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Optical genetic mapping defines regions of chromosomal variation in serovars of *S. enterica* subsp. *enterica* of concern for human and animal health

M. P. SAUNDERS[1,2], G. WU[1], M. ABUOUN[1], Z. PAN[1,2], M. ANJUM[1] AND M. J. WOODWARD[1]*

[1] *Department of Food and Environmental Safety* (*FES*), *Veterinary Laboratories Agency* (*Weybridge*), *Addlestone, Surrey, UK*
[2] *School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK*

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SUMMARY

Infections involving *Salmonella enterica* subsp. *enterica* serovars have serious animal and human health implications; causing gastroenteritis in humans and clinical symptoms, such as diarrhoea and abortion, in livestock. In this study an optical genetic mapping technique was used to screen 20 field isolate strains from four serovars implicated in disease outbreaks. The technique was able to distinguish between the serovars and the available sequenced strains and group them in agreement with similar data from microarrays and PFGE. The optical maps revealed variation in genome maps associated with antimicrobial resistance and prophage content in *S.* Typhimurium, and separated the *S.* Newport strains into two clear geographical lineages defined by the presence of prophage sequences. The technique was also able to detect novel insertions that may have had effects on the central metabolism of some strains. Overall optical mapping allowed a greater level of differentiation of genomic content and spatial information than more traditional typing methods.

Key words: Animal pathogens, *Salmonella*, *Salmonella enterica*, *Salmonella* Typhimurium, veterinary pathogens.

INTRODUCTION

Salmonella is an important zoonotic pathogen linked with serious animal and human disease. In animals, *Salmonella* infections can be responsible for chronic diarrhoea with associated weight loss and poor production, as well as abortion and in severe cases death. It is one of the most common causes of foodborne infections in man [1] and non-typhoidal *Salmonella* accounts for an estimated 1·4 million infections annually in the USA with 15 000 hospitalizations and 400 deaths in 2001 [2, 3]. There were also 155 540 confirmed cases of human salmonellosis reported in the European Union in 2007 [4]. *Salmonella enterica* subsp. *enterica*, which contains over 2500 serovars [5], is responsible for the majority of infections.

Epidemic serovar strains have emerged in different host species, regions and at different times. During the 1980s and 1990s *S.* Typhimurium DT104, associated with a multidrug resistance (MDR), emerged in cattle. During the same period, *S.* Enteritidis phage type (PT)4 and related types emerged in poultry. In the UK *S.* Enteritidis and *S.* Typhimurium accounted for over 66% of human isolations [6] and are the prevalent causes of human inflammatory gastroenteritis [7]. MDR *S.* Newport has emerged as a problem serovar in

* Author for correspondence: Dr M. J. Woodward, Department of Food and Environmental Safety (FES), Veterinary Laboratories Agency (Weybridge), Addlestone, Surrey, KT15 3NB, UK. (Email: m.j.woodward@vla.defra.gsi.gov.uk)

cattle and humans in the USA [8], whereas in the UK this serovar does not express resistance to antibiotics and is associated with tenfold less human infections [4, 9]. *S.* Dublin is the most common serotype associated with abortion in cattle, and has been consistently the most prevalent serovar in cattle in the UK [10] but is not associated with severe human infections.

During *Salmonella* outbreaks it is important to undertake epidemiological tracing, for which a number of molecular techniques such as random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), and multi-locus sequence typing (MLST) are available (for review see [11]). Of these PFGE is the most commonly used, sorting strains by the size and banding patterns of the total genomic content after digestion of genomic DNA with infrequent cutting restriction endonucleases. PFGE can indicate acquisition or loss, but not location, of large genomic fragments by fragment size analyses [12].

A more research-oriented molecular method is comparative genomic hybridization (CGH) using microarrays. This has become a powerful tool for the interrogation of differences between closely related bacterial species. *Salmonella* has been a target for CGH microarray [13, 14] with comparisons made both between subspecies and within them. These studies have led to the identification of the core and variable components of *S. enterica* subspecies I [15]. However, the outputs of the array data are limited by the genes printed on the array such that novel genes cannot be detected nor can genomic rearrangements be identified [15].

