diluted and plated on LB agar plates with and without a supplement of kanamycin (50 μg ml\(^{-1}\)) followed by the number of c.f.u. being counted. The experiment was performed in triplicate.

**Gene expression studies.** The expression of the genes sb46, sb49, sb50 and sb54, and the entire region sb7–sb11 (absent in LT2), was analysed. The S. Typhimurium SL1344 wild-type was cultured in LB and fresh blood under aerobic and anaerobic conditions, respectively. When performing aerobic conditions, the LB and blood samples were taken at the time points corresponding to the late exponential phase and stationary phase in both media. When using anaerobic conditions, the LB and blood samples were tested after 12 h and 24 h post-inoculation, respectively. For the LB samples, total RNA was isolated using an RNeasy mini kit (Qiagen). For the blood erythrocyte lysis (allowing further bacterial RNA extraction), blood samples were washed with two volumes of 0.7 % (w/v) NaCl and two volumes of cold sterilized MilliQ water, followed by centrifuging the samples for 15 min at 6000 r.p.m. The wash step was repeated using only two volumes of 0.7 % (w/v) NaCl. Finally, bacterial RNA was extracted from the recovered pellets by using the kit mentioned above. Eluted RNA samples were treated with DNase I (Promega) to remove any traces of DNA. The quality of the total RNA was examined by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm. RNA samples were co-converted to cDNAs by reverse transcriptase-PCR (RT-PCR) by using SuperScript II transcriptase (Invitrogen). cDNAs were used directly as templates for

### Table 2. Primers designed and used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>ST64B region</th>
<th>Sequence*</th>
<th>Amplicon size (bp)</th>
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<td>Precursors for the screening in <em>Salmonella</em> collections and RT-PCR</td>
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<tr>
<td>Sb7–11-F</td>
<td>sb7–sb11</td>
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<tr>
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</table>

*The restriction endonuclease sites engineered into the primer sequences are underlined: AAGCTT, HindIII; TCTAGA, XbaI. Letters in italics indicate primer sequences that annealed with the aph resistance cassette from pKD4 (Datsenko & Wanner, 2000). The expected amplicon for this cassette is 1015 bp.*
Comparison of the genomes of S. Typhimurium SL1344, 14028s, D23580 and LT2, and presence of prophage ST64B genes in the chromosomes of other serovars of Salmonella

When comparing the S. Typhimurium genomes of the strains SL1344, 14028s, D23580 and LT2, a region with significant similarity to the ST64B prophage was found to be rudimentary in LT2. Results from the genomic comparison with the BLAST atlas software are shown in Fig. 1(a). A total of 48 out of the 56 ORFs (similarity above 80 %) of the prophage ST64B (Fig. 1b) were exclusively found in the SL1344, 14028s and D23580 chromosomes. Of these, 19 encoded proteins of unknown function (not associated with phage functions: capsid, tail, morphogenesis, lysis etc.), as determined by using the Uniprot and NCBI databases. The BLASTN results of all 19 investigated genes showed that one of them, sb46, was interrupted by fragments of the virulence gene ssph2, commonly found in the chromosome of Salmonella (Miao & Miller, 1999; Saitoh et al., 2005). sb49, sb50 and sb55 were only found in the S. Typhimurium genomes. However, the remaining 16 ORFs encoding unknown functions showed similarity above 50 % to genes present in chromosomes of other S. enterica serovars, including highly invasive serovars such as S. Typhi, S. Choleraesuis and S. Dublin. The ‘in silico’ studies also revealed that the prophage ST64B was integrated within the t-RNA-encoding gene serU of SL1344, 14028s and D23580, as it was described for S. Typhimurium strain DT64 (Mmolawa et al., 2003), and adjacent to the umuCD operon. Additional information about the exact position of the selected ST64B genes within the Salmonella chromosomes used for the comparison analysis is shown in Table 3.

