Identification of genetic and phenotypic differences associated with prevalent and non-prevalent *Salmonella* Enteritidis phage types: analysis of variation in amino acid transport

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In this study, differences at the genetic level of 37 *Salmonella* Enteritidis strains from five phage types (PTs) were compared using comparative genomic hybridization (CGH) to assess differences between PTs. There were approximately 400 genes that differentiated prevalent (4, 6, 8 and 13a) and sporadic (11) PTs, of which 35 were unique to prevalent PTs, including six plasmid-borne genes, *pefA*, *B*, *C*, *D*, *srgC* and *rck*, and four chromosomal genes encoding putative amino acid transporters. Phenotype array studies also demonstrated that strains from prevalent PTs were less susceptible to urea stress and utilized L-histidine, L-glutamine, L-proline, L-aspartic acid, gly-asn and gly-gln more efficiently than PT11 strains. Complementation of a PT11 strain with the transporter genes from PT4 resulted in a significant increase in utilization of the amino acids and reduced susceptibility to urea stress. In epithelial cell association assays, PT11 strains were less invasive than other prevalent PTs. Most strains from prevalent PTs were better biofilm formers at 37 °C than at 28 °C, whilst the converse was true for PT11 strains. Collectively, the results indicate that genetic and corresponding phenotypic differences exist between strains of the prevalent PTs 4, 6, 8 and 13a and non-prevalent PT11 strains that are likely to provide a selective advantage for strains from the former PTs and could help them to enter the food chain and cause salmonellosis.

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INTRODUCTION

Infection with nontyphoidal *Salmonella* (NTS) species is one of the leading causes of diarrhoea in developed

countries. Epidemiological studies have shown that relatively few *Salmonella* serovars/strains are responsible for the bulk of NTS infections in humans. In fact, serovars Enteritidis and Typhimurium are currently the most prevalent causes of human inflammatory gastroenteritis, often referred to as food poisoning (Gatto *et al.*, 2006; Rodrigue *et al.*, 1990). The current worldwide human epidemic of *Salmonella* Enteritidis is thought to have started in the 1980s, although some have suggested a much earlier date in the 1960s after the introduction of *S. Enteritidis* into poultry flocks from rodents (Baumler *et al.*, 2000). Irrespective of the exact starting point, the incidence of *S. Enteritidis* isolation from patients has increased dramatically since the 1980s (Ward *et al.*, 2000). In 2007, a total of 155 540 confirmed cases of human salmonellosis were reported in the European Union, with 64.5 % of cases caused by *S. Enteritidis* (European Food Safety Authority, 2008). Increases in *S. Enteritidis* incidences were also reported in the USA starting from 1999, with more than

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Abbreviations: AUC, area under the curve; CGH, comparative genomic hybridization; MLST, multilocus sequence typing; PT, phage type.

The microarray data discussed in this paper are available from ArrayExpress under accession number E-MEXP-1720.

Eight supplementary tables, listing conserved and variant genes identified from CHG microarray of 28 *S. Enteritidis* strains, genes identified to be unique in non-prevalent PT strains from CGH microarray, PCR validation of selected genes, MLST sequence types of 23 *S. Enteritidis* strains, and primers used in this study, are available with the online version of this paper.

1400 cases in 2006 (Centers for Disease Control and Prevention, *Salmonella* annual summaries; <http://www.cdc.gov/ncidod/dbmd/phlisdata/salmonella.htm>).

In 1987, a phage typing scheme for *S. Enteritidis* was described (Ward *et al.*, 1987). The scheme is based on differences in susceptibility to a panel of reference bacteriophages that separates most *S. Enteritidis* phage types (PTs) into two distinct lineages, PT4-like strains (including PT1, PT4, PT4b, PT6, PT6a, PT7 and PT24) and PT8-like strains (including PT2, PT8, PT13, PT13a and PT23) (Guard-Petter, 1999). Recent studies have also revealed genomic differences between these two groups. Three prophage regions are mainly responsible for the difference, and include a 38 kb ST64B region, an 18 kb Fels-2 region and a few genes belonging to prophages ST27 and ST35 (Porwollik *et al.*, 2005). *Salmonella* phage typing has become the recognized method by which epidemiologists attribute *Salmonella* outbreaks or epidemics to a specific strain type (Boccia *et al.*, 2004; Lukinmaa *et al.*, 2006; Usera *et al.*, 1994). Within the last decade, *S. Enteritidis* PT4 and PT6 have been the most prevalent in Europe (Echeita *et al.*, 2005; Gillespie *et al.*, 2005), whereas in the USA and Canada, PT8 and PT13a are the most common types (Brown *et al.*, 1999; White *et al.*, 2007). In contrast, PTs such as PT11 have rarely been associated with human infection. Data from the UK Health Protection Agency (HPA) database show that over the past 10 years, *S. Enteritidis* human infections in the UK have mainly been due to PT4, PT6 and PT8 strains, which caused 40 104, 5804 and 5722 cases respectively, but only 916 cases were caused by PT11 isolates during the same time period (Christopher Lane, HPA Centre for Infections, personal communication). Also, a search within the *Salmonella* strain collection database at the UK Veterinary Laboratories Agencies (VLA) Enteric Reference Unit identified only 42 PT11 strains isolated in the past 10 years, in contrast to over 2000 PT4 strains isolated during this period from animals. However, it has been noted that different PTs become prominent at different times in a periodic manner. Therefore, the aim of this study was to analyse prevalent and sporadic/incidental strains from human infection within the Enteritidis serovar to gain insight into factors associated with epidemic strains prevalent in human infection.

We therefore chose a panel of *S. Enteritidis* strains from both prevalent and non-prevalent PTs (4, 6, 8, 13a and 11) gathered in the past 12 years to assess genomic differences by comparative genomic hybridization (CGH). A *Salmonella* pan-genome microarray covering seven *Salmonella* genomes (Cooke *et al.*, 2007) was used for this study. We also characterized these strains using other approaches, such as multilocus sequence typing (MLST) and PFGE. Experiments to correlate phenotypic differences with genetic differences were also conducted in order to gain further insight into how genome variations contribute to the apparent differences in the prevalence of the various PTs.