A limitation of all currently used methods for typing, including CGH array approaches, is the inability to identify accurately major alterations to the genome such as deletions, insertions, inversions or other rearrangements that may be intimately associated with the emergence of a particular bacterial strain. While next-generation whole-genome sequencing is becoming readily available, it is currently not practical to use this technology to screen emergent strains routinely.

In this study we wished to assess a novel technology, optical genetic mapping, that generates a physical map of the bacterial chromosome for the comparison of pre-epidemic and epidemic strains of *Salmonella*. This method has the capacity to combine greater resolution, positional information and the identification of novel insertions to an extent that is lacking in PFGE and CGH. In brief, specific restriction endonucleases cut the DNA of a bacterium that has been immobilized on a derivatized glass slide. The restriction fragments are fluorescently labelled *in situ* which are then visualized and photographed. The mass of each fragment is determined by the intensity of fluorescence and partial genome maps are developed. These are assembled by overlapping segments into a genome optical map using alignment software [16].

In the current study we selected strains of interest from the four serovars Typhimurium, Newport, Enteritidis, and Dublin to be screened by the optical mapping technique. For *S.* Typhimurium these spanned the period of the epidemic for phage-type DT104 and had variant resistance profiles from before, during and after the epidemic period. The *S.* Enteritidis isolate chosen had a phage type (PT11) that is less frequently associated with human disease than other phage types such as PT4 which is commonly associated with human disease. Representative UK and USA *S.* Newport strains were chosen to compare geographical variation within the same serovar. Finally recent *S.* Dublin strains isolated from cattle showing clinical symptoms were chosen to monitor an outbreak in progress.

METHOD

Bacterial strains

Sources of the *Salmonella enterica* strains used in this study are listed in Table 1. *In-silico* maps of those sequenced: *S.* Dublin CT_02021853 (GenBank CP001144.1), *S.* Enteritidis P125109 (GenBank AM933172), *S.* Typhimurium NCTC13348 (Sanger Institute) [17], *S.* Typhimurium LT2 (GenBank AE006468), *S.* Newport SL254 (GenBank CP001113), *S.* Gallinarum 287/91 (GenBank AM933173), *S.* Agona SL483 (GenBank CP001138), *S.* Paratyphi B (GenBank CP000886), *S.* Schwarzengrund CVM19633 (GenBank CP001127), *S.* Choleraesuis SC-B67 (GenBank AE017220), *S.* Heidelberg SL476 (GenBank CP001120), *S.* Paratyphi A AKU_12601 (GenBank FM200053), *S.* Paratyphi A ATCC9150 (GenBank CP000026), *S.* Typhi CT18 (GenBank AL513382), *S.* Typhi Ty2 (GenBank AE014613), *S. enterica* sbsp. *arizonae* 62:z4,z23:– (GenBank CP000880), were used as reference mapped strains.

Optical mapping

Optical maps were prepared by OpGen (USA) following the method presented in Zhou *et al.* [18]. In brief, following gentle lysis and dilution, high-molecular-mass genomic DNA molecules were

