Salmonella paratyphi C: Genetic Divergence from Salmonella choleraesuis and Pathogenic Convergence with Salmonella typhi

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Abstract

Background: Although over 1400 Salmonella serovars cause usually self-limited gastroenteritis in humans, a few, e.g., Salmonella typhi and S. paratyphi C, cause typhoid, a potentially fatal systemic infection. It is not known whether the typhoid agents have evolved from a common ancestor (by divergent processes) or acquired similar pathogenic traits independently (by convergent processes). Comparison of different typhoid strains with non-typhoidal Salmonella lineages will provide excellent models for studies on how similar pathogens might have evolved.

Methodologies/Principal Findings: We sequenced a strain of S. paratyphi C, RKS4594, and compared it with previously sequenced Salmonella strains. RKS4594 contains a chromosome of 4,833,080 bp and a plasmid of 55,414 bp. We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 152 pseudogenes (149 in the chromosome and 3 in the plasmid). RKS4594 shares as many as 4346 of the 4,640 genes with a strain of S. choleraesuis, which is primarily a swine pathogen, but only 4008 genes with another human-adapted typhoid agent, S. typhi. Comparison of 3691 genes shared by all six sequenced Salmonella strains placed S. paratyphi C and S. choleraesuis together at one end, and S. typhi at the opposite end, of the phylogenetic tree, demonstrating separate ancestries of the human-adapted typhoid agents. S. paratyphi C seemed to have suffered enormous selection pressures during its adaptation to man as suggested by the differential nucleotide substitutions and different sets of pseudogenes, between S. paratyphi C and S. choleraesuis.

Conclusions: S. paratyphi C does not share a common ancestor with other human-adapted typhoid agents, supporting the convergent evolution model of the typhoid agents. S. paratyphi C has diverged from a common ancestor with S. choleraesuis by accumulating genome novelty during adaptation to man.

Citation: Liu W-Q, Feng Y, Wang Y, Zou Q-H, Chen F, et al. (2009) Salmonella paratyphi C: Genetic Divergence from Salmonella choleraesuis and Pathogenic Convergence with Salmonella typhi. PLoS ONE 4(2): e4510. doi:10.1371/journal.pone.0004510

Editor: Malcolm James Horsburgh, University of Liverpool, United Kingdom

Received: October 25, 2008; Accepted: December 15, 2008; Published: February 20, 2009

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Funding: This work was supported by a Canadian Institutes of Health Research grant to RNL; and a grant of Harbin Medical University, National Natural Science Foundation of China grants (NSFC30370774, NSFC30870098), a 985 Project grant of Peking University Health Science Center, a Specialized Research Fund for the Doctoral Program of Higher Education (No. 20030001029), and a Discovery Grant from Natural Sciences and Engineering Research Council of Canada to SLL. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Salmonella are important human and animal pathogens [1,2], and over 1400 serovars have the potential to cause human gastroenteritis, which is essentially a self-limited disease. However, a few Salmonella serovars, such as S. typhi and S. paratyphi A, B and C, elicit typhoid, which is a serious systemic infection with high mortality rates [3,4]. S. paratyphi C as a typhoid agent [5–7] is not reported as frequently as S. typhi or S. paratyphi A or B, partly because this pathogen shares the antigenic formula 6,7,c:1,5 with S. choleraesuis and S. typhimurium and clinical identification of Salmonella isolates usually does not go beyond serotyping, although molecular methods are available to reliably distinguish S. paratyphi C from other Group C members [8]. As the gastroenteritis-causing and typhoidal Salmonella serovars are all closely related, sharing up to about 90% of their genetic contents [9–14], it is natural to ask how typhoid pathogenicity has developed in just a few of the thousands of Salmonella serovars. Specifically, are these similar pathogens the products of divergent (recent common ancestry) or convergent (common pathogenic
traits incorporated into different genetic backgrounds) evolutionary processes?

Speculations have been made regarding these questions. The overwhelming genetic similarity (homologous genes having over 97% DNA sequence identity) and sharp pathogenic differences (self-limited local infections vs potentially fatal systemic infections) among the *Salmonella* serovars suggest horizontal acquisition of different pathogenic traits by these closely related bacteria. Whole genome comparisons at the physical map level reveal blocks of genomic insertions in different *Salmonella* lineages [15–22].

Genomic sequence comparisons identified 11–13% unique genes between *S. typhi* and *S. typhimurium* [9,10,12]. These results indicate that the *Salmonella* core genome provides a genetic framework for specific pathogenic determinants to be housed: acquisition of gastroenteritis- or typhoid-causing traits may “direct” the bacteria towards fundamentally distinct pathogens.