METHODS

Bacterial strains, growth and DNA extraction. The bacterial strains used in this study (Table 1) included 42 human and animal isolates of *Salmonella* and seven sequenced laboratory strains [*Salmonella* bongori 12419 (ATCC 43975), *Salmonella* Typhi CT18, *Salmonella* Typhimurium SL1344 (NCTC 13347), DT104 (NCTC 13348) and LT2 (ATCC 700220), *Salmonella* Gallinarum 287/91 (NCTC 13346), *S. Enteritidis* PT4 (NCTC 13349)]. Bacteria were cultured in Luria-Bertani (LB) broth overnight at 37 °C. Genomic DNA was extracted from overnight cultures using either a Qiagen Tissue DNA extraction kit or a Puregene kit (Gentra Systems) following the manufacturer's instructions.

Microarray experimental procedure. The design and construction of the pan-*Salmonella* array was an extension of a previous array (Thomson *et al.*, 2004), incorporating specific genes found in newly sequenced *Salmonella* genomes (Cooke *et al.*, 2007). For each strain, genomic DNA was extracted from at least two cultures grown independently. The genomic DNA from seven sequenced laboratory strains was mixed equally for the universal control mix (Cy5-labelled control channel), with each strain contributing a seventh (i.e. 0.286 µg) of the total DNA. The DNA (2 µg) for every test strain was labelled with Cy3 dye (test channel). DNA labelling, hybridization and washing protocols were essentially performed as previously described (Wu *et al.*, 2008). However, differences included resuspension of Cy3/Cy5-labelled DNA in a hybridization buffer composed of 5 × SSC, 6 × Denhardt's, 60 mM Tris-HCl (pH 7.6) and 0.12 % Sarkosyl (Sigma, filter-sterilized), and the use of tRNA (from yeast, Sigma R8508) for blocking.

The microarray slides were scanned using the Genepix 4000B scanner (Axon Instruments). The images obtained were quantified with Genepix Pro software (Axon Instruments). Of the 12 strains from PT4, six strains (4, 5, 6, 47, 48 and 49) were tested in duplicate, while the rest (strains R2, R3, R5, R7, R8 and R9) were tested once due to the high consistency between results from strains in this phage group. At least two duplicate hybridization experiments were performed for the remaining test strains.

Normalization, analysis and validation of CGH microarrays. As described above, we produced CGH microarrays using an equal mix of genomic DNA from the seven sequenced *Salmonella* strains represented on the microarray (universal control) in the control channel. This enabled all spots present on the microarray to have a positive control following hybridization. We validated this novel approach of using a universal control by hybridizing genomic DNA from each sequenced strain in turn against the universal control. A collection of methods from the LIMMA package (Bioconductor; www.bioconductor.org) were applied to the microarray data (Supplementary Table S1). Present and absent/divergent genes for each method were selected using a threshold over the signal distribution, as described by Carter *et al.* (2008). Sensitivities and specificities for each normalization method were calculated by comparing the set of present and absent/divergent genes to the scores from BLASTN results of the PCR probe against the corresponding control sequenced strain. An illustration of this is given in Supplementary Table S1, which shows all sensitivities and specificities for all the normalization methods applied for strain SL1344. Supplementary Table S2 summarizes sensitivities and specificities for all seven sequenced strains using the two best-suited normalization methods. The best normalization method (Bc1, median) was applied to all test strains.

PCR validation. PCR amplification was performed on selected genes present in the prevalent and non-prevalent PTs. All PCRs were conducted on a GeneAmp PCR System 9700 (Applied Biosystems) in

Table 1. Bacterial strains used in this study

The strains were collected from different regional centres in the UK, both at the Health Protection Agency (HPA) and the Veterinary Laboratories Agency (VLA); source information has been given where available. NA, Not available.

Strain	Serotype	PT	Source	Year	Host
01	Enteritidis	PT13a	Coventry HPA	1998	Human
02	Enteritidis	PT13a	USA	Unknown	Poultry
03	Enteritidis	PT13a	Preston HPA	1998	Human
04	Enteritidis	PT4	Nottingham HPA	1998	Human
05	Enteritidis	PT4	Bristol Royal Infirmary	1996	Human
06	Enteritidis	PT4	UK	1997	Poultry
47	Enteritidis	PT4	Colindale HPA	1994	Human
48	Enteritidis	PT4	Colindale HPA	1997	Human
51	Enteritidis	PT4	Colindale HPA	1998	Human
R2	Enteritidis	PT4	Stonegate UK	2002	Poultry
R3	Enteritidis	PT4	Humphrey, Wolfhanger UK	2006	Poultry
R5	Enteritidis	PT4	Humphrey, Wolfhanger UK	2003	Poultry
R7	Enteritidis	PT4	Cotswold House 8 UK	2002	Poultry
R8	Enteritidis	PT4	Cotswold House 8 UK	2006	Poultry
R9	Enteritidis	PT4	Sutton UK	2002	Poultry
07	Enteritidis	PT6	UK	1999	Poultry
08	Enteritidis	PT6	Norwich HPA	1995	Human
09	Enteritidis	PT6	Birmingham Children's Hospital	1996	Human
10	Enteritidis	PT8	UK	1996	Poultry
11	Enteritidis	PT8	USA	1999	Egg
12	Enteritidis	PT8	St James's University Hospital, Leeds	1998	Human
41	Enteritidis	PT11	Colindale HPA	1995	Human
42	Enteritidis	PT11	Colindale HPA	1995	Human
43	Enteritidis	PT11	Colindale HPA	1996	Human
44	Enteritidis	PT11	Colindale HPA	1996	Human
45	Enteritidis	PT11	Colindale HPA	2000	Human
46	Enteritidis	PT11	Colindale HPA	2000	Human
PE1	Enteritidis	PT11	VLA Weybridge	2006	Canine
PE2	Enteritidis	PT11	VLA Weybridge	2005	Feline
PE3	Enteritidis	PT11	VLA Weybridge	2004	Horse
PE4	Enteritidis	PT11	VLA Weybridge	2003	Sheep
PE5	Enteritidis	PT11	VLA Weybridge	2002	Hedgehog
PE6	Enteritidis	PT11	VLA Weybridge	2000	Chicken
PE11	Enteritidis	PT11	VLA Weybridge	2001	Rodent
PE19	Enteritidis	PT11	VLA Weybridge	2007	Hedgehog
PE31	Enteritidis	PT11	VLA Weybridge	1998	Other animals
PE33	Enteritidis	PT11	VLA Weybridge	2007	Cattle
S13	Enteritidis	PT3	VLA Weybridge	2008	Dog
L364	Enteritidis	PT12	VLA Weybridge	2009	Cat
S6198	Enteritidis	PT12	VLA Weybridge	2008	Chicken
S6236	Enteritidis	PT12	VLA Weybridge	2008	Chicken
S475	Enteritidis	PT22	VLA Weybridge	2008	Chicken
PT4 (NCTC 13349)	Enteritidis	PT4	Sanger Centre, UK	NA	Sequenced laboratory strain
LT2 (ATCC 700220)	Typhimurium	NA	Sanger Centre, UK	NA	Sequenced laboratory strain
SL1344 (NCTC 13347)	Typhimurium	NA	Sanger Centre, UK	NA	Sequenced laboratory strain
DT104 (NCTC 13348)	Typhimurium	DT104	Sanger Centre, UK	NA	Sequenced laboratory strain
<i>S. Gallinarum</i> 287/91 (NCTC 13346)	Gallinarum	NA	Sanger Centre, UK	NA	Sequenced laboratory strain
CT18	Typhi	NA	Sanger Centre, UK	NA	Sequenced laboratory strain
<i>S. bongori</i> 12419 (ATCC 43975)	Bongori	NA	Sanger Centre, UK	NA	Sequenced laboratory strain