Distribution of four ST64B regions encoding unknown functions among strains of S. Typhimurium and other Salmonella serovars

The presence of four selected prophage ST64B regions (sb7–sb11, sb46, sb49–sb50 and sb54), including nine genes detected only in the sequenced chromosomes of S. Typhimurium strains SL1344, 14028s and D23580, was tested by PCR in a collection of 310 S. Typhimurium strains (Table 1). All the 99 isolates from blood together with the positive control, S. Typhimurium SL1344, showed the bands expected for all four prophage ST64B regions analysed (Table 1). In contrast, the genes were less frequently found in the 211 strains collected from other sources. Thus, the region sb7–sb11 was detected in 108 (51.2 %) strains, 180 (85.3 %) carried the gene sb46, 60 (28.4 %) harboured the region sb49–sb50 and 167 (79.1 %) harbour the sb54 gene. Fifteen of these non-blood isolates, containing the four regions tested, were associated with an outbreak in Mauritius caused by consumption of marlin mousse (Issack et al., 2009). An additional strain associated with this outbreak was collected from blood, and consequently considered as a blood isolate. A high variability in the distribution of the prophage ST64B genes was observed among the animal strains for the regions sb7–sb11, sb46 and sb54. It is noteworthy that sb49–sb50 was absent in all 22 pig isolates, while 23 (46 %) of the poultry strains carried the region.

In addition, the screening for the presence of the four prophage ST64B regions was performed in a collection of 90 strains of different serovars, and a high variability was detected (Table 1). The four S. Choleraesuis strains tested carried sb46 only. The five S. Dublin strains and the single S. Typhi included in the study shared the genotype: sb7–sb11/sb46/sb54. None of the isolates carried the region sb49–sb50, which therefore appeared only to be carried by strains of S. Typhimurium. The region sb7–sb11 was only present in 8 strains (8.9 %) of four serovars: S. Dublin (n = 5), S. Typhi (n = 1), S. Schwarzengrund (n = 1 out of 3) and S. Paratyphii B var. Java (n = 1 out of 2). Only 10 strains (11.1 %) of five serovars, S. Dublin (n = 5), S. Typhi (n = 1), S. Saintpaul (n = 2 out of 3), S. Brandenburg (n = 1 out of 2) and S. Albanys (n = 1), were positive for sb54. Of note, the gene sb46 [22 strains (24.4 %) of 15 serovars] was the most frequently distributed among the different serovars. Finally, 52 isolates (57.7 %) assigned to 29 different serovars, including the 7 S. Virchow strains, did not carry any of the regions.

sb49 and sb50 are not involved in invasion of a cell culture model of human epithelium

The role of the genes sb49 and sb50, as well as the combined region sb49–sb50, dominant among the S. Typhimurium blood isolates in invasion of the Int407 human epithelial cell line was investigated. The infections were performed with the mutant strains LT2 + sb49, LT2 + sb50, LT2 + sb49−sb50, ΔSL1344/sb49, ΔSL1344/sb50, ΔSL1344/sb49–sb50 and ΔST64B, as well as the wild-type isolates LT2 and SL1344. S. Typhimurium 4/74 and the isogenic strain S. Typhimurium invH201::TnphoA (Watson et al., 1995) were used as controls in the experiments. The latter has a mutation in the SPI-1 invH gene, reducing its invasion capability (Watson et al., 1995). Results showed that neither the genes sb49 and sb50 nor the entire ST64B region were involved in invasion.
of the human epithelium (Table 4). Thus, the cloning of either of the genes sb49 or sb50, or the whole region sb49–sb50, into the LT2 strain did not increase the rate of invasion significantly ($P > 0.05$). Similarly, comparable levels of invasion were observed for the SL1344 wild-type strain and the isogenic mutants $\Delta$SL1344sb49, $\Delta$SL1344sb50, $\Delta$SL1344sb49–sb50 and $\Delta$[ST64B] ($P > 0.05$), indicating that the loci were not important for cell invasion.

### Comparison of growth between the S. Typhimurium isogenic strains SL1344 and $\Delta$[ST64B] in plasma and human blood reveals a slightly better bloodstream survival of $\Delta$[ST64B]

To determine whether the prophage ST64B favours *Salmonella* growth in blood and plasma, assays with the

![Table 3](http://jmm.sgmjournals.org)

