

Genomic Comparison of the Closely Related *Salmonella enterica* Serovars Enteritidis and Dublin

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Abstract: The Enteritidis and Dublin serovars of *Salmonella enterica* are closely related, yet they differ significantly in pathogenicity and epidemiology. *S. Enteritidis* is a broad host range serovar that commonly causes gastroenteritis and infrequently causes invasive disease in humans. *S. Dublin* mainly colonizes cattle but upon infecting humans often results in invasive disease. To gain a broader view of the extent of these differences we conducted microarray-based comparative genomics between several field isolates from each serovar. Genome degradation has been correlated with host adaptation in *Salmonella*, thus we also compared at whole genome scale the available genomic sequences of them to evaluate pseudogene composition within each serovar.

Microarray analysis revealed 3771 CDS shared by both serovars while 33 were only present in Enteritidis and 87 were exclusive to Dublin. Pseudogene evaluation showed 177 inactive CDS in *S. Dublin* which correspond to active genes in *S. Enteritidis*, nine of which are also inactive in the host adapted *S. Gallinarum* and *S. Choleraesuis* serovars. Sequencing of these 9 CDS in several *S. Dublin* clinical isolates revealed that they are pseudogenes in all of them, indicating that this feature is not peculiar to the sequenced strain. Among these CDS, *shdA* (Peyer's patch colonization factor) and *mgIA* (galactoside transport ATP binding protein), appear also to be inactive in the human adapted *S. Typhi* and *S. Paratyphi A*, suggesting that functionality of these genes may be relevant for the capacity of certain *Salmonella* serovars to infect a broad range of hosts.

Keywords: Comparative genomics, Host specificity, Pseudogenes, *Salmonella*, *S. Dublin*, *S. Enteritidis*.

1. INTRODUCTION

Infection with non-typhoidal *Salmonella enterica* is a major cause of food-borne disease in humans worldwide [1-3]. Animals and their products are regarded as the main sources of this pathogen, although it may also be present in other potential sources, such as fresh vegetables [4-6]. From over 2500 different serovars of *Salmonella enterica* (defined by their surface antigenic properties, both somatic O antigen and flagellar H antigens) about 50 are significant pathogens of animals and humans. Acute infections in humans can develop in one of four ways: enteric fever, gastroenteritis, bacteremia, or extraintestinal focal infection [7]. As with other infectious diseases, the course and outcome of the infection depend on a variety of factors, including the inoculating dose, the immune status of the host, and the genetic background of both the host and the infecting organism.

Although *S. enterica* serovars are genetically very similar, they differ significantly in host range and disease

spectrum. *S. enterica* serovars may be classified as ubiquitous, host-restricted or host-specific. Ubiquitous serovars, which include Typhimurium and Enteritidis, most commonly produce self-limiting gastrointestinal infections in a wide range of hosts. Host-specific serovars, such as Typhi in humans or Gallinarum in fowl, cause severe systemic diseases in their specific hosts. A few *Salmonella* serovars, such as Choleraesuis and Dublin, have a narrow host range and are classified as host-restricted [8].

Host-restricted and host-specific serovars are generally more prone to cause invasive disease than ubiquitous serovars [9, 10]. Globally, human extra-intestinal salmonellosis is generally associated with those serovars that are also associated with gastroenteritis, as is the case with *S. Enteritidis* and *S. Typhimurium*. However, certain serovars are more prone to cause invasive infections than others, as is clear when the percentage of isolates from bacteremia related to total cases (invasive index) is calculated [7, 11]. For *S. Typhimurium* and *S. Enteritidis*, the invasive index ranges from 1 to 7% [11, 12], while for *S. Dublin* different reports indicate that the invasive index ranges from 50% to 70% [7, 11, 13-15]. Loss of gene function through pseudogene accumulation has been indicated as a hallmark of host-

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specific pathogenic bacteria as compared to their host-generalist relatives [16-22].

