Comparative Analysis of *Salmonella* Plasmids and Mobile Elements by Whole Genome Sequencing

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ABSTRACT

Salmonellosis is an important global public health issue. The genus *Salmonella* includes more than 2,500 different serotypes. While mobile elements play an important role in *Salmonella* evolution, most investigations have focused on plasmids, from commonly isolated *Salmonella* serotypes, that harbor antimicrobial resistance and virulence genes. In order to assess the mobile element repertoire of *Salmonella* in a less biased fashion, we used whole genome sequencing with the SOLiD™ next generation sequencing system to characterize mobile elements and plasmids in 16 uncommon human disease associated *Salmonella* serotypes. Putative large plasmids were identified, subsequent to *de novo* assembly, by finding scaffolds that do not match any *Salmonella* chromosome. One IncI1 plasmid of approximately 120 kb and one element of approximately 91 kb were found in serotype Inverness; the 91 kb element shows characteristics of a mobile element, which was also identified in serotypes Rubislaw and Urbana. These elements lack genes encoding any previously described proteins for self-replication and partitioning, but contains genes for conjugative transfer proteins and a type IVB pili operon homologous to the SPI-7 encoded pili operon of *S.* Typhi. Putative IncI1 plasmids harboring virulence genes were identified in Mississippi and Urbana isolates. A serotype Montevideo isolate was found to contain two plasmids, one IncW plasmid carrying an integron class 1 element that encodes sulfonamide and aminoglycoside resistance. And one IncH12 plasmid encoding resistance to several heavy metals, disinfectants, and tetracycline. We have thus identified novel putative mobile elements in *Salmonella* and shown the feasibility of using full genome sequencing and *de novo* assembly for rapid initial characterization of bacterial mobilome.

INTRODUCTION

Salmonella enterica is a foodborne pathogen which causes an estimated 1.4 million human cases annually in the US (Mead et al., 1999). It is a highly diverse pathogen with more than 2,500 different serotypes of *Salmonella* described. Current comparative genomic research shows that *Salmonella* is characterized by a high genomic plasticity. It has further been demonstrated that mobile elements play an important role in the evolution of *Salmonella*. Thus far the study of plasmids has been mainly focused on multidrug resistant *Salmonella* isolates and virulence plasmids. In this study we describe the identification of conjugative plasmids and other mobile elements in a selection of isolates that was not biased towards highly virulent or multidrug resistant isolates. These plasmids carry antibiotic resistance and virulence genes and suggest a larger repertoire of transmissible genetic elements in *Salmonella* than previously recognized. These elements may allow for rapid emergence of new pathogenic strains and the identification and comparison of these elements may prove helpful in outbreak investigations.

MATERIALS AND METHODS

Isolates. Sixteen *Salmonella* isolates were selected for whole genome sequencing. These isolates represent the serotypes Senftenberg, Rubislaw, Gaminara, Hvittingfoss, Minnesota, Urbana, Alachua, Adelaide, Wandsworth, Johannesburg, Baildon, Mississippi, Inverness, Montevideo, Uganda and Give.

Genome sequencing and assembly. Genomes were sequenced using the SOLiD™ system (Applied Biosystems, Foster City). Mate-paired libraries with approximately 1.5 kb inserts were constructed and deposited on one quarter of a flowcell. Then, 25 bp reads were obtained from each of the F3 and R3 tags. After correcting errors in colorspace reads using a modified version of the spectral alignment tools from the EULER-USR package (Chaisson, et al., 2009), de novo assembly was performed using the SOLiD™ de novo pipeline, which employs the Velvet assembly engine (Zerbino & Birney, 2008). Scaffolds were aligned to two reference genomes (S. Typhimurium LT2 and S. Enteritidis) and concatenated into pseudogenomes. Scaffolds that did not match the chromosomes of the reference genomes considered to be putative plasmids or strain specific transposable elements

Whole genome alignments. Automated annotation was performed with the RAST server (http://rast.nmpdr.org; Aziz et al. 2008) and whole genome alignments were performed using the Mauve Genome Alignment Software (Darling et al., 2004). Circular representations of the plasmids were generated with DNA Plotter (Carver et al., 2009).

RESULTS AND DISCUSSION

Two plasmids carrying antimicrobial resistance genes were identified in the same isolate of *Salmonella* Montevideo. Two different plasmids were identified in *S.* Montevideo: (i) a conjugative plasmid of 53 kb encoding antimicrobial resistance genes and (ii) a large (299 kb) conjugative plasmid of the IncH12 incompatibility group (table 1, table 2 and figure 1). The large plasmid is similar to plasmids found in an APEC *E. coli* strain (pAPEC-O1-R) and an *Enterococcus cloacae* strain (pEC-IMPQ) and encodes multiple heavy metal and disinfectant resistance genes. Despite the large size of these plasmids, they encode several mechanisms for plasmid stability, which explains the maintenance of these large plasmids within their host. The presence of several resistance genes in these two conjugative plasmids is an advantage for survival in farm environments, and the potential to be transferred to other strains is a potential public health concern.