a total volume of 20 µl with final concentrations of 1.5 mM MgCl₂ and 200 µM each dNTP, 1.0 U *Taq* DNA polymerase and 10 ng template DNA (HotStarTaq Plus Master Mix kit, Qiagen). The thermal cycling conditions were as follows: an initial denaturation at 95 °C for 10 min, followed by 30 cycles at 95 °C for 30 s, T_m for 45 s and 72 °C for 1 min, completed by a final extension for 10 min at 72 °C. The presence of PCR amplicons was examined by electrophoresis on a 1.5 % agarose gel along with a 100 bp and a 1 kb DNA Ladder (Promega) as markers. Primers used and annealing temperatures are listed in Supplementary Table S3).

MLST. Seven housekeeping genes were amplified using the protocols at the MLST database at the Environmental Research Institute, University College Cork (<http://mlst.ucc.ie/>). The genes amplified were: *thrA*, *purE*, *sucA*, *hisD*, *aroC*, *hemD* and *dnaN*. The PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and both strands were sequenced. The Lasergene v6 (DNASTAR, Inc.) software was used to align and analyse sequences. Allelic profiles and sequence type (ST) designations were assigned using the MLST database (<http://mlst.ucc.ie/>).

Adhesion and invasion assay. Strains 1, 2, 3, 4, 5, 7, 8, 9, 11, 10, 12, 42, 43, 45, 47, 48 and 51 (Table 1), and the sequenced laboratory strain PT4, which represented five PTs, were selected for adhesion and invasion assay. The standard protocol was followed as previously described (Dibb-Fuller *et al.*, 1999). Bacterial cultures grown overnight at 37 °C in LB under aerobic conditions were centrifuged and the pellet was resuspended in pre-warmed Eagle's modified essential medium (EMEM; Sigma) supplemented with 1 % L-glutamine (Sigma) and 1 % non-essential amino acids (Sigma). The culture was adjusted to 10⁸ c.f.u. ml⁻¹. Duplicate plates of 10-day-old Caco-2 human intestinal epithelial cells (well differentiated) were set up for association assays with bacterial cultures (Bolton *et al.*, 2000). Adhesion and invasion assays were performed in four replicates per strain and repeated at least two times. The mean for each strain was calculated and strains representing each PT were grouped and compared. For statistical analyses, counts were log₁₀-transformed and following a standard F test, a two-tailed *t* test was carried out.

Biofilm forming assay and colony morphology. The ability of strains to form biofilms was assessed according to Woodward *et al.* (2000). Plates were read at 570 nm using an MRX Revelation absorbance reader (DYNEX, Magellan Biosciences). Blank values were obtained from wells that contained medium alone. The biofilm assay for each strain at each condition was performed at least twice with four technical repeats. Colony morphology and Congo red binding were analysed essentially as described previously (Allen-Vercos *et al.*, 1997). Congo red binding was observed when colonies developed a deep red colour within ~72 h incubation.

Phenotype microarray and bacterial growth kinetics. Strains were grown on LB agar plates at 37 °C for 18 h. To prepare the inoculum, colonies from each strain were suspended in medium base IF-0A (Biolog) and the turbidity was adjusted to 85 %. For PM3, the medium base was supplemented with 20 mM sodium succinate, 2 µM ferric citrate and 2.5 mg thiamine ml⁻¹. Each well of the Biolog plates was inoculated with 100 µl inoculum of each strain. Tetrazolium violet was used as a redox dye to indicate via cell respiration the utilization of nutrient or biochemicals in plates. Cell respiration actively reduces tetrazolium dye and produces a strong purple colour. Plates were incubated at 37 °C for 48 h. Phenotypic data were recorded with an OmniLog instrument, which captures a digital image of the plates every 15 min, and colour change values were quantified. Kinetic data were analysed with OmniLog-PM software. Kinetic graphs were plotted by timescale and colour

changes. The area under the curve (AUC) was calculated to assess substrate utilization by each strain. At least two individual repeats were performed for each strain.