The Enteritidis (O: 1, 9, 12: gm: -) and Dublin (O:1, 9, 12: gp: -) serovars share antigenic properties and are phylogenetically closely related, yet they seem to differ significantly in pathogenic potential [23, 24]. *S. Enteritidis* commonly causes gastroenteritis but rarely causes invasive disease in humans. *S. Dublin* usually infects cattle causing abortion and systemic infection, but occasionally can be found infecting other hosts such as pigs and humans. On the rare occasions when it infects humans it often results in bacteraemia with severe disease and high mortality [25-27]. Characterization of the mechanisms underlying these differences is central to a more general understanding of the invasiveness of salmonellae. To date only one complete genome of a *S. Enteritidis* strain (P125109, hereafter referred as PT4) and two *S. Dublin* isolates (CT_02021853 and 3246) have been sequenced and annotated and are publicly available [28], [<http://www.ncbi.nlm.nih.gov/genomeprj/19467>] [29].

To gain new insights into genetic differences that could help to understand the basis of such marked different pathogenic behaviors, here we describe a comparative study between *S. Enteritidis* and *S. Dublin*. We conducted microarray-based comparative genomics between four *S. Dublin* clinical isolates and the core genome resulting of the comparative genome analysis of 29 *S. Enteritidis* isolates previously reported by us [30]. Further the pseudogene content of each serovar was also evaluated using the available genome sequences.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

Twenty-nine *S. enterica* serovar Enteritidis isolates from diverse origins in Uruguay were previously characterized by microarray and phenotypic assays [30, 31]. Seven *S. enterica* serovar Dublin isolates from human infections in Uruguay were used in this study (Table 1).

Isolates were maintained frozen at -80°C in LB containing 25% glycerol. Bacteria were cultured in LB broth, or on LB containing 1.6% agar, or Tryptic Soy Agar. All isolates were identified as *Salmonella enterica* using standard biochemical tests and microbiological methods.

Serovar was determined by the slide agglutination test for O antigen and the tube agglutination test for H antigen, using commercially available anti-O and anti-H antisera (Difco, France). Differentiation between *S. Enteritidis* and *S. Dublin* was confirmed by PCR for the detection of genetic regions specific for Enteritidis [32] and by sequencing the *fliC* gene, which differs between these serovars.

2.2. Comparative Genomic Hybridization Analysis (CGH)

Four *S. Dublin* strains were analyzed by CGH using the *Salmonella* generation IV microarray [30, 33, 34] with PT4 DNA [28] as reference. The array is non-redundant and contains coding sequences from the following eight genomes: *S. enterica* serovar Typhi (*S. Typhi*) CT18, *S. Typhi* Ty2, *S. Typhimurium* LT2 (ATCC 700220), *S. Typhimurium* DT104 (NCTC 13348), *S. Typhimurium* SL1344 (NCTC 13347), *S. Enteritidis* PT4 P125109 (NCTC 13349), *S. Gallinarum* 287/91 (NCTC 13346), and *S. bongori* 12419 (ATCC 43975). Total DNA (including plasmid DNA) was extracted from each strain using a Genome DNA extraction kit (Promega) and quantified by agarose gel electrophoresis. Labeled DNA from *S. Enteritidis* PT4 (control sample) and one of the query *Salmonella* strains (experimental sample) were mixed in equal volumes and concentrations and hybridized to the microarray slides as previously described [30]. Data were normalized to the median value, and the total list of 6,871 genes was filtered by removing those spots with a high background and those without data in at least one of the replicates (three slides per strain, duplicate features per slide). After filtering, a list of 5,695 genes was obtained that corresponded to genes that presented a valid signal in at least one of the strains analyzed. Data analysis was performed on Excel files, following criteria previously described [30].

Genes assigned as absent/divergent in all *S. Dublin* isolates were compared to the core genome of *S. Enteritidis* as defined in our previous study [30]. Genes detected as present in all *S. Dublin* isolates but absent in *S. Enteritidis* PT4 were compared with the *S. Enteritidis* dispensable genome as well as with the fully sequenced *Salmonella* isolates available in the NCBI database. Genes encoded in plasmids were not considered in this analysis.