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Table 1. *Strain list*

Strain identifier	Serotype	Phage type	Host species	Country	Resistances	Isolation date	Genome size
S00674-09	Dublin	—	Animal	UK	—	2009	4815408
S00680-09	Dublin	—	Animal	UK	—	2009	4845772
S00697-09	Dublin	—	Animal	UK	—	2009	4788185
P3854860 [24]	Enteritidis	PT11	Human	UK	—	—	4779606
P4722210	Typhimurium	DT104	Human	UK	ACSSuSpT	1997	4879212
P5289060	Typhimurium	DT104	Human	UK	SSp	2000	4874723
P5066840	Typhimurium	DT104	Human	UK	ACSSuSpTTmNxCp	1999	4908531
H042080120	Typhimurium	DT104	Human	UK	—	2004	4800297
H042120222	Typhimurium	DT104	Human	UK	—	2005	4857190
52520256	Typhimurium	DT104	Human	UK	SSuSp	2005	4926183
P0977470	Typhimurium	DT104	Human	UK	—	1986	4865085
S01760-03	Typhimurium	DT104	Porcine	UK	ACSSuSpT	2003	4891270
1341/96	Typhimurium	DT104	Human	UK	ACSSuSpT	1996	4909383
S05161-02	Newport	—	Bovine	USA	ACSSuT	2002	4809653
S05143-02	Newport	—	Bovine	USA	ACSSuT	2002	4783859
S05136-02	Newport	—	Bovine	USA	ACSSuT	2002	4816759
S04075-05	Newport	—	Bovine	UK	—	2005	4615177
L01169-07	Newport	—	Bovine	UK	—	2007	4582371
S03730-03	Newport	—	Bovine	UK	—	2003	4616773
S06233-03	Newport	—	Bovine	UK	—	2003	4659358
CT_02021853 [41]	*Dublin*	—	*Animal*	*USA*	—	—	4842908
P125109 [42]	*Enteritidis*	*PT4*	*Human*	*UK*	—	—	*4685848*
NCTC13348 [17]	*Typhimurium*	*DT104*	*Human*	*UK*	*ACSSuSpT*	—	*4933631*
LT2 [43]	*Typhimurium*	*LT2*	*Human*	*USA*	—	—	*4857432*
SL254 [23]	*Newport*	—	*Human*	*USA*	*ACSSuT*	—	*4827641*
287/91 [42]	*Gallinarum*	—	*Animal*	*Brazil*	—	—	*4658697*
SL483[44]	*Agona*	—	—	—	—	—	*4798660*
SPB7 [45]	*Paratyphi B*	—	*Human*	—	—	—	*4858887*
CVM19633[46]	*Schwarzengrund*	—	*Human*	—	*MDR*	—	*4709075*
SC-B67 [47]	*Choleraesuis*	—	*Human*	—	*MDR*	*2002*	*4755700*
SL476 [48]	*Heidelberg*	—	*Human*	—	*MDR*	—	*4888768*
AKU_12601[49]	*Paratyphi A*	—	*Human*	—	—	—	*4581797*
ATCC 9150 [50]	*Paratyphi A*	—	*Human*	—	—	—	*4585229*
Ty2 [51]	*Typhi*	—	*Human*	—	—	—	*4791961*
CT18 [52]	*Typhi*	—	*Human*	*Vietnam*	*ACSuTmT*	—	*4809037*
Arizonae [53]	*62:z4,z23:–*	—	—	—	—	—	*4600800*

Strains in italics have been genome sequenced and the sequences were used to derive *in silico* optical genetic maps.

spread and immobilized onto derivatized glass slides and digested with *Nco*I. The DNA digests were stained with YOYO01 fluorescent dye, and photographed using a fluorescent microscope interfaced with a digital camera. Automated image-analysis software located and sized fragments, and assembled multiple scans into whole-chromosome optical maps.

RESULTS

Genome alignment relationships

The OpGen MapSolver software was used to create an unweighted pair group method with arithmetic mean (UPGMA) to create a phylogenetic tree of the optical maps from the available *in-silco Salmonella* sequences and those generated from the 20 test strains (Fig. 1). The clustering predominantly grouped the test strains with their respective 'control' sequenced strains. The sequenced *S*. Dublin and three *S*. Dublin test strains showed the least variation, with 0·3% difference within the group. The nine *S*. Typhimurium DT104 test strains showed little variation also with 0·8% difference within the group regardless of resistance profile. The exception to this is strain H042080120, which is an atypical sensitive DT104 and groups closer to the LT2 strain. The seven *S*. Newport test strains clustered into two groups

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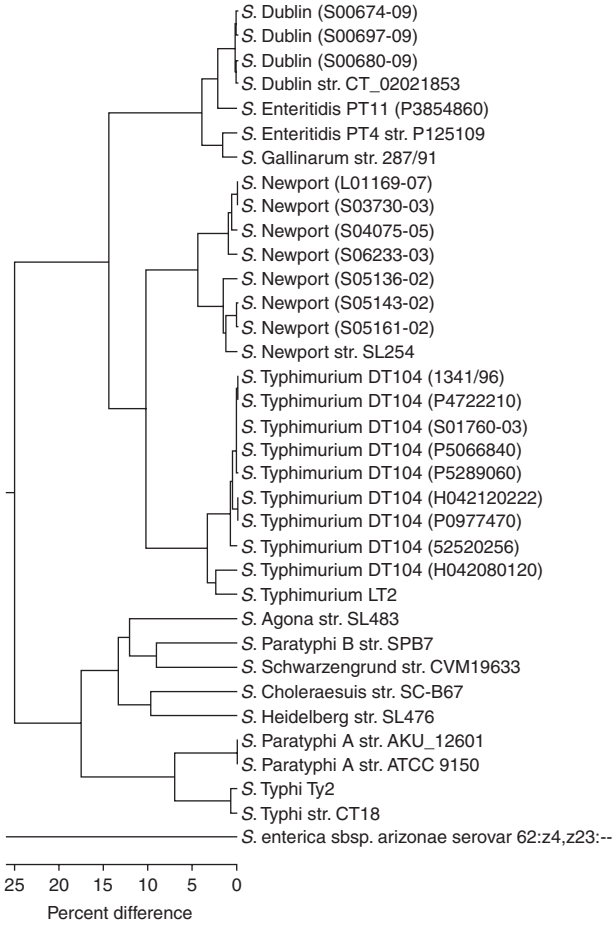