Bacterial growth kinetics were monitored by using a FLUOstar OPTIMA plate reader (BMG Labtech). Strains were grown in 3 ml LB broth at 37 °C for 16–18 h. Bacterial cultures were inoculated into 96-well Iwaki plates to a final concentration that was 0.1 % of the original inoculum. Each strain was tested in triplicate under each condition. Control medium (no bacteria) was also tested in triplicate. The plate was then incubated in the FLUOstar OPTIMA at 37 °C for the desired time period. OD₆₀₀ was measured and saved every 15 min. M9 minimal medium with 1 mM proline as sole nitrogen source was used for the proline utilization test. LB-G broth containing 3 % urea was used for the urea stress test. At least three biological repeats were performed for each strain. The AUC was calculated and a two-tailed *t*-test was carried out to assess the statistical significance.

Inactivation of amino acid transporter genes and complementation. A knockout mutant of genes SEN1417–1420 in the sequenced PT4 strain (NCTC 13349) was constructed by insertional inactivation using the method of Datsenko & Wanner (2000). Essentially, the gene region of interest (SEN1417–1420) was disrupted by inserting a kanamycin-resistance gene, thus preventing the gene from functioning (Fig. 4a). The kanamycin-resistance gene was PCR-amplified with primers containing a 40 bp region homologous to the target gene. The amplified kanamycin-resistance gene was transferred into the wild-type PT4 strain with a temperature-sensitive helper plasmid pKD46. The helper plasmid promoted homologous recombination between the kanamycin-resistance gene and the target gene. Chromosomal allelic exchange between the wild-type and the mutant copy then resulted in an isogenic mutant. The helper plasmid was then eliminated by increasing cultivation temperature to 37 °C.

In order to validate knockout mutants, primers from both outside and inside the knockout region were designed. Primers specific to helper plasmid pKD46 were also used to verify the elimination of the plasmid. PCRs were performed as described above. Primers used and annealing temperatures are listed in Supplementary Table S3. CGH was also used to validate the mutant strain. The same CGH slide and hybridization method were used as described above.

Complementation of the target gene region was performed in both the PT4 mutant strain and a representative PT11 strain. TA Cloning (Invitrogen, TOPO TA cloning kit) was used to clone the target gene region following the manufacturer's instructions. The pCR2.1 vector without insertion of target gene was also transferred into the PT4 mutant strain and the PT11 strain as a control.

RESULTS

Similarities and differences in *S. Enteritidis* genome content

In order to identify genetic differences between prevalent and non-prevalent/sporadic strains associated with human infection, a total of 27 *S. Enteritidis* strains were tested by CGH microarray (Table 1). This included 12 strains from PT4, nine from PTs 6, 8 and 13a (prevalent PTs), and six from PT11 (non-prevalent PT). Prior to the study, the PT of all strains was reconfirmed. The genetic differences, based on the genome content of the seven *Salmonella* sequenced strains, showed that 3734 genes were present within all *S. Enteritidis* strains tested. This meant that

~95 % of the genome of the sequenced PT4 strain (NCTC 13349) represented on the microarray was conserved (Supplementary Table S4), and that 80 % of the conserved genes were found within the core *Salmonella enterica* subspecies I genome, as previously described (Anjum *et al.*, 2005), while 20 % were genes from the sequenced *Salmonella* genomes present on the current microarray (Supplementary Table S4).

A total of 412 genes were variably present in the different *S. Enteritidis* PTs included in the study (Supplementary Table S5). This gene set was used to perform a hierarchical clustering using the Pearson correlation coefficient in GeneSpring (v7), which clustered the strains into two main groups, A and B. Group A included all strains from the prevalent PTs tested in our study and was further subdivided into PT8-like and PT4-like subgroups. Group B contained only strains from non-prevalent PT11 (Fig. 1). PFGE profiles also clustered strains into the same groups and subgroups (data not shown).

Group A: prevalent PTs 4, 6, 8 and 13a

Our data showed that of all 27 strains tested from the prevalent PTs 4, 6, 8 and 13a shared more similarities to each other than to strains of the non-prevalent PT11 (Fig. 1). There were 35 genes that were present in all prevalent PT strains, but absent in all strains from non-prevalent PT11 (Table 2). More than 75 % of these genes were mobile genes, including 10 plasmid genes and 18 prophage genes. The plasmid genes included a group of *pefBCD* (plasmid-encoded fimbriae) genes, *srgC* (SdiA-regulated gene), pSLT023–026 and conjugative transfer genes pSLT075–076. Prophage genes included a degenerate Gifsy-2 prophage region (SEN0910–0912), a 12 kb prophage SE14 region (SEN1379–SEN1395, genome coordinates 1 469 682–1 481 286) and three genes (SEN1390 and SEN1385–1386) identified within an unknown degenerate lysogenic bacteriophage region. Seven chromosomal genes were also present exclusively in prevalent PTs. These genes were mainly located in a 4.5 kb region (SEN1415–1420, genome coordinates

Fig. 1. Main genetic variations (412 genes) in 27 test strains representing five PTs. Two main groups were separated by genome content. The genomic relationship between strains was calculated by average-linkage hierarchical clustering using Pearson's correlation coefficient as part of the GeneSpring microarray analysis software v5.0 (Silicon Genetics). Those genes that can be considered to be absent/divergent with the greatest degree of certainty lie at the blue end of the scale, around a $\log_{10}(\text{Cy5} : \text{Cy3})$ ratio of 0. Those genes that are present/conserved with the greatest degree of certainty lie in the yellow region and have a $\log_{10}(\text{Cy5} : \text{Cy3})$ ratio of around 1. The red end of the scale indicates an increased hybridization with the test strain and may be due to the presence of genes with multiple orthologues; black represents missing data.

Table 2. Genes identified by CGH microarray to be unique in prevalent PT strains

A systematic name or orthologue is given where the information is available. NA, Not available.