Table 1. Description of the *S. Dublin* Isolates Analyzed in This Work

Strain Designation	Year of Isolation	Origin ^a	CGH ^b	9 CDS Sequence ^c
SDU1	1995	blood	+	+
SDU2	2004	blood	+	+
SDU3	2006	blood	+	+
SDU4	2008	blood	-	+
SDU5	2000	feces	+	+
SDU6	2005	feces	-	+
SDU7	2008	feces	-	+

+: tested. -: non-tested. ^a Correspond to human samples. ^b Comparative genomics hybridization. ^c Nucleotide sequence of CDS as described in text and Table 2.

2.3. Web Based Comparative Genomics

The sequences and annotations of the *Salmonella* genomes analyzed here were obtained from the data available at NCBI [<http://www.ncbi.nlm.nih.gov/>]. Nucleotide sequences were analyzed using the sequence visualization and annotation tool Artemis version 10 [35]. The search for homologous genes and regions was performed using Blast-n and Blast-p online at the NCBI website.

2.4. Pseudogene Screening in *S. Dublin* Isolates

The sequences of nine CDS detected as pseudogenes in the *S. Dublin*, *S. Gallinarum* and *S. Choleraesuis* sequenced

strains were evaluated in all 7 *S. Dublin* isolates included in this work. Genomic DNA was extracted from the bacterial strains using DNeasy blood and tissue kit (Qiagen). Specific primers for amplification and sequencing were designed based on the sequences of the corresponding regions in the genomes of *S. Enteritidis* PT4 and *S. Dublin* CT_02021853 (Table 2). PCRs were conducted using a 10:1 mix, in terms of units, of *Taq* Polymerase and *Pfu* Polymerase (Fermentas) and the PCR products were sequenced. Sequences were analyzed and aligned using BioEdit Sequence Alignment editor version 7.0.9.0, 2007.

Table 2. Description of the Primers used for Amplifying and Sequencing the 9 CDS Described as Pseudogenes in *S. Gallinarum*, *S. Dublin* and *S. Choleraesuis* Fully Sequenced Strains

Gene in <i>S. Enteritidis</i>	Primer Sequence (5'-3')	
SEN0042	TATTCAAAACTTGCTTAGAAAGTAGAG	Forward
	CGGGTCTTGTGCATAAATGG	Reverse
	GGAAAGTAATGTTGTCCGCTG	Reverse2
SEN0784	GTGGTAAACATATGTAAATGTTATTTTC	Forward
	AATGTGATTCAGGCTGTGCT	Reverse
SEN2182	AGACCGGATAACGTATTTCTTTTGCC	Forward
	ATCCGCCCTCTTTCAGCCAGGTC	Reverse
	GTGATTGTCCCGACGACTTCTC	Reverse2
SEN2493	TCCAGTTTGCTTCGTGAACG	Forward
	CACTGGCGATGTGACGATT	Forward2
	CAATTCGGCGTAATGACGTT	Forward3
	ATCAACCGGTTTGTCAATTCG	Reverse
	TACCGTCCCAGTCGCCGTTG	Reverse2
SEN2783	GTGAGGTATATCAACAAAAAGACCA	Forward
	TCCAGAGGCAATCCAGGA	Forward2
	TGTGCAGGCGCCGTTG	Forward3
	ACGGACGGGGAGCCAGG	Reverse
	CAACCTCTTTCGTGTATCAACC	Reverse2
SEN2806	GTGCTGGTAGGCGATATTAAG	Forward
	CTTCCCGACGCGGTAT	Forward2
	AACCTGCATTTTCAGTCACTACAG	Reverse
SEN3461	TTTGGCACGGCTGGCGACAT	Forward
	GAATGCCCTGTGGTGGATT	Forward2
	CGTGCCGGAACTATAACAG	Forward3
	AGCACCGACCCGCCCAACA	Reverse
	GCCGCGCAAACCGTAGTTCA	Reverse2
SEN3672	GGCCTGGTCACGTCTGTAAC	Forward
	CTCTCTTTTGTCTTCGGTATCC	Forward2
	TATGACGGTTTGATGACAATGG	Reverse
SEN4290	AACGCTTGAGGATTTAATAGAA	Forward
	CTGATTCAGTACCGTCAGTG	Reverse