<table>
<thead>
<tr>
<th>ST64B genes</th>
<th>14028s (start–end)</th>
<th>D23580 (start–end)</th>
<th>SL1344 (start–end)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sb7</td>
<td>2108909–2109106</td>
<td>2081013–2081210</td>
<td>2055073–2055270</td>
<td>197</td>
</tr>
<tr>
<td>sb8</td>
<td>2108583–2108903</td>
<td>2080687–2081007</td>
<td>2054747–2055067</td>
<td>320</td>
</tr>
<tr>
<td>sb9</td>
<td>2108182–2108583</td>
<td>2080286–2080687</td>
<td>2054346–2054747</td>
<td>401</td>
</tr>
<tr>
<td>sb10</td>
<td>2107698–2108207</td>
<td>2079802–2080311</td>
<td>2053862–2054371</td>
<td>509</td>
</tr>
<tr>
<td>sb11</td>
<td>2107141–2107698</td>
<td>2079245–2079802</td>
<td>2053305–2053862</td>
<td>557</td>
</tr>
<tr>
<td>sb46</td>
<td>2120348–2121205</td>
<td>2092452–2093309</td>
<td>2066512–2067369</td>
<td>857</td>
</tr>
<tr>
<td>sb49</td>
<td>2118717–2119337</td>
<td>2090821–2091441</td>
<td>2064881–2065509</td>
<td>620</td>
</tr>
<tr>
<td>sb50</td>
<td>2118327–2118683</td>
<td>2090431–2090787</td>
<td>2064991–2064847</td>
<td>356</td>
</tr>
<tr>
<td>sb54</td>
<td>2115726–2116142</td>
<td>2087830–2088246</td>
<td>2061890–2062306</td>
<td>416</td>
</tr>
</tbody>
</table>

strains SL1344 and $\Delta$[ST64B] were performed by comparing their growths in LB, human plasma and human blood (Fig. 2). Both isolates showed the same growth behaviour in LB, reaching stationary phase at 6–8 h post-inoculation (Fig. 2a). Similarly, when growing in plasma, they followed the same trend (Fig. 2b). In this case, a decrease in the c.f.u. (almost 1 log$_{10}$) during the first 2–3 h was observed. This decrease is lower than the decrease observed in human serum for the *Salmonella* strains LT2 and D23580, which exhibited a reduction of 2–3 log$_{10}$ at the end of 3 h (Goh & MacLennan, 2013). The same detected decrease in c.f.u. was observed when the isolates were grown in heat-treated serum, suggesting that this reduction was not associated with a heat-labile factor, such as complement proteins (not shown). After this time period, the exponential phase started, and stationary phase was reached at 10–12 h post-inoculation (Fig. 2b).

The growth pattern was evaluated in fresh human blood from three individuals. Since the response was partly sample-dependent, all three results are shown (Fig. 2c, d, e). For the first 8–12 h a drastic reduction was observed for both the wild-type and the mutant strains, with no difference between them. From 8–12 h post-inoculation, the $\Delta$[ST64B] strain showed a slightly reduced growth potential in all samples; however, the difference with the wild-type strain was not statistically significant ($P > 0.05$). In two out of three samples the mutant showed a slightly longer exponential phase after the reduction period, and in one out of the three samples it reached a lower maximum c.f.u. (Fig. 2c, d, e). When growth experiments were performed with blood that had been incubated for 8 h at 37$\,^\circ$C, corresponding to the killing phase indicated above, no killing was observed and again the mutant showed a growth defect (Fig. 2f).

### Growth of the S. Typhimurium isogenic strains SL1344 and $\Delta$[ST64B] under direct competition in human blood and effect of the addition of iron

Since the SL1344 and $\Delta$[ST64B] isogenic strains showed constant but insignificant differences during growth in blood, we repeated the growth experiments in an assay
where growth in whole human blood was performed in direct competition. This is a more sensitive method for fitness evaluation, since sample to sample variation is eliminated. The growth of both isolates was followed over a period of 48 h and any difference between them was observed (data not shown). For the first 8–12 h the c.f.u. counts of both strains dropped below the initial inoculum (from $\sim10^7$ to $\sim10^5$ c.f.u. ml$^{-1}$) as expected in blood. The