Gene	Product	Prophage or plasmid, related or not	Systematic name or orthologue	Notes and references
PSLT024	Hypothetical protein	Plasmid gene		
PSLT025	Putative periplasmic protein	Plasmid gene		
PSLT026	Putative cytoplasmic protein	Plasmid gene		
PSLTpefB	Plasmid-encoded fimbriae; regulation	Plasmid gene	PSLT019	The <i>pef</i> operon is composed of genes encoding plasmid-encoded fimbriae and has been demonstrated to play an important role in epithelial cell adhesion and intestinal persistence (Baumler <i>et al.</i> , 1996; van der Velden <i>et al.</i> , 1998; Weening <i>et al.</i> , 2005).
PSLTpefC	Plasmid-encoded fimbriae; usher protein	Plasmid gene	PSLT017	
PSLTpefD	Plasmid-encoded fimbriae; chaperone	Plasmid gene	PSLT016	
PSLTrepA2	DNA replication	Plasmid gene	PSLT023	
PSLTsrgC	SdiA-regulated gene	Plasmid gene	PSLT008	<i>srg</i> genes together with the <i>rck</i> gene are downstream adjacent to the <i>pef</i> operon, and have been reported to be regulated by SdiA, which is a transcriptional regulator and recognized to be involved in quorum sensing and the regulation of many virulence genes (Ahmer <i>et al.</i> , 1998).
PSLTtraJ	Conjugative transfer: regulation	Plasmid gene	PSLT075	
PSLTtraY	Conjugative transfer: <i>oriT</i> nicking	Plasmid gene	PSLT076	
SEN0910	Hypothetical protein Ent638DRAFT_3652	Within a degenerate Gifsy-2 prophage		
SEN0911	NA	Within a degenerate Gifsy-2 prophage		
SEN0912	Gifsy-2 prophage probable tail fibre protein	Within a degenerate Gifsy-2 prophage		
SEN1379	LygA, putative phage-encoded exoDNase	Within prophage SE14 region		A 12 kb region, reported to be absent from PT6a and PT35 strains (Porwollik <i>et al.</i> , 2005).
SEN1380	LygB, predicted phage protein	Within prophage SE14 region		
SEN1381	Putative phage-encoded cell division inhibitor protein	Within prophage SE14 region		
SEN1382	LygC, putative phage membrane protein	Within prophage SE14 region		
SEN1383	LygD, predicted phage protein	Within prophage SE14 region		
SEN1388	Predicted phage protein	Within prophage SE14 region		
SEN1390	Putative bacteriophage protein	Within prophage SE14 region		
SEN1391	Putative antitermination protein	Within prophage SE14 region		
SEN1392	NA	Within prophage SE14 region		
SEN1393	Tellurite resistance protein (<i>E. coli</i>)	Within prophage SE14 region		
SEN1394	Putative prophage membrane protein	Within prophage SE14 region		
SEN1395	Conserved phage protein	Within prophage SE14 region		
STY0176	Dihydrolipoamide acetyltransferase component (E2) of pyruvate dehydrogenase	Not related	SEN0157	
STY1434	Putative membrane protein	Not related	SEN1415	

Table 2. cont.

Gene	Product	Prophage or plasmid, related or not	Systematic name or orthologue	Notes and references
STY1435	Putative amino acid ABC transporter	Not related	SEN1417	Putative prokaryotic-type polar amino acid ABC transporter
STY1436	ABC transporter ATP-binding subunit	Not related	SEN1418	
STY1437	Putative ABC amino acid transporter permease	Not related	SEN1419	Transmembrane domain (TMD)
STY1438	Putative periplasmic amino acid-binding protein	Not related	SEN1420	
STY1513	Putative isomerase	Not related	SEN1504	
STY2051	Putative bacteriophage protein	Within a degenerate prophage region	SEN1390*	
STY2057	Hypothetical protein	Within a degenerate prophage region	SEN1386	
STY2058	Putative membrane protein	Within a degenerate prophage region	SEN1385	

*SEN1390 in the systematic name indicates that STY2051 has a region homologous to SEN1390.

1 500 659–1 505 193) and harbour a group of putative amino acid ABC transporter genes.

Group B: non-prevalent PT11

From the CGH microarray data, all strains belonging to this PT clustered together, and there were 146 genes uniquely present in PT11 strains (Fig. 1). Of these, 70 were prophage-related genes and included genes homologous to bacteriophages ST104, P2, 186, ST64B, Gifsy-1 and Gifsy-2, and a few unknown prophage regions (Supplementary Table S6). There were also 76 non-prophage genes exclusively present in PT11 strains that included 25 *S. Typhimurium* LT2 plasmid genes containing members of the *tra* operon (*traBCEFGHTRX*), *ssbE* and *psiB*, and 16 *S. Typhi* CT18 genes, which mainly encoded hypothetical proteins but included a ClpB-like protein (Supplementary Table S6).

PCR validation

PCR analysis was performed on a selection of genetic differences found between prevalent and non-prevalent PTs in order to validate the microarray data, and the results are listed in Supplementary Table S7. In addition to the *pef* genes present on the microarray (*pefB*, *pefC*, *pefD* and *srgC*), the remaining genes from the *pef* operon (*pefA*, *pefI*) and some from the adjacent cluster (*srgA*, *srgB* and *rck*) were also included in this analysis. PCR was also performed on the four hypothetical amino acid ABC transporter genes (SEN1417–1420). In order to avoid strain selection bias due to the limited number of non-prevalent PT11 strains tested on CGH microarray, another 10 PT11 strains (PE1–PE33, Table 1) of different host origin were also included. Furthermore, five strains from PTs 3, 12 and 22, which are also rarely associated with human infections, were tested. PCR results for all eight genes chosen from the microarray (*pefB*, *pefC*, *pefD*, *srgC* and SEN1417–1420) were consistent with the microarray data, i.e. present in tested prevalent PTs but absent from all PT11 strains. However, these eight genes were found to be present in the further five strains selected from PTs 3, 12 and 22. Examination of the extra five genes showed that *pefI*, which is known to be a putative regulator of *pef* fimbrial expression (Collighan & Woodward, 2001), and adjacent genes *srgA* and *srgB*, were PCR positive for all strains from both prevalent and non-prevalent PTs. The *pefA* and *rck* genes were positive for all prevalent PT strains and the additional five strains from PT3, 12 and 22 (with exception of the *rck*-negative PT3 strain), but negative for all PT11 strains.