Fig. 1. UPGMA clustering of *in-silco* optical maps and 20 test strains. Strains grouping close to their sequenced reference strain are shown (note the distance between *S. arizonae* and the other *Salmonella* strains is 93·7%).

which will be discussed below. The *S.* Enteritidis test PT11 strain P3854860 clustered closer to the *S.* Dublin strains than to the control *S.* Enteritidis PT4 strain P125109, which was closer to the sequenced Gallinarum strain with a 1·8% difference.

S. enterica subsp. *arizonae* (subsp. IIIa) serovar 62:z4,z23:– showed 93·7% divergence from the serovars from subsp. I. The branch with serovars Agona, Choleraesuis, Heidelberg, Paratyphi A, Paratyphi B, Schwarzengrund, Typhi showed 25% divergence form that branch containing Dublin, Enteritidis, Gallinarum, Newport, and Typhimurium.

The *in-silico* optical maps of the sequenced *S.* Typhimurium LT2 and MDR DT104 were compared (Fig. 2). The regions identified as mobile elements and their genomic coordinates for LT2 by Hermans *et al.* [19] and for DT104 by Cooke *et al.* [20], are highlighted. The variation between the strains is predominantly in these regions associated with mobile elements, notably prophages. *S.* Typhimurium LT2 contains the regions of the bacteriophages Fels-1 and Fels-2 that are absent from DT104. Fels-1 has been shown to contain the genes *sodCIII* (superoxide dismutase), *nanH* (neuraminidase) and *grvA*. DT104 contains prophage 1 (ST104), prophage 3, prophage 4 and the *Salmonella* Genomic Island 1 (SGI1) that are absent from the LT2 strain. This later region contains the genes that confer antibiotic resistance to DT104 strains [21]. Both strains show the presence of Gifsy-1 and Gifsy-2, two lambda-like phages that have been associated with virulence [22].

Variation within the *S.* Typhimurium test strains

The upper part of Figure 3 shows regions of variation between the *S.* Typhimurium test strain 52520256 and the sequenced DT104. A 40-kb insert downstream from prophage 5 was identified, that is absent from the sequenced strain. This insert is located upstream of genes related to purine metabolism (*purM* and *purN*) and *ppk* (polyphosphate kinase) and downstream of *guaB* (inositol-5-monophosphate dehydrogenase).

Strains 52520256 (Fig. 3) and P5289060 (not shown) are antibiotic resistant but have different profiles from the penta-resistant profile of the sequenced strain. These differences in resistance profile were visible by optical mapping, with partial deletions in the region of SGI-1 in the test strains, between bases 4115969 and 4124741 for strain 52520256 and between bases 4112582 and 4122042 for strain P5289060. This region encompasses the second 'resistance cassette' of the genomic island including, for 52520256, the genes *tet*(*G*), *groE1*/*int1*, $bla_{\text{PSE-1}}$, *qacE*Δ*1*, *sul1* and *tnpA*, and for P5289060 the genes *floR, tetR, tet*(*G*), *groE1*/*int1*, $bla_{\text{PSE-1}}$ and *qacE*Δ*1* [21].

The lower part of Figure 3 shows the total absence of the SGI-1 region from two strains, P0977470 and H04212022, both of which are sensitive to the panel of antibiotics that the sequenced strain is resistant to. Aside from the variation in SGI-1, the insertion in 52520256 and the atypical strain H042080120, the optical mapping technique revealed the largely clonal nature of strains from the phage-type DT104.