3. RESULTS

3.1. Microarray-based Comparative Genomics of *S. Enteritidis* and *S. Dublin* Isolates

The genetic content of the 4 *S. Dublin* isolates was evaluated by microarray and a core genome (i.e. genes present in all strains) was defined. To explore the genetic determinants underlying the phenotypic differences between *S. Dublin* and *S. Enteritidis*, we compared the core genome of *S. Dublin* with the previously defined core genome of *S. Enteritidis* [30]. We found 3771 genes shared by both serovars, whereas 33 genes were only present in *S. Enteritidis* strains (Table 3) and 87 genes were only present in *S. Dublin* isolates (Table 4). The regions of difference found by CGH analysis are similar to the regions of difference obtained from comparison of the genomes of the two sequenced strains PT4 and CT_02021853 (results not shown). From these 120 (33 + 87) genes which are exclusive of one serovar or the other, 53 are bacteriophage-encoded.

As shown in Table 3 four DNA regions and seven single genes were present only in *S. Enteritidis*. Region En1 (SEN083-SEN085) encodes two putative secreted proteins and one sulphatase. BLAST analysis revealed that this region has homologues in several fully sequenced serovars of *Salmonella*, including *S. Gallinarum*, *S. Typhi*, *S. Paratyphi A*, *S. Paratyphi B*, *S. Choleraesuis*, *S. Typhimurium*, *S. Agona*, *S. Newport* and *S. Heidelberg*. Region En2 (SEN1379-1395), corresponds to phage SE14 [28], that includes genes encoding for DNA nucleases and membrane proteins, and was previously postulated to be a region of difference between *S. Enteritidis* and all other *Salmonella*

serovars [30, 36]. Region En3 (SEN1432-1435) corresponds to a genomic island previously described as ROD13 [28] that encodes for idonate dehydrogenase, gluconate dehydrogenase, proteins involved in sugar transport, and proteins similar to those required for hexonate uptake. This genomic island is present in the *S. Gallinarum* genome sequence, but is absent from all other salmonellae sequenced to date. Region En4 (SEN1500-1506) corresponds to part of another genomic region, named ROD14 [28], and encodes for a putative transcriptional regulator akin to the LacI family, and other regulatory proteins probably involved in drug efflux. This region is present in the genome sequences from various *S. Typhimurium* strains, but is degraded in the *S. Gallinarum* and PT4 genome sequences.

Six regions and six isolated genes are present only in *S. Dublin* (Table 4). Region Du1 comprises thirteen genes previously annotated within the genome of *S. Gallinarum* (SG1032-1044) which include proteins that are members of the Rhs family, Clp proteases and exported proteins. Region Du2 (SG1182-1195 and SG1211-1219) corresponds to part of the Gifsy-2-like prophage remnant present in the genome of *S. Gallinarum* [28]. Region Du3 corresponds to genes found in SPI-6 from *S. Typhi* CT18.

Regions Du4, Du5 and Du6, correspond to prophages found in the genome sequence of *S. Typhi* CT18 [16]. Single genes present only in *S. Dublin* strains include a membrane transport protein (SG3368), a putative glycolate oxidase (STY1444) and several phage-related proteins.

Microarray methodology allowed us to detect only presence or absence/divergence of genes, but not small

Table 3. Regions (Reg) and Single Genes (Sing) that form the *S. Enteritidis* Core Genome but Appear as Absent/Divergent in *S. Dublin* Strains

	Gene Range	Homologous	Function/Gene Prediction
Reg En1	SEN0083-0085	CT18, TY2, LT2, DT104, SL1344, SBG, SPA, SGAL	probable secreted proteins, sulfatase
Reg En2	SEN1379-1395 (1387 present)	STY (SOME)	part of PHAGE SE14, <i>ligA</i> , <i>B</i> , <i>C</i> , <i>D</i> , <i>F</i> , <i>ydaD</i>
Reg En3	SEN1432-1435	SGAL	ROD13 genomic island, idonate and gluconate dehydrogenase, sugar transport
Reg En4	SEN1500-SEN1506	LT2, SL1344, (CT18 and SBG some)	part of ROD14 genomic island
Sing En1	SEN0196	SBG	<i>fhuA</i> , ferrichrome iron receptor
Sing En2	SEN0281	NO	<i>safa</i> , fimbrial subunit
Sing En3	SEN0356	SGAL	putative autotransporter
Sing En4	SEN1515	CT18, TY2, LT2, DT104, SL1344, SBG, SPA, SGAL	Ni/Fe-hydrogenase 1 b-type cytochrome subunit HyaC2
Sing En5	SEN1539	CT18, TY2, LT2, DT104, SL1344, SBG, SPA, SGAL	<i>dcp</i> , dipeptidil carboxipeptidaseII
Sing En6	SEN2167	CT18, TY2, LT2, DT104, SL1344, SBG, SPA, SGAL	conserved hypothetical protein
Sing En7	SEN2420	SGAL	putative exported protein