MLST

In order to interpret the evolutionary relationships of different PTs, MLST was performed on seven housekeeping genes on 23 strains representing the five PTs. Our data indicated that all strains selected from the prevalent PTs 4, 6, 8 and 13a belonged to ST11, whilst all six strains from PT11 belonged to ST183. This was due to a single base pair variation in the *purE* locus, resulting in *purE* allele type 6 for strains from PTs 4, 6, 8

and 13a, and *purE* allele type 60 for PT11 strains (see Supplementary Table S8). This is concordant with data from the MLST database, whereby strains of PTs 4, 6, 8, 9 and 13a belong to ST11, but PT11 strains belong to ST183.

Adhesion and invasion of human epithelial cells by different PTs

CGH microarray data highlighted significant variation in *pef* genes between prevalent PT strains and non-prevalent PT11 strains. This raises the question of whether adhesion and/or invasion of *S. Enteritidis* into epithelial cells is different between these groups. A panel of 18 *S. Enteritidis* strains representing the five different PTs were screened in adhesion and invasion assays using human intestinal epithelial cells (Caco-2 cells). Following infection of Caco-2 cells, bacterial counts were determined and the percentage of invading bacteria with respect to the total number of adhered bacteria was calculated. The data showed that strains from all PTs invaded as well as *S. Typhimurim* SL1344 (>80 % of the total adhered bacteria), with the exception of PT11 strains. The PT11 strains showed significantly reduced invasion (<75 % of the total adhered bacteria) of Caco-2 cells compared with the other PTs and SL1344 ($P < 0.05$). In Fig. 2, the mean for each PT is shown, and error bars show $2 \times \text{SEM}$ to represent the 95 % confidence interval within the group. Of the prevalent PTs, PT4 strains showed the highest level of invasion. Similar data were also obtained using Hep-2 cells (data not shown).

Biofilm formation correlates with thin aggregative fimbriae (*agf*/curli) expression and colony morphology

Of the 10 genes unique to prevalent PTs strains shown by microarray data and PCR validation, *srgC* and *rck* are both

SdiA-regulated genes. SdiA is involved in quorum sensing or biofilm formation and regulates many virulence-associated genes (Ahmer *et al.*, 1998). Furthermore, virulence in *S. Enteritidis* has been correlated with biofilm production, especially at low salt concentrations on abiotic surfaces (Bonafonte *et al.*, 2000; Jain & Chen, 2007; Korber *et al.*, 2002; Mireles *et al.*, 2001; Solano *et al.*, 1998, 2001). Therefore the ability of a selection of 21 *S. Enteritidis* strains to form biofilms at two different temperatures (28 and 37 °C) in de-salted LB medium was tested. Biofilm formation did not generally correlate with a particular PT, except for PT11 strains (Fig. 3a). Five strains were poor biofilm formers ($\text{OD}_{570} < 0.3$) at both 37 and 28 °C (PT13a_1, PT13a_3, PT8_10, PT8_11 and PT4_47), whilst the rest of the 17 strains were poor biofilm formers at one temperature but moderate ($\text{OD}_{570} 0.3\text{--}0.6$) to good ($\text{OD}_{570} > 0.6$) biofilm formers at the other temperature. The PT11 strains showed the most significant increase in biofilm formation when the temperature was decreased from 37 to 28 °C, whilst it was the converse in most prevalent PT strains (Fig. 3a). Expression of the thin aggregative fimbriae (*agf*; also called curli) is one of the key factors in biofilm formation. We detected curli expression by Congo red binding and colony morphology at 25 and 37 °C. It was noted that PT11 strains generally formed bigger and 'lacier' red colonies at both temperatures, in particular at the lower temperature, than strains from the prevalent PTs (Fig. 3b). At 37 °C, some strains from the prevalent PTs formed larger colonies, although they were generally less 'lacy' than those formed by PT11 strains (Fig. 3b).

Phenotype array

A group of amino acid transporter genes was discovered to be present in prevalent PT strains using microarray analysis

Fig. 2. *S. Enteritidis* association with the human adenocarcinoma cell line Caco-2 (described in Methods). The adhesion and invasion differences among five PTs from *S. Enteritidis* were compared. Bacterial counts were determined as described in Methods and the values shown are mean percentage invasion derived from the $\log_{10}$ -transformed invasion and adhesion values. Error bars, $2 \times \text{SEM}$.

Fig. 3. (a) Comparison of the biofilm formation abilities (binding to polystyrene) of 21 *S. Enteritidis* strains representing five PTs. LB-G-NaCl medium (LB medium without both glucose and sodium chloride) was used, and the incubation temperatures were 37 and 28 °C. OD₅₇₀ values were determined as described in Methods. (b) Growth of the same set of strains on CFA Congo red plates for 72 h at 25 and 37 °C. Colony morphology and Congo red binding abilities were compared and are displayed below the corresponding biofilm formation values.

and PCR. To determine the function of these genes in *S. Enteritidis* strains, 12 strains representative of five PTs were tested for nitrogen utilization by using Phenotype Microarray PM3 plates. Of the 95 different nitrogen substrates tested, the most outstanding differences between PT11 strains and the prevalent PT strains were in the utilization of five amino acids and two dipeptides (Table 3). Both PT11 strains clearly demonstrated a reduced ability to utilize L-histidine, L-glutamine, L-

proline, L-aspartic-acid, L-cysteine, gly-asn and gly-gln compared with strains selected from the prevalent PTs, which showed good or moderate utilization of most of these substrates. Another phenotype microarray plate (PM9) was also used to test strain susceptibility to osmotic stress. Both PT11 strains were more susceptible to high concentrations of sodium chloride (5–6%) and urea (2–3%) than prevalent PT strains (results not shown).