**Fig. 2.** Comparison of the growth of the isogenic strains SL1344 (●) and Δ[ST64B] (■) in different media. (a, b) Strains grown in LB (a) and human plasma (b). The number of viable salmonellae was determined by serial dilution on LB agar every 2 h from 0 to 24 h. Data are the means ± SD of three experiments. (c, d, e) Strains grown in human blood from three individuals. c.f.u. counts were determined after 0, 12, 14, 18, 22, 24 and 48 h. Time points were selected on the basis of preliminary results (data not shown). Data from three experiments are independently illustrated and therefore error bars are not shown. (f) Strains grown in human blood previously incubated at 37 °C for 8 h. c.f.u. counts were determined after 0, 12, 14, 18, 24 and 48 h. One single experiment is shown. Statistical significances of differences were calculated with a paired t-test.
behaviour was the same in every dilution step, and at the end of the 48 h growth period both isolates had reached a population of $\sim 10^9$ c.f.u. ml$^{-1}$.

Iron represents one of the limiting nutrients in blood, and when bacteria reach the bloodstream during septicaemia the carriage of genes involved in iron uptake might provide an additional survival advantage. To investigate whether the prophage ST64B could be involved in iron uptake, both strains were grown under direct competition in blood supplemented with 10 $\mu$M FeCl$_3$ for 48 h. No significant difference ($P>0.05$) was detected between the growth of the isolates (data not shown). The growth pattern was very similar to that observed for both strains when growing under direct competition in blood not supplemented with FeCl$_3$. Therefore, the addition of iron, apparently, did not affect their growth in human blood.

The selected prophage ST64B genes are not expressed during growth in human blood

Expression of the genes sb46, sb49, sb50 and sb54, and the entire region sb7–sb11, was investigated during growth in LB (control) and human blood under aerobic and anaerobic conditions. It was not possible to detect expression of any of the genes tested regardless of the conditions or the moment when the sample was taken. The included control was positive.

DISCUSSION

In the current study we have identified a region of $\sim 40$ kb, corresponding to the prophage ST64B in the genomes of virulent strains of S. Typhimurium, which is rudimentary in the chromosome of the attenuated isolate LT2. The prophage ST64B has been speculated to be involved in horizontal gene transfer, although its precise role in the evolution and epidemiology of Salmonella remains unknown (Mmolawa et al., 2003). The presence of the entire prophage was found by PCR to be restricted to S. Typhimurium as described for other phages identified in this serovar (Figueroa-Bossi et al., 2001; Brüssow et al., 2004). This observation is supported by a previous study where comparative genomics revealed that ST64B was absent in serovars other than S. Typhimurium and species other than Salmonella (Kröger et al., 2012).

The prophage ST64B (annotated as SLP203) has been previously reported in the chromosomes of five S. Typhimurium strains (S. Typhimurium SL1344, 14028s, D23580, UK-1 and 4/74) (Kröger et al., 2012). In the current study ST64B was shown to be widespread in strains of this serovar, and also to be strongly associated with isolates originating from blood. Among the latter ($n=99$), at least 25 strains were assigned to the multilocus sequence typing ST313 (Leekitcharoenphon et al., 2013), and all of them carried the entire prophage. S. Typhimurium ST313 is strongly associated with invasive disease in Africa (Kingsley et al., 2009).

The region sb49–sb50 was present in all of the blood isolates of S. Typhimurium and only in 28.4 % of the non-blood isolates included in this study. The presence of sb49–sb50 was also tested in 14 blood isolates of S. Typhimurium collected in developed countries (11 from Spain, 2 from Argentina and 1 from Taiwan) (data not shown). All of the Spanish isolates carried the region, while in the rest of the three isolates sb49–sb50 was not identified. It is of note that this region was only found in S. Typhimurium, indicating a potential serovar Typhimurium specificity. The gene sb46, which showed similarity to the Salmonella virulence gene spsH2, was the most frequently distributed among the S. Typhimurium isolates (100 % of the blood and 85.3 % of the non-blood-isolates), and this gene was also identified in Salmonella strains of other serovars (24.4 %). In support of an association between the presence of ST64B and an invasive phenotype, it is of note that this gene was present in strains of the serovars S. Dublin, S. Choleraesuis and S. Typhi, which are considered to be highly invasive in humans (Bolton et al., 1999; Deng et al., 2003; Sirichote et al., 2010).