CT18: *S. Typhi* CT18, TY2: *S. Typhi* Ty2, LT2: *S. Typhimurium* LT2, DT104: *S. Typhimurium* DT104, SL1344: *S. Typhimurium* SL1344, SBG: *S. bongori*, SPA: *S. Paratyphi A*, SGAL: *S. Gallinarum*.

Table 4. Regions (Reg) and Single Genes (Sing) that are Present in all *S. Dublin* Strains but Absent in the *S. Enteritidis* Sequenced and Analyzed Isolates

	Gene Range	Homologous	Gene Description
Reg Du1	SG1032-1044	NO	<i>clpB</i> , Rhs proteins, conserved hypot proteins
Reg Du2a	SG1182-1195	SOME SDT, SOME STY	Gyfsi-2 like prophage, phage proteins and cel division inhibitor <i>kil</i>
Reg Du2b	SG1211-1219	STM, SDT, SL	Gyfsi-2 like prophage, phage proteins
Reg Du3a	STY0289-0294	STM, SDT, SL, SPA, TY2, SOME GAL	SPI6, hypothetical and <i>clpB</i> heat shock protease like protein
Reg Du3b	STY0302-0310	STM, SDT, SL, SPA, TY2	SPI6, hypothetical conserved, membrane and lipoproteins
Reg Du3c	STY0320-0323	STM, SDT, SL, SPA, TY2	SPI6, hypothetical and RHS proteins
Reg Du4	STY1020-1036	TY2, SOME STM, SDT, SL	<i>S. Typhi</i> prophage 10, DNA binding and phage proteins, methyltransferase
Reg Du5	STY2043-2045	SOME SDT	<i>S. Typhi</i> degenerate bacteriophage, putative endolysin
Reg Du6	STY3662-3671	TY2, SOME STM	Phage proteins, regulatory protein CII, DNA adenine methylase
Sing Du1	SG1227	STM, SDT, SL	phage tail protein
Sing Du2	SG3368	STY, STM, SDT, SL, SBG, SPA	possible membrane transport protein
Sing Du3	STY0602	SDT, SBG, SPA	phage integrase
Sing Du4	STY1444	TY2, STM, SDT, SL, SBG, SPA	putative glycolate oxidase
Sing Du5	STY2690	TY2, STM, SDT, SL	hypothetical protein
Sing Du6	STY3029	NO	transposase

CT18: *S. Typhi* CT18, TY2: *S. Typhi* Ty2, LT2: *S. Typhimurium* LT2, DT104: *S. Typhimurium* DT104, SL1344: *S. Typhimurium* SL1344, SBG: *S. bongori*, SPA: *S. Paratyphi* A, SGAL: *S. Gallinarum*.

variations in gene sequences. Considering that pseudogene accumulation has been postulated to be involved in host restriction and adaptation, we decided to compare the pseudogene content among the available genomic sequences of both serovars and then evaluate if the Uruguayan *S. Dublin* clinical isolates harbour a particular set of these pseudogenes.

3.2. Pseudogene Analysis

Analysis of the genomes available in the NCBI database for *S. Dublin* CT_02021853 and *S. Enteritidis* PT4 strains, show that they have 289 CDS and 111 CDS annotated as pseudogenes respectively. From the 289 *S. Dublin* pseudogenes, 7 have no homologues in the *S. Enteritidis* sequence, and 32 correspond to intergenic regions. Among the others, 38 are homologous with 29 pseudogenes in *S. Enteritidis*, whereas the other 212 pseudogenes in *S. Dublin* correspond to 177 active genes in *S. Enteritidis*. Conversely, there are 83 *S. Enteritidis* pseudogenes that appear to be functional in *S. Dublin* CT_02021853. We analyzed the pseudogenes specific of each serovar, and grouped them in different classes according with their homology with functional CDS (Table 5).