Similar IncI1 plasmids were identified in *Salmonella* serotypes Inverness, Urbana and Mississippi. Similar IncI1 plasmids were identified in three isolates representing different uncommon serotypes (table 1, figure 2). These plasmids encode virulence genes (e.g., hemolysin, adhesins) and genes for the type IV secretion system (table 2). In addition, these IncI1 plasmids have some regions of homology with plasmids in *Serratia* sp. (pADAP) and *S.* Typhimurium (pSLT-BT). Currently, there are no sequences of plasmids with similar backbone characteristics available, suggesting a considerable repertoire of conjugative plasmids among *Salmonella* serotypes.

***Salmonella* serotypes Urbana, Rubislaw and Inverness encode a Pilus type IV operon in a putative mobile element.** A type IVB pilus operon is present in the *Salmonella* Pathogenicity Island 7 (SPI-7) of *Salmonella* Typhi. This operon encodes an important virulence determinant which is used by *S.* Typhi to enter human intestinal epithelial cells (Zhang et al., 2000). We identified this operon in isolates representing *Salmonella* Inverness, Rubislaw and Urbana (table 2). When we compared the region surrounding this operon, we identified that a part of SPI-7 is present in these isolates. In the same region genes involved in conjugative transfer, plasmid stability genes and an integrase were found.

Figure 1. Circular representation of plasmids identified in *Salmonella* Montevideo. pS5403-1 is 53 kb and encodes antimicrobial resistance genes, the cluster of genes outside this plasmid represents a comparison of an integron class 1 homologous to an integron inserted in pSN254, shaded triangles represent similar regions. pS5403-1 is 299 kb and encodes heavy metals and antimicrobial resistance genes. Inner circles represents CG%. Genes were color coded, as follow: resistance, pink; plasmid transfer, blue; transposition/IS, orange; replication, green; plasmid stability, light blue; metabolism, brown; hypothetical proteins, grey.

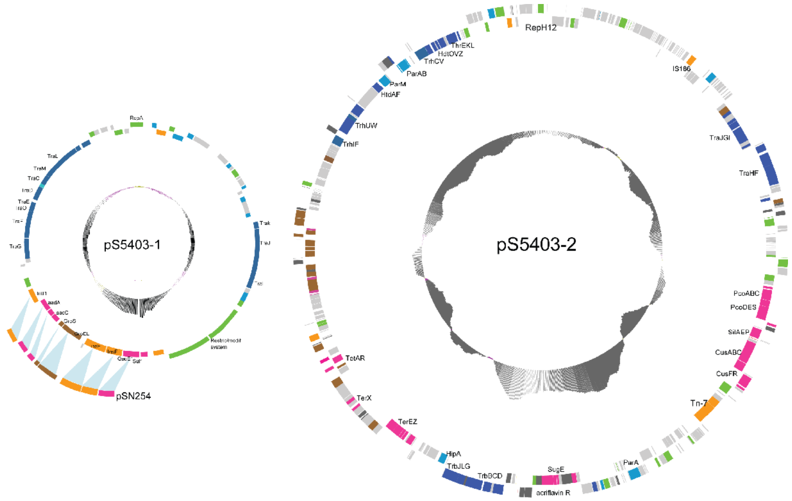


Table 1. Basic characteristics of plasmids and putative ICE identified by the *novo assembly*

Serotype Isolation source Year	Montevideo Human 2004		Inverness Human 2005		Urbana Human 2008		Mississippi Human 2005	Rubislaw Human 2005
Element	Plasmid pS5403-1	Plasmid pS5403-2	Plasmid pR83668	Putative ICE R83668	Plasmid pR82977	Putative ICE R82977	Plasmid pA4633	Putative ICE A4653
Size (bp)	53,632	299,644	121,190	91,394	123,020	130,973	122,532	103,056
G+C content (%)	52.53	48	50.58	53.99	50.0	52.4	50.70	52.85
Replication type	IncW	IncH12	IncI1	-	IncI1	-	IncI1	-
No. conjugative transfer genes	15	21	35	4	34	5	34	5
IS/transposases	6	2	5	3	5	5	6	5

Table 2. Antimicrobial resistance, virulence genes and elements for plasmid stability encode in plasmids and putative ICE