Variation within the *S.* Newport test strains

Figure 4 shows the comparison of the optical maps from the sequenced *S.* Newport strain, a representative USA strain and a representative UK strain. It shows three general regions of divergence and their

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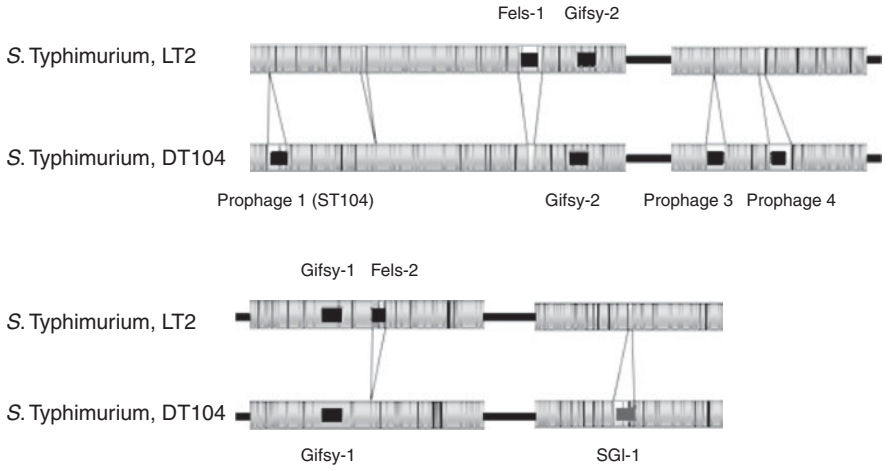

Identifier	Genome position, base pair number	
DT104 (Cooke *et al.* [20])		
Prophage 1 (ST104)	365 588	406 646
Prophage 2 (Gifsy-2)	1 079 201	1 124 674
Prophage 3	1 954 176	1 995 393
Prophage 4	2 110 069	2 149 002
Prophage 5 (Gifsy-1)	2 797 168	2 845 750
SGI1	4 083 657	4 129 583
LT2 (Hermans *et al.* [19])		
Prophage Gifsy-1	2 726 772	2 777 314
Prophage Gifsy-2	1 096 831	1 144 131
Prophage Fels-1	962 001	1 005 353
Prophage Fels-2	2 844 427	2 878 156

Fig. 2. Comparison of optical maps of sequenced *Salmonella enterica* subsp. *enterica* serovar Typhimurium strains LT2 and DT104. Prophage regions identified by Hermans *et al*. [19] and Cooke *et al*. [20] highlighted in black, the *Salmonella* Genomic Island 1 (SGI1) containing MDR genes, in dark grey. Genomic location of prophages listed in the bottom part of the figure. The figure shows that the majority of variance between strains is due to prophage regions.

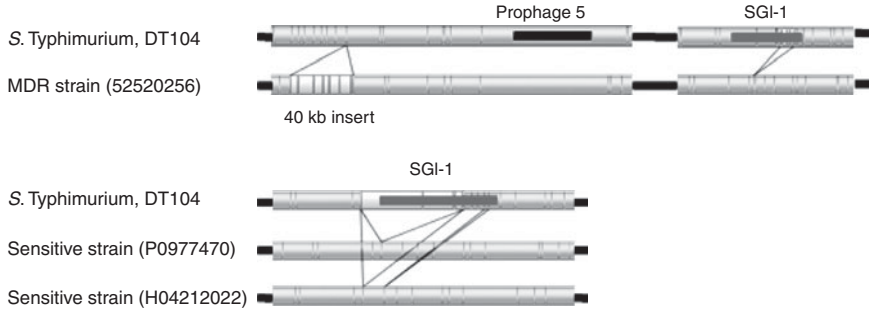

Fig. 3. Comparison of sequenced DT104 strain to three DT104 field isolates. Resistant strain 52520256 shows a 40-kB insert downstream of prophage 5 and a variant SGI1 region with part of the region missing. Two sensitive strains P0977470 and H04212022 show the absence of the SGI1 region.

associated genes obtained from the annotated strain information [23]. The UK (S04075) strain lacks a portion of the Gifsy-2 phage, a portion of the Gifsy-1 phage and a region associated with the genes *cpxR, fieF* and *sodA*.