S. Enteritidis, *S. Dublin* and *S. Gallinarum* form a related cluster of serovars but with marked differences in host-specificity, thus we also included *S. Gallinarum* in the pseudogene analysis. There is a single annotated genome sequence for this serovar that contains 309 pseudogenes [28] and among them only 21 are also annotated as pseudogenes

in *S. Dublin* but not in *S. Enteritidis* (Table 6). This group of CDS includes nine that are also inactive (7) or completely absent (2) in the other host-restricted serovar *S. Choleraesuis* [37] and are described in Table 6.

Table 5. Distribution of the *S. Enteritidis* or *S. Dublin* Specific Pseudogenes among Different Functional Classes

	Pseudogenes SEN (%) ^a	Pseudogenes SDU (%) ^b
Surface	20.48	37.43
metabolism	10.84	22.91
regulatory	1.20	10.06
transposase	15.66	1.68
hypothetical	14.46	18.99
Virulence	3.61	1.12
ribosomal	0.00	1.12
Phage	26.51	1.12
Other	7.23	5.59

^a: distribution of the 83 *S. Enteritidis* specific pseudogenes. ^b: distribution of the 177 *S. Dublin* specific pseudogenes.

Overall, the presence of these nine pseudogenes could be regarded as potential distinguishing markers of host-

Table 6. List of 21 CDS that are Predicted to be Pseudogenes in *S. Dublin* and *S. Gallinarum* but Active Genes in *S. Enteritidis* PT4

Gene	Choleraesuis Pseu/Absent ^a	Gene Description
SEN0042	YES	putative transport protein
SEN0325	NO	possible transmembrane regulator
SEN0621	NO	putative sigma54 dependent transcriptional regulator
SEN0784	YES	hypothetical protein
SEN1194	NO	putative membrane transport protein
SEN1331	NO	conserved hypothetical protein
SEN1335	NO	putative membrane protein
SEN1524	NO	putative membrane protein
SEN2173	NO	putative transcriptional regulator
SEN2182 ^b	YES	<i>mglA</i> , galactoside transport ATP binding protein
SEN2493 ^b	YES	<i>shdA</i> , Peyer's patch colonization and shedding factor
SEN2611	NO	putative type I secretion protein, SPI9 ATP-binding protein
SEN2783	YES	conserved hypothetical protein
SEN2806	YES	<i>ygcY</i> probable glucarate dehydratase
SEN3461	YES	<i>lpfC</i> , outer membrane usher protein
SEN3537	NO	<i>rfaZ</i> (<i>waaZ</i>) LPS core biosynthesis protein
SEN3571	NO	<i>yicJ</i> sodium galactoside family symporter
SEN3672	YES	probable PTS system permease
SEN3954	NO	<i>nfi</i> , putative endonuclease V
SEN4259	NO	hypothetical protein
SEN4290	YES	Type I restriction-modification system methyltransferase

^a YES indicates that the corresponding gene is a pseudogene or is absent in the genome of *S. Choleraesuis* SC-B67. NO indicates that corresponds to an active gene. ^b indicates that corresponds to a pseudogene in the sequences of *S. Typhi* CT18 and Ty2 as well as in *S. Paratyphi A* ATCC 9150 and *S. Paratyphi A* AKU_12601, as analyzed by Holt *et al.* [22].

restricted serovars, thus we decided to evaluate their sequences in all *S. Dublin* Uruguayan isolates obtained from human infections (4 strains analyzed by CGH as described above plus 3 other isolates, Table 1). We found that all 7 isolates have these 9 CDS inactivated as pseudogenes, either by the same point mutations that are present in the fully sequenced *S. Dublin* CT_02021853 strain (7 of the 9 CDS) or by a different deletion as is the case of the CDS homologous to SEN2493 and SEN4290. Recently the genome sequence of another *S. Dublin* strain (*S. Dublin* 3246), was publicly released (GenBank: CM001151) [29]. We found that all 9 CDS are also pseudogenes in this strain. Further, in all but one of them the inactivation is due to the same changes than in *S. Dublin* CT_02021853. Interestingly, the exception is the CDS corresponding to SEN4290, which possess the same deletion than the Uruguayan strains analyzed here.