The strong association of the prophage ST64B, or parts of its DNA regions, with blood isolates of S. Typhimurium and other invasive serovars led us to hypothesize that the prophage might be involved in virulence, either at the stage of invasion or during the stage of bacteremia. We first considered a role in the initial interaction with the intestinal epithelium, i.e. the presence of the prophage could render strains hyperinvasive; however, isogenic strains with or without the region sb49–sb50 or the entire prophage ST64B did not differ in their ability to invade human epithelial cells. This is in line with a published study, where it was shown that the prophage was not required for virulence in a murine model (Alonso et al., 2005).

Bacteremia, characterized by the presence of pathogenic bacteria in the bloodstream, is a major cause of morbidity and mortality worldwide (Mylotte & Tayara, 2000). Since the prophage ST64B had been found to be associated with isolates from blood, we examined the relevance of ST64B for growth in human blood. Inactivation of ST64B caused a slight, although statistically insignificant, reduction in the ability to grow in blood following an initial phase of identical fast killing. Since the results were only indicative and non-significant, we investigated the phenomenon by performing more sensitive growth competition assays between wild-type and mutated strains. Again, no significant difference between the isolates was observed and addition of iron to the blood medium during the competition assays did not affect the growth performance either (data not shown). Thus, a potential role of ST64B in iron uptake, as well as growth ability, in human blood could be ruled out.

From a theoretical point of view, the observed marked presence of the prophage in blood isolates may have resulted from an accidental co-selection with factors that confer a selective advantage in systemic infections, i.e. the
prophage might happen to be co-localized with hitherto undiscovered genes responsible for an enhanced ability to survive in blood. However, no obvious gene candidates that could be involved in bacteraemia were found in the regions adjacent to the prophage in the investigated strains (data not shown). An increased ability to confer systemic disease can also be caused by other factors, such as complement resistance and increased intracellular survival and multiplication in phagocytic cells. In the current study we ruled out complement resistance as a factor, since growth in heat-inactivated and normal plasma was identical; however, further studies are needed to investigate the interaction with phagocytic cells.

Competition assays may lead to incorrect conclusions in the case where the wild-type strain can compensate for the defect of the mutant. Relysogenization of ST64B might take place, resulting in the loss of both the kanamycin-resistance cassette used for selection and the prophage-associated phenotype (Figueroa-Bossi & Bossi, 2004). It has previously been observed that co-culturing of a ST64B-carrying strain with an isogenic isolate lacking the prophage leads to the formation of active forms of the virus due to the reversion of the frameshift mutation in the gene sb21 that prevents the tail assembly. Furthermore, newly lysogenized strains released active ST64B particles spontaneously and at high frequencies (Figueroa-Bossi & Bossi, 2004). Currently we cannot demonstrate that ST64B relysogenization has not occurred during the competition assays.

In support of our observation that ST64B does not enhance growth ability in blood, we found that none of the prophage genes that we investigated were expressed during growth of S. Typhimurium SL1344 in blood under aerobic and anaerobic conditions. The contribution of the selected genes to the survival of Salmonella in blood cannot be totally discarded based on this, since the genes might be expressed before the bacteria reach the bloodstream. However, putting all results together, they strongly argue against a possible function of ST64B in growth in blood.

In the present work, we observed that S. Typhimurium suffers a drastic population decrease during the first 8–12 h of growth in blood, possibly because nutrients required for survival (iron, oxygen etc.) are scarce during this time period (Simmonds & Harkness, 1981; Aguiló et al., 2000). After this period, growth of the strain was initiated. The drastic decrease in growth was not caused by a heat-sensitive factor in the blood, since the exact same growth pattern was observed when we first incubated the blood at 37 °C for 8 h and then inoculated with our strains. The action of complement was not responsible for the decrease either, since the same reduction was observed when plasma was heat treated and the complement, therefore, inactivated. A previous study has reported that human sera from three individuals killed the isolates LT2 and D23580 by 2–3 log10 at 180 min (Goh & MacLennan, 2013). In contrast, the SL1344 strain used in this work was only reduced by 1–2 log10 in 180 min by plasma, suggesting that this strain is not quite as sensitive to the bactericidal activity of serum as the strains mentioned above.

Conclusions

Comparative genomics and PCR results showed an association of the prophage ST64B with blood isolates of S. Typhimurium; however, studies with cell cultures and growth assays in human blood did not indicate a role of the whole prophage, nor the selected regions studied, in virulence.

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