Other specific differences are revealed. The UK (S04075) strain also has an additional ~50-kb insert, upstream of Gifsy-2, that is absent from the sequenced and USA strain. The same strain, as well as the UK strain S03730-03, has a further ~40-kb insert downstream of the location of Gifsy-1. There are also variations for the USA test strain (S05136) compared to the sequenced and UK strain in a region associated with the genes *amn* and *arsB*.

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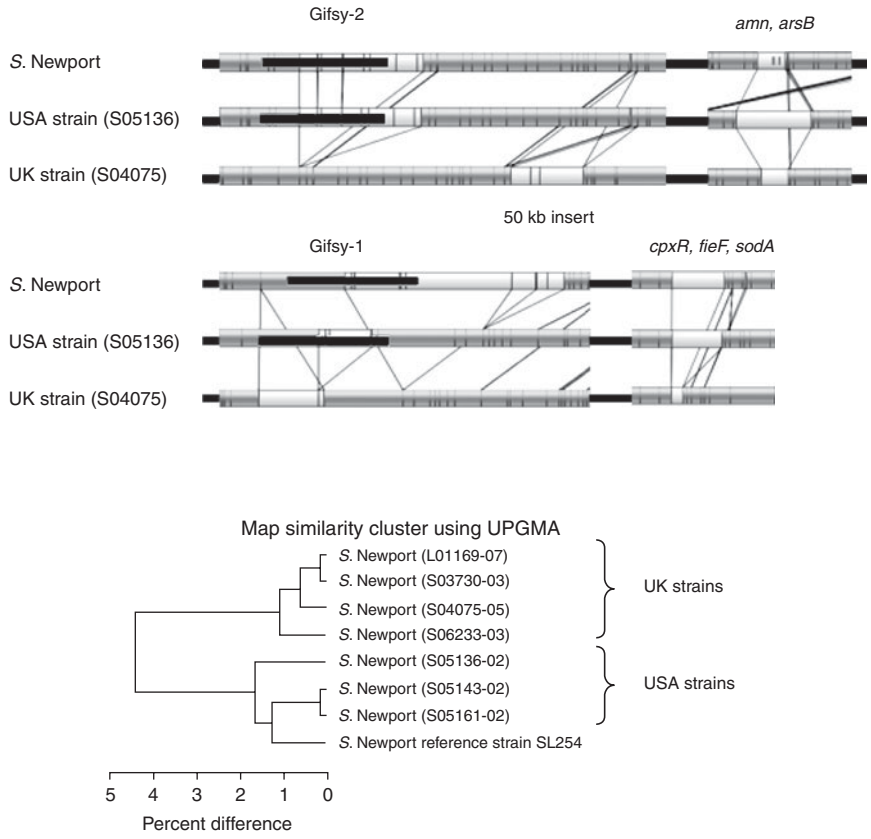

Fig. 4. *Top panels*: Comparison between optical maps from sequenced *S*. Newport, strain S05136 (USA) and strain S04075 (UK). The absence of portions of Gifsy-2, Gifsy-1 and genes *cpxR, fieF* and *sodA*, from the UK strain is illustrated; also shown is variation in the region around *amn* and *arsB* genes. *Lower panel*: UPGMA clustering of sequenced *S*. Newport strain with seven field strains. Strains L01169-07, S03730-03, S04075-05 and S06233-03 from the UK. Strains S05136-02, S05143-02 and S05161-02 are like the sequenced strain from the USA. UK and USA strains group into two distinct groups.

The general variation between UK and USA strains is shown by UPGMA clustering in the lower part of Figure 4. It shows that there is a clear geographical grouping to the strains, with the four UK strains (on top) grouping away from the USA strains and the sequenced strain, which also had a USA origin.

Variation between the *S*. Enteritidis test strains and sequenced PT4

Figure 5 shows the comparison between the sequenced *S*. Enteritidis strain, which is from PT4, and the test strain which is PT11. The optical maps show that there are several insertions in the PT11 genome which are absent from PT4. These insertions contribute to the fact that the PT11 genome is around 100 kb greater than the PT4 genome [24]. The UPGMA clustering, in the lower part of Figure 5, shows that PT11 groups closer to the *S*. Dublin sequenced and test strains than to the sequenced *S*. Enteritidis PT4 strain. The PT4 strain grouped closer to *S*. Gallinarum.