Table 3. Nitrogen utilization of strains from different PTs

The nitrogen utilization of strains from different PTs was compared. Nutrient utilization, indicated by bacterial respiration, was classified as negative (–, AUC <6000), indistinct (+/–, 6000 <AUC <10000) and positive (+, AUC >10000). AUC was calculated with OmniLog-PM software.

Substrate	PT13a			PT4			PT6		PT8		PT11	
	1	3	5	PT4	48	51	8	9	10	12	42	45
L-Histidine	+	+	+	+	+	+	+	+	+	+	–	–
L-Glutamine	+/-	+	+	+	+	+	+	+	+	+	–	–
L-Proline	+/-	+	+	+	+	+	+	+	+	+	–	–
L-Aspartic acid	–	+	+	+	+	+	+	+	+	+/-	–	–
L-Cysteine	–	–	+	+	–	+	+	+	–	+/-	–	–
Gly-asn	+	+	+	+	+	+	+	+	+	+	–	–
Gly-gln	+	+	+	+	+	+	+	+	+	+	–	–

Fig. 4. (a) Comparison of the SEN1417–1420 regions in the PT4 and PT11 genomes. SEN1415–1420 are present in PT4, but absent in PT11. The SEN1417–1420 region in PT4 was disrupted by inserting a kanamycin-resistance gene. (b) PCR validation of mutant strain ZP is shown. Lanes (from left): 1 and 2, ZP and PT4 amplified with primers outside the knockout region; 3 and 4, ZP and PT4 amplified with primers outside and inside the knockout region; 5 and 8, 1 kb DNA ladders (Promega); 6 and 7, ZP and PT4 amplified with pKD46-specific primers. (c) PT4 and ZP genetic variations in the SEN1417–1420 region. Red/yellow indicates present genes; blue indicates absent/divergent genes.

Inactivation of amino acid transporter genes and complementation

In order to elucidate the effects of the amino acid transporter genes on amino acid utilization and osmoprotection, inactivation and complementation of the amino acid transporter genes were conducted in both PT4 and PT11 representative strains. Both PCR and microarray analysis validated the mutant, with correct insertion of the kanamycin-resistance gene (Fig. 4b) and no other rearrangement within the genome (Fig. 4c). Knockout of the SEN1417–1420 region of PT4 generated no significant effects on amino acid utilization in either the phenotype microarray or bacterial growth tests (Fig. 5a). However, complementation of these genes in a PT11 strain generated a significant increase in amino acid utilization (Fig. 5b).

Similarly, when both mutant and complemented strains were subjected to urea stress, inactivation of the amino acid transporter genes did not have a significant effect on the PT4 strain (Fig. 6a). On the other hand, the complemented PT11 strain was significantly better at coping with urea stress (Fig. 6b).

DISCUSSION

The recent epidemic caused by *S. enterica* serovar Enteritidis was not recognized until it was well established. Interventions such as vaccination of poultry, which may have been a prime factor in control, were not investigated until many years after onset. However, to intervene early requires an understanding of the basis of bacterial

Fig. 5. Comparison of bacterial growth in M9 minimal medium with 1 mM proline as both carbon and nitrogen source. (a) At least three individual repeats were performed for each strain. The AUC was calculated and Student's *t* test was applied to validate the significance of the difference ($P=0.0003$). PT4, PT4 sequenced strain; 42, PT11 representative strain; ZP, PT4 strain SEN1417-20::kana. (b) At least three individual repeats were performed for each strain. The AUC was calculated and Student's *t* test was applied to validate the significance of the difference ($P=0.0086$). ZPc+i, ZP complemented with SEN1417-20; ZPc, ZP complemented with control plasmid without insertion; 42c+i, 42 complemented with SEN1417-20; 42c, 42 complemented with control plasmid without insertion.

pathogenesis and its epidemiology. It may be argued that human epidemic strains (i.e. those highly prevalent in human infection) are 'more fit' than non-epidemic strains (i.e. those less prevalent in human infection), and therefore may carry genetic factors that enhance fitness. Therefore, the main purpose of this study was to compare genetic and other related factors of *S. Enteritidis* strains from different PTs associated with prevalent and sporadic human infections.

Most strains tested on an *S. enterica* CGH microarray based on seven known genomes were from human infection, although a number of strains from the most prevalent PTs were of poultry and egg origin, sources to which human infection has been attributed. Other strains from PTs 11, 3, 12 and PT22 included for PCR gene verification were of animal origin. The CGH microarray data in our study confirmed the earlier observation of Morales and Porwollik and their co-workers of genetic variations between the PT4 and PT8 groups, which are mainly in Fels-2 and ST64B, two prophage regions (Morales *et al.*, 2005; Porwollik *et al.*, 2005). PT11 strains in our study displayed distinct genetic properties which separated PT11 strains from both PT4 and PT8 groups, and included 70 genes in prophages ST104, P2, 186, ST64B, Gifsy-1 and Gifsy-2, and 76 non-prophage genes. Plasmid profiling (data not shown) revealed that PT11 strains possess a large plasmid (~80 kb), whereas strains from PTs 4, 6, 8 and 13a possess

the virulence or *spv* plasmid (~58 kb) (Gulig *et al.*, 1993). In addition, PFGE and MLST separated PT11 from other PTs. Collectively, these data suggest that PT11 has evolved as a unique PT lineage.