Among the human-adapted typhoid agents, comparison has been mostly focused on *S. typhi* and *S. paratyphi A* for elucidation of the genetic basis that might have led them to become fundamentally distinct pathogens. This pair of typhoid agents have greatly different sets of pseudogenes [11], suggesting separate immediate ancestries and thus favoring the convergence hypothesis of typhoid pathogenesis evolution. Later, the convergence model was again supported by a different approach, i.e., a Bayesian changepoint model, which points out a high level of recombination between *S. typhi* and *S. paratyphi A* [23]. However, neither approach was conclusive about the evolution of the typhoid agents, largely due to the difficulty to have the divergent and convergent genomic events to be unambiguously distinguished. To reveal with greater confidence the genomic features common to the typhoid agents but not seen in non-typhoidal *Salmonella* pathogens for the elucidation of the genetic basis of the typhoid pathogenicity, we included additional typhoid agents as well as non-typhoidal salmonellae in the genomic comparisons. In this study, we determined the complete genome sequence of a strain of *S. paratyphi C*, RKS4594, and compared it with other published *Salmonella* genome sequences. Our analysis demonstrates that *S. paratyphi C* may have diverged from a common ancestor with *S. choleraesuis*, which is primarily a swine pathogen [13] but may also occasionally cause invasive infections in humans [24–27], relatively recently by adapting to a different niche and converged with *S. typhi* by accumulating genomic changes, including acquisition of genomic insertions and loss of certain genes.

**Results**

**Overall features of *S. paratyphi C* and global comparisons with other *Salmonella* genomes**

*S. paratyphi C* RKS4594 was a clinical isolate and representative of a specific electrophoretic type, ET Pe-2, as determined by multiplex electrophoresis; it was included in the set of the *Salmonella* Reference collection B strains [SARB49] [28]. This *S. paratyphi C* strain contains a chromosome of 4,833,080 bp and a plasmid of 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1). We predicted 4,640 intact coding sequences (4,578 in the chromosome and 62 in the plasmid) and 55,414 bp (Fig. 1 and Table 1).

**Figure 1. Map of the *S. paratyphi C* RKS4594 genome.** (A) The chromosome. Circles range from 1 (outer circle) to 7 (inner circle): 1 and 2, genes on forward and reverse strand, respectively; 3, pseudogenes; 4, genes that are conserved among all six sequenced strains compared in this study; 5, rRNA operons (blue), and prophages and SPIs (brown); 6, G+C content, with values greater than average in green and smaller in red; and 7, GC skew (G−C/G+C), with values greater than zero in green and smaller in red. All genes displayed in circles 1 and 2 are colored by NCBI COG (Clusters of Orthologous Groups) function category: information storage and processing, cyan; cellular processes and signaling, yellow; metabolism, magenta; poorly characterized, black. The locations of two prophages, Gifsy2 and Gifsy1, are indicated by arrows, which are recombination sites inverting a large chromosomal segment (ca. 1600 kb). (B) The virulence plasmid pSPCV. This plasmid comprises spv operon (cyan), pet operon (yellow), tra operon (magenta), and other regions (grey). The outermost arcs are additional regions of pSLT (a virulence plasmid from *S. typhimurium* LT2) compared with pSPCV. The inner black arc represents the conserved region of pKDSC50 (a virulence plasmid from *S. choleraesuis* SC-867), pSPCV and pSLT. The gene content of pKDSC50, the most reduced of the three virulence plasmids, is virtually equal to the black arc.

doi:10.1371/journal.pone.0004510.g001
We then looked further into the phylogenetic relationships of S. paratyphi C with five other sequenced Salmonella strains through systematic sequence comparisons.

We aligned sequences of common regions of the six Salmonella genomes and calculated genetic distances to estimate their relatedness. The resulting phylogenetic tree (Fig. 2) reveals a greater distance from S. choleraesuis, indicating their very recent divergence, and a much greater distance from S. paratyphi A, S. typhi or S. typhimurium. These data, again, strongly suggest that the typhoid-associated pathogenicity has evolved by convergent processes in different Salmonella genetic backgrounds.

Comparison between S. paratyphi C and S. choleraesuis

The demonstrated recentness of the divergence between S. paratyphi C and S. choleraesuis suggests that we may still have an opportunity to track the evolutionary events that might have contributed to the evolution of an human-adapted typhoid agent. For this, we made systematic sequence comparisons between S. paratyphi C RKS4594 and S. choleraesuis SC-B67 [13].

We first focused on large genomic segments that differ between S. paratyphi C RKS4594 and S. choleraesuis SC-B67, as they are supposed to be laterally acquired and contain large numbers of genes that may have facilitated the pathogenic divergence process. Two regions, SPI-7 (SPC_0869–SPC_0908) and SPA-3-P2SPC (SPC_0869–SPC_0908), are present in S. paratyphi C RKS4594 but absent from S. choleraesuis SC-B67. SPI-7 has only been found in S. typhi, S. paratyphi C and human-isolates of S. dublin [29]. In S. typhi, SPI-7 comprises four parts: type IVB pilus operon, SopE prophage, Vi biosynthetic operon, and a 15 kb phage-like segment [9,29], whereas in many S. paratyphi C and S. dublin strains, only parts of type IVB pilus operon are present with the SopE bacteriophage and the 15 kb phage-like segment being entirely absent [29,30]. Despite its differences in structure among these bacteria, SPI-7 seems to have been acquired by S. typhi and S. paratyphi C fairly recently at around the same time, long after the emergence of S. typhi and S. paratyphi C. We made this speculation based on the fact that the sequence divergence of SPI-7 between S. paratyphi C and S. typhi (0.0006) is considerably lower than their chromosomal divergence (0.0179).