DISCUSSION

The optical mapping technique has been used to compare representative strains from four serovars of epidemiological interest to the *in-silico* maps produced from the available sequenced strains. The technique has the advantage of allowing a greater degree of discrimination between strains, and to identify novel insertion regions as well as providing a backbone for sequencing projects.

With the serovar Typhimurium the technique was able to discriminate between multidrug resistant and sensitive strains, to the degree that it could distinguish the strains with variant *Salmonella* Genomic Islands. Since the composition of the classical *S*. Typhimurium DT104 SGI1 has been described [21] other examples with different resistance profiles have been identified. Variants SGI-A to SGI-O have been described in Typhimurium and also in *Proteus mirabilis*, and a variant genomic island with a different lineage, termed SGI2 has also been described [25–27]. In

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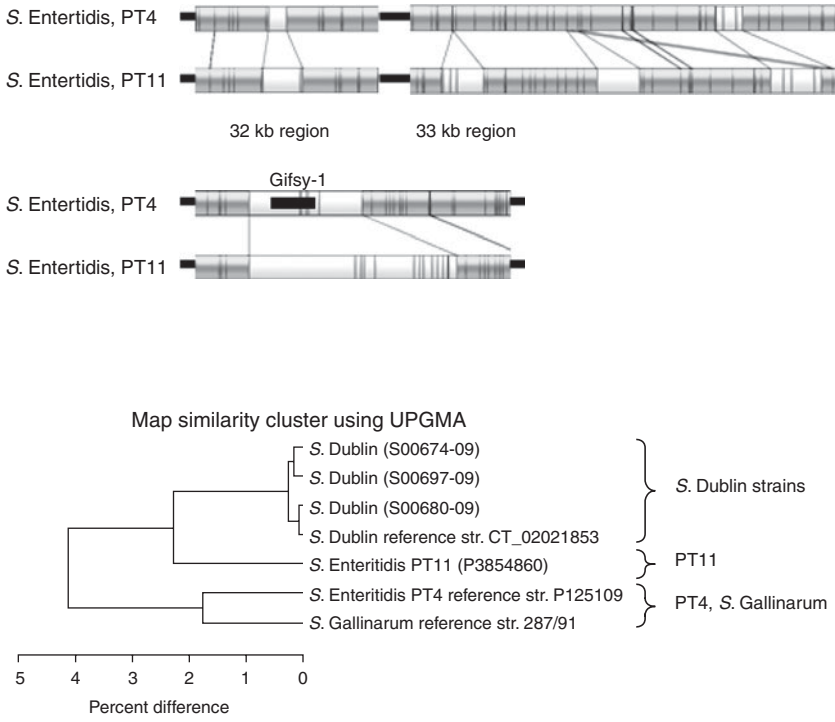

Fig. 5. *Top panels*: Comparison of optical maps of sequenced *S.* Enteritidis PT4 and strain P3854860 (PT11). Additional regions present in PT11 strain, including around Gifsy-1 phage are illustrated. *Lower panel*: UPGMA clustering of *S.* Enteritidis strains with sequenced *S.* Gallinarum and the four *S.* Dublin strains. The PT11 strain is more similar to the *S.* Dublin strains than to the PT4 strain.

addition, a secondary attachment for SGI1 has been discovered via R1 plasmid-mediated transformation with *S.* Typhimurium LT2. This is located in the intergenic region between the chromosomal *sodB* and *purR* genes [28]. The ability of optical mapping to quickly identify variation in SGI1-related regions demonstrates its ability to monitor the evolving nature of chromosomally based antibiotic resistance.

A 40-kb insert was identified in UK strain 52520256, between the genes *ppk* and *guaB*. The gene *ppk* codes for a polyphosphate kinase and the disruption of its transcription may have implications for the fitness of the strain; *ppk* mutants have been shown to have reduced survival and sensitivity to weak organic acids [29, 30]. Polyphosphate is synthesized in response to high salt levels, nitrogen limitation and amino-acid starvation. Polyphosphate also stimulates ATP-dependent proteolysis of certain ribosomal proteins after a shift from a rich to minimal media [31]. Phenotypic screening of strain 52520256 through the Phenotypic MicroArray (Biolog, USA) system revealed a dysfunctional metabolism for the majority of sole nitrogen and phosphate sources (data not shown) and this may be due to the interference with *ppk* by the genomic insertion. This is a topic for future study. The importance of identifying and assessing the impact of such insertions on metabolism is important when considering epidemicity. Such regions could subsequently be the target of sequencing and allow the progression of bacterial pathogenicity to be monitored.