DISCUSSION

S. Enteritidis and *S. Dublin* are two closely related serovar with marked differences in pathogenic traits and epidemiological behavior, thus it is reasonable to assume

that genomic comparison between them could shed some light on the molecular basis of these differences. A single previous report described a microarray-based genome comparison [38], and here we conducted a similar analysis using a different set of field isolates and microarray chip. Further, we now report a comparison of the full genome sequences of *S. Enteritidis* and *S. Dublin* particularly looking at differences in pseudogene composition between them.

Our comparative genome hybridization study predicted 33 genes specific to *S. Enteritidis* and 87 specific to *S. Dublin*. The analysis revealed four genetic regions and seven single genes that seem to be exclusive of *S. Enteritidis* core genome, as well as six regions and six single genes specific for *S. Dublin*. These results corroborate and extend the previous report where 3 *S. Dublin* and 24 *S. Enteritidis* strains were compared [38]. This report described the same four regions specific for *Enteritidis* but only one of the six *S. Dublin* regions found by us. This particular region, that we denominated Du3, corresponds to regions B24, B25_a and B25_b as of the earlier report. Region Du3 corresponds to genes found in SPI-6 from *S. Typhi* CT18. This region encodes a ClpB heat-shock protease-like protein, as well as

different membrane proteins and lipoproteins that belong to the T6SS encoded in this island. Interestingly, this region includes a gene in the *rhs* family (STY0321) that has no homologue in the CT_02021853 genome sequence.

Among the other regions specific for *S. Dublin* described here, Region Du1 was recently proposed to be a pathogenicity island (SPI-19) identified in *S. Gallinarum*, *S. Dublin*, *S. Weltevreden* and *S. Agona* that encodes a type-6 secretion system (T6SS) [39]. In *S. Enteritidis*, an internal deletion has eliminated most of the island. Region Du2, includes various bacteriophage regulatory proteins, recombinases, transposases, and structural proteins. It also includes one gene (SG1186) previously annotated as encoding a putative phage-encoded cell division inhibitor protein belonging to the *kil* super-family and associated with the capacity to inhibit the essential *ftsZ* cell-division gene [40]. *ftsZ* expression is altered during the intracellular phase of infection with *S. enterica*, a process that is independent of *sulA*, a known inhibitor of *ftsZ* [41]. Genes encoding proteins belonging to the same super-family are also present in several *S. Typhi* genome sequences, as well as in other enterobacteria (e.g. different STEC strains, *Shigella flexneri*, *Shigella dysenteriae* and others) as revealed by Blast-p analysis, suggesting a possible role for these proteins in pathogenesis. Regions Du1 and Du2 were not represented in the microarray used by Porwollik and collaborators [38], thus we cannot exclude that these regions were also present in the strains studied there, but simply not found because of the particular microarray used. Regions Du4, Du5 and Du6, correspond to prophages found in the genome sequence of *S. Typhi* CT18 [16]. Region Du4 comprises 17 genes from a lambdoid bacteriophage that include several CDS encoding for DNA binding proteins. Region Du5 includes 3 genes that are part of a degenerate bacteriophage; one of these (STY2044) encodes a putative endolysin similar to several lysozymes from *E. coli* and *Shigella* strains. Region Du6 spans 10 genes including a DNA adenine methylase (STY3667), regulatory proteins and endonucleases. These 3 regions of difference were not found in the earlier report, despite the CDS been present in the microarray. Instead, that work reported differences in other prophage-derived genes. Thus, it could be that the genomes of the particular set of strains used in both studies possess different prophage composition. The analysis of Du4-Du5-Du6 in both *S. Dublin* sequenced isolates, revealed that regions Du5 and Du6 are very conserved in both strains whereas region Du4 is almost complete in CT_02021853 but incomplete and less conserved in strain 3246, supporting the hypothesis of different content in phage genes among *S. Dublin* isolates.