Interestingly, of the 35 genes found to be unique to strains from prevalent PTs and absent from PT11 strains, 10 plasmid genes, including *pefABCD*, *srgC* and pSLT023-026, were also found to be present in epidemic multi-antibiotic-resistant *S. Typhimurium* DT104 strains, but absent in non-epidemic DT208 strains (Kang *et al.*, 2006). However, five strains from rare PTs 3, 12 and 22 were also positive for these genes in PCR validation. This suggests that other epidemicity factors play important roles in these cases. The *pef* genes are known to be virulence-associated, and are often involved in adhering to epithelial cells, and biofilm formation on HEp-2 cells and chicken intestinal epithelium (Baumler *et al.*, 1996; Ledebor *et al.*, 2006; van der Velden *et al.*, 1998; Weening *et al.*, 2005). *Salmonella* contains at least 13 putative fimbrial operons on its chromosome, including *bcf*, *sti*, *stf*, *saf*, *stb*, *fim* (SEF21, type 1 fimbriae), *agf* (*csg*, SEF17, curli), *stc*, *ste*, *std*, *lpf*, *sef* (SEF14) and *sth*. Except for the plasmid-borne *pef* operon, all strains from both prevalent and non-prevalent PTs were found to possess these fimbrial operons. Of all fimbrial operons identified, *fim*, *sef*, *agf*, *lpf* and *pef* have been studied most extensively. It has been demonstrated that the *sef* operon is expressed copiously at 37 °C and is the dominant factor in

Fig. 6. Comparison of bacterial growth in LB-G medium with 3% urea. (a) At least three individual repeats were performed for each strain. The AUC was calculated and Student's *t* test was applied to validate the significance of the difference ($P=0.014$). PT4, PT4 sequenced strain; 42, PT11 representative strain; ZP, PT4 strain SEN1417-20::kana. (b) At least three individual repeats were performed for each strain. The AUC was calculated and Student's *t* test was applied to validate the significance of the difference ($P=0.00025$). ZPc+i, ZP complemented with SEN1417-20; ZPc, ZP complemented with control plasmid without insertion; 42c+i, 42 complemented with SEN1417-20; 42c, 42 complemented with control plasmid without insertion.

adherence to inanimate surfaces. On the other hand, *agf* and *fim* are expressed at ambient temperature (25 °C), where *agf* is the dominant factor (Woodward *et al.*, 2000). Interestingly, a homologue of the plasmid gene *pefI* (putative regulatory gene for the *pef* operon) has been found in the *sef* operon on the *S. Enteritidis* chromosome (Collighan & Woodward, 2001), indicating a possible synergy in their regulation. Phenotypic characterization of our strains showed that PT11 strains were poor biofilm formers at 37 °C but had enhanced biofilm-forming ability at 25–28 °C, whilst the opposite was true for most of prevalent PT strains. It is possible that the presence of *pef* genes in prevalent PTs enhances biofilm formation at 37 °C. Another factor that probably contributes to biofilm expression at 37 °C in the prevalent PTs is the *srg* operon. The *srg* operon, which includes the *rck* gene (resistant to complement killing) and *srgA*, *B* and *C*, is positively

regulated by SdiA. SdiA often acts as a receptor for *N*-acylhomoserine lactones (AHLs) produced by other bacteria, and plays an important role in biofilm formation and quorum sensing (Ahmer *et al.*, 1998). Furthermore, it has been shown that expression in LB motility agar of the *rck* (or *srg*) operon, regulated by SdiA and the presence of AHLs, is only observed at 37 and 42 °C but not at 22 and 30 °C (Smith & Ahmer, 2003). Thus, the absence of the *rck/srg* operon, in conjunction with *agf* gene expression, likely enhances biofilm formation in PT11 strains at ambient temperature. Similarly, the increased adhesiveness and decreased invasiveness of PT11 strains of human epithelial cells at 37 °C in comparison with other PTs may be influenced by the absence of the *pef* and *srg* operons.

BLAST results for the integral membrane proteins of the identified amino acid transporter genes (SEN1417–1420) revealed that these genes encode a polar amino acid transport system. Eleven types of polar amino acid ABC transporters have been identified in bacteria (Hosie & Poole, 2001). Nine of the 11 are solute-specific transporters, such as the histidine transporter (Nikaido *et al.*, 1997) and the arginine transporter (Wissenbach *et al.*, 1995). Results from the phenotype array showed that strains from the prevalent PTs, which harbour the amino acid transporter genes (SEN1417–1420), utilize at least five amino acids (L-histidine, L-glutamine, L-proline, L-aspartic-acid and L-cysteine) more effectively than PT11. This indicates that SEN1417–1420 encodes a 'broad solute specificity' amino acid transporter that can transport a wide range of acidic, basic and aliphatic L-amino acids, although these are relatively rare (Hosie & Poole, 2001).

Proline accumulation has been shown to be an important strategy that bacteria use to cope with osmotic stress (Balaji *et al.*, 2005; Grothe *et al.*, 1986). The *proU* operon (encoding a transport system for proline and glycine betaine) promoter is induced by low water potential in *Escherichia coli* and *Salmonella* (Wright & Beattie, 2004). Similarly, glutamate has also been shown to be necessary for *S. Typhimurium* to grow optimally in a high-osmolarity environment (Csonka *et al.*, 1994). We insertionally inactivated the amino acid transporter genes (SEN1417–1420) in a PT4 strain and confirmed the mutation by thorough PCR and CGH analysis. Surprisingly, the knockout mutant did not have a significant effect on amino acid utilization in PT4. However, a PT11 strain complemented with these genes showed a significant increase in proline utilization, as well as reduced susceptibility to urea stress. The different effects of these amino acid transporter genes between PT4 and PT11 strains suggest that duplication of gene functions exists in PT4, but not in PT11. The lack of functionality in PT11 strains may render PT11 strains less competitive particularly in the gastrointestinal tract and this requires further analysis. On the other hand, an extra transport system for these amino acids in prevalent PT strains is likely to contribute towards better survival under stress conditions such as in poultry faeces, an environment with a

high urea content. Furthermore, it has been reported that the transport of amino acids or metal ions is important for *S. Enteritidis* to persist in egg albumin (Clavijo *et al.*, 2006), a major route of infection in humans. Thus, the presence of the four amino acid transporter genes could allow better survival for strains from the prevalent PTs in such environments over PT11 strains. In fact the majority of animal-associated PT4 and PT6 strains isolated in the past 10 years were from poultry or eggs, whilst the majority of PT11 strains originated from a variety of hosts, with the highest numbers being from canines, rodents and hedgehogs (VLA Enteric Reference Unit). Further investigation is required to determine whether these amino acid transporter genes contribute to host adaptation and provide a selective advantage to enable PT4, PT6, PT8 and PT13a to enter the food chain to cause human salmonellosis.

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