The technique also confirmed that the variation between DT104 and LT2 lies in the previously reported phage regions [19, 20] and this is suggestive of their role in the evolution of bacterial pathogens by the horizontal gene transfer facilitated by mobile genetic elements. Fels-1 and Fels-2 are absent from DT104 and prophages 1 (ST104), 3, 4 and SGI1 are absent from LT2. Fels-1 contains *sodCIII*, a superoxide dismutase and *nanH*, a neuraminidase, and its presence has been suggested as a factor for increasing virulence [32, 33].

Optical mapping allowed the differentiation between *S.* Newport strains from UK and USA origin and flagged up regions of difference that may have been missed by more conventional techniques. In the case of USA MDR Newport strains the resistance profile is given by a plasmid (of around 150 kb) which is missing in the UK strains [34]. The reasons why this plasmid is only present in strains with a USA origin is

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not known; however, the variations in phage may be of some importance. Currently the optical mapping technique in unable to detect, interrogate and compare plasmids as their genome size is too small. Therefore other techniques should be used in conjunction with the method when considering the impact of plasmids on epidemicity, antibiotic resistance and virulence.

The UK strains lack some of the Gifsy-1 and -2 phage regions. Gifsy-2 has been shown to contain genes involved in *Salmonella* virulence in mice. *Sse*I codes for a type III effector protein, *sodC-1* for a superoxide dismutase and the gene *gtgE* which has also been associated with virulence [22]. Curing *S*. Typhimurium of Gifsy-2 has been shown to reduce the ability of the bacteria to cause systemic disease in mice [35].

In addition the USA strains are shown to have a region containing the genes *sodA, fieF* and *cpxR*. The *sodA* gene encodes superoxide dismutase, *fieF* encodes a cation efflux pump and *cpxR* (with *cpxA*, a membrane sensor), makes up a two-component regulatory system which has been implicated in response to osmolarity in *E. coli* [36], porin expression in antibiotic resistance [37] and virulence in Typhimurium [38]. The presence of the additional superoxide dismutases, *sodA* and *sodC-1*, may confer a greater ability for the strains from the USA lineage to survive in the intracellular environment of the host, as they provide protection against the oxidative burst of host cell defences. There was also variation around genes *amn* and *arsB* codes for a arsenical resistance protein [39].

The presence in the USA strains of regions and genes associated with intracellular survival and increased pathogenicity may go some way to explaining their greater prevalence in cases of salmonellosis.

Recent studies by Pan *et al*. [24] showed by MLST, CGH and various phenotypic analyses that *S*. Enteritidis PT11 possibly belongs to a distinct clonal lineage compared to *S*. Enteritidis phage types 4, 8, 9a, and 13. These four phage types are prevalent in terms of human and poultry isolations [10] and appear to be closely related [40]. It is possibly significant that the one representative PT11 selected for study showed a distinct optical genetic map and was more closely related to *S*. Dublin than the sequenced PT4. Given this data, this PT11 strain has been selected for full genome sequencing (Hayward *et al*., unpublished observations).

The *S*. Dublin strains that were mapped showed a large degree of homogeneity, both between themselves and when compared to the sequenced strain. The test strains had a UK origin while the sequenced strain came from the USA. This lack of variation shows that the serovar displays a level of genetic stability, and is perhaps indicative of the level of its adaptation to and success within its bovine host environment.

Overall the optical mapping of serovars from *S. enterica* subsp. *enterica* has demonstrated the usefulness of this technique when considering strains with animal and human health implications. It allows the presence of novel insertions and genomic rearrangements to be detected. It can also distinguish variations in genomic islands that can be connected to a change in resistance profiles. When used in conjunction with a backbone of a previously sequenced strain it can allow the monitoring of phage content and therefore the possible acquisition of pathogenic determinants. The greater level of discrimination over more traditional typing and research methods makes this an important addition to the arsenal of tools available to the microbiologist.

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DECLARATION OF INTEREST

None.

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