Features	Plasmid pS5403-1	Plasmid pS5403-2	Plasmid pR83668	Putative ICE R83668	Plasmid pR82977	Putative ICE R82977	Plasmid pA4633	Putative ICE A4653
Plasmid stability	*ArdBK, CcgAIC, StbAC, EcoR124IIMSR*	*parMR, parAB,* DNA-methylases	*klcA, parAB, psiAB, hok, relE/parE, kfrAs,* DNA-methylase	Type II restriction endonuclease	*klcA, parAB, psiAB, hok, relE/parE, vagCD,* DNA-methylase	Type II restriction endonuclease	*klcA, parAB, psiAB, hok, relE/parE, kfrAs,* DNA-methylase	Type II restriction endonuclease
Antimicrobial resistance	*sul1,qacEΔ1, aacC, aadA, strAB*	*pcoABCDE, pcoSR, silAEP, cusBCF, cusRS, sugE, NodT, terEZW, tetAR*	-	-	-	-	-	-
Virulence	-	-	Hemolysin, *hilC,* adhesin, *ldaA, fedA, iroN*	Hemolysins, adhesins, *yapH,* Pil operon	Hemolysin exporter, *hilC,* adhesin, *myfA, vagDC*	Hemolysins, adhesins, *yapH,* Pil operon, *sopE*	Hemolysin, *hilC,* adhesin, *ldaA, fedA,iroN*	Hemolysins, adhesins, *yapH,* fimbria, Pil operon

These results suggest that Type IVB pilus operon on serotypes Inverness, Rubislaw and Urbana could have been acquired by horizontal gene transfer. Studies have shown precise excision of SPI-7 (Bueno et al., 2004), however no proof of *in vitro* transfer has been found. With presence of a similar conjugative plasmid in serotypes Inverness and Urbana and this putative mobile element, we can speculate that the IncI1 plasmid could be a helper plasmid involved in the mobilization of this element; however further research is needed to prove this. Amino acidic comparison of the PilS, PilQ and PilV ORFs, showed that this operon is highly conserved on Typhi. However the Pilus operon in the newly sequenced genomes is more divergent, and the operons of Inverness and Urbana cluster together (figure 3). The separate placement of the Inverness and Urbana isolates is interesting because these isolates contain the putative IncI1 helper plasmid.

Figure 2. Circular representations of IncI1 plasmids identified in *Salmonella* serotypes Urbana (second circle), Inverness (third circle) and Mississippi (outer circle). Plasmids were aligned against the Urbana plasmid. Inner circle represents the CG%, outside genes clusters represent insertions. Genes were color coded, as follow: virulence, red; plasmid transfer, blue; transposition/IS, orange; replication, green; plasmid stability, light blue; metabolism, pink; hypothetical proteins, grey. Arrows indicate the corresponding plasmid.

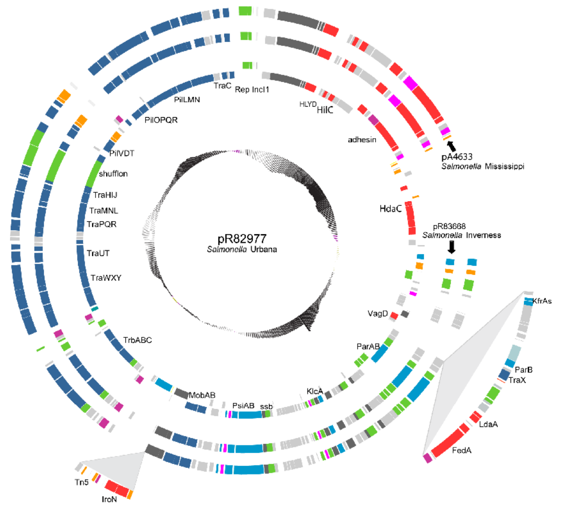
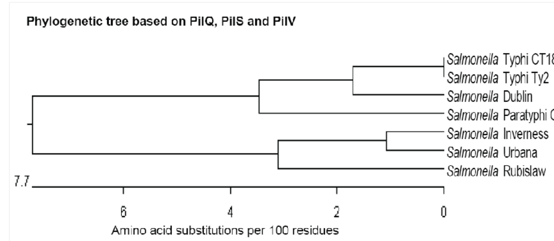


Figure 3. UPGMA tree based on the amino acidic sequences of PilS, PilV and PilQ proteins encoded in SPI-7 in *Salmonella* serotypes Typhi, Paratyphi C and Dublin and in Pilus operon in serotypes Inverness, Rubislaw and Urbana



CONCLUSIONS

Plasmids and mobile elements carrying antimicrobial resistance and virulence genes are widely distributed in uncommon *Salmonella* serotypes.

Putative novel mobile elements in *Salmonella* were identified by whole genome sequencing with the SOLiD™ next generation sequencing technology.

Identification and characterization of plasmids and mobile elements in *Salmonella enterica* is essential to understand the emergence of new pathogenic serotypes and will help in outbreak investigations

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