Among the seven single genes that are for the first time described here as absent in *S. Dublin* strains, *safA* (SEN0281) and *dcp* (SEN1539) are of special interest. *safA* is the first gene of the *saf* fimbrial operon and encodes a lipoprotein. The operon forms part of the degraded pathogenicity island SPI-6 in the *S. Enteritidis* chromosome. This operon is not annotated in the *S. Dublin* genome sequences available. However, Blast analysis revealed that this is a region highly conserved at a nucleotide level between PT4 and both *S. Dublin* sequenced isolates. There are several stop codons in the *S. Dublin* sequence homologous to *safA*, suggesting that this gene is in process

of degradation. The fact that we cannot detect *safA* by CGH in the *S. Dublin* Uruguayan isolates may be related with this. The *dcp* gene encodes for dipeptidyl-carboxypeptidase II, which is highly conserved among the Enterobacteriaceae. This gene has been described previously as a frequent site for SNPs in *S. Enteritidis* [42], and it is absent from the CT_02021853 sequence.

Overall, the CGH analyses did not detect clear differences in genes that have been previously reported as required for virulence to explain the differences in pathogenicity of both serovars. However, the presence/absence of a gene, as detected by this methodology, does not inform about its expression, thus these results should be interpreted with caution.

The high number of pseudogenes detected in CT_02021853 suggests that this mechanism might be relevant in the process of host adaptation of this serovar, as well as in the different epidemiological and pathogenic behavior of *S. Dublin* when compared with *S. Enteritidis*. As we describe in Table 5, we observed a differential distribution of functionality amongst the CDS inactive in *S. Enteritidis* and *S. Dublin*. More than 40% of the pseudogenes specific for *S. Enteritidis* correspond to CDS related to phages or transposases but only 12% with those involved in metabolism and regulatory proteins. Conversely, among the pseudogenes specific for *S. Dublin* 33% correspond to CDS encoding proteins involved in central metabolism or regulatory proteins and 37% to CDS related to surface structures but only 3% to phages and transposases. These observations may be relevant to understand the host restriction of *S. Dublin*.

We found 21 CDS that appear to be active genes in the broad host-range *S. Enteritidis* but pseudogenes in the host-restricted *S. Dublin* and in the host-specific *S. Gallinarum*. From this set of CDS, 9 are pseudogenes as well in the other host-restricted serovar *S. Choleraesuis* suggesting that their inactivation could be relevant as genetic determinants of host adaptation. These nine CDS correspond to two hypothetical proteins (SEN0784 and SEN2783), one putative transport protein (SEN0042), the gene encoding the outer membrane usher protein LpfC (SEN3461), one probable phosphotransferase system permease (SEN3672), one gene encoding a putative Type I restriction modification system protein (SEN4290), and the gene encoding a probable glucarate dehydratase 2 (SEN2806 or *ycgY*). The other two genes that complete this list are *mglA* (SEN2182) and *shdA* (SEN2493), which are pseudogenes in *S. Typhi* CT18 and Ty2 as well as in *S. Paratyphi A* ATCC 9150 and *S. Paratyphi A* AKU_12601 [22]. ShdA is involved in colonization of Peyer's patches by *S. Typhimurium* and in shedding of the bacteria after infection [43-45]. MglA is a galactoside transport ATP binding protein. The roles of these genes in the broad host-range of *S. Enteritidis* remain to be established.

All these nine CDS are pseudogenes in the seven *S. Dublin* clinical isolates evaluated in this work, as well as in the other fully sequenced isolate *S. Dublin* 3246, suggesting that the loss of their functionality is not a consequence of random mutation. Two of these 9 pseudogenes in the Uruguayan isolates have lost their functionality by mutations

that are different from those seen in the sequenced strain CT_02021853 suggesting that this loose of functionality involves a process of convergent evolution.

In conclusion, our results show several genetic differences that may help to explain why such close related organisms can nevertheless behave with such marked differences. Comparison of larger numbers of field strains at full genome scale is becoming increasingly feasible, and may provide new insights into the genetic basis of host adaptation.

CONFLICT OF INTEREST

None declared.

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