SPA-3-P2SPC is highly similar to SPA-3-P2 of S. paratyphi A in sequence but has a different insertion site in the chromosome of S. paratyphi C RKS4594. Although SPI-7 and SPA-3-P2SPC constitute the main genetic differences between S. paratyphi C and S. choleraesuis, they do not exist in all human-adapted typhoid agents (e.g., SPI-7 is not present in S. paratyphi A, SPA-3-P2 or SPA-3-P2SPC is not present in S. typhi), suggesting that they are not necessarily a determinant for the typhoid pathogenesis. Other prophages and pathogenicity islands found in S. paratyphi C RKS4594 are summarized in Table S2.

We then made systematic sequence comparisons of the remaining parts of genomes between S. paratyphi C RKS4594 and S. choleraesuis SC-B67. These two strains have accumulated distinct sets of mutations, which is striking considering their very short divergence history. This is first reflected by their different sets of pseudogenes (Table S3). Although S. paratyphi C and S. choleraesuis have similar numbers of pseudogenes, with 152 in the former and 156 in the latter, only 55 are common to both. These findings may reflect distinct selection pressures on S. paratyphi C and S. choleraesuis when they were adapting to different niches.

The distinctness of accumulated mutations between S. paratyphi C and S. choleraesuis is also reflected by the exceptionally high nonsynonymous (dN) over synonymous (dS) substitution ratio (dN/dS, ca. 0.62; Table 2), as compared to those between S. paratyphi C and S. typhimurium, S. typhi or S. paratyphi A, which are in the range of 0.13–0.15 (Table 2). The two sequenced S. typhi strains also have a high dN/dS ratio (Table 2), but the mechanisms might be different.

To reveal the actual nucleotide substitutions that would lead to amino acid changes, we aligned the sequences coding for 3238 proteins common to all six Salmonella genomes compared and

![Figure 2. Phylogenetic tree for the six sequenced Salmonella strains, based on whole-genome sequences (all conserved regions among the six genomes are concatenated and aligned for tree construction).](doi:10.1371/journal.pone.0004510.g002)
identified 2335 amino acids that are different between S. paratyphi C and S. choleraesuis. Since as many as 2222 of the 2335 amino acids are identical in S. typhi, S. paratyphi A and S. typhimurium, we assumed these amino acids to be the state in the ancestors of S. paratyphi C, S. choleraesuis, S. typhi, S. paratyphi A and S. typhimurium. Of these 2222 amino acids that are common to S. typhi, S. paratyphi A and S. typhimurium, S. paratyphi C has 1147 (the other 1073 being different from those in S. typhi, S. paratyphi A, S. typhimurium and S. choleraesuis) and S. choleraesuis has 1028 (the other 1194 being different from those in S. typhi, S. paratyphi A, S. typhimurium and S. paratyphi C), suggesting differential selection pressures to “force” S. paratyphi C and S. choleraesuis to have these distinct sets of particular amino acids selected for their eventual adaptation to different niches. Of special interest is a list of nine amino acids in S. paratyphi C RKS4594 that are different from their counterparts in S. typhi, S. paratyphi A and S. paratyphi C (Table S4), possibly reflecting a need of these particular amino acids by the human-adapted Salmonella lineages for dwelling in the host. These features again strongly indicate S. paratyphi C as an ideal model in studies to elucidate the molecular mechanisms of human adaptation during the evolution of a typhoid agent from its host-generalist ancestor.

**Table 2. dN and dS values in pairs of compared genomes.**

| Genomes compared | dN         | dS         | dN/dS      |
|------------------|------------|------------|------------|
| SPC vs SC        | 0.00131(=0.00967) | 0.00209(=0.00940) | 0.62453    |
| SPC vs STM       | 0.00459(=0.03428) | 0.03416(=0.14710) | 0.13451    |
| SPC vs CT18      | 0.00642(=0.03156) | 0.04564(=0.14594) | 0.14074    |
| SPC vs Ty2       | 0.00641(=0.03156) | 0.04568(=0.14611) | 0.14029    |
| SPC vs SPA       | 0.00726(=0.04701) | 0.04762(=0.18746) | 0.15252    |
| CT18 vs Ty2      | 0.00016(=0.00329) | 0.00029(=0.00601) | 0.57240    |

Footnote: SPC, S. paratyphi C RKS4594; SC, S. choleraesuis SC-867; STM, S. typhimurium LT2; Ty2, S. typhi Ty2; CT18, S. typhi CT18; SPA, S. paratyphi A ATCC9150.

doi:10.1371/journal.pone.0004510.t002

Common gains and losses of genes among the sequenced typhoid agents

We examined possible common gains and losses among the sequenced human-adapted typhoidal strains, relative to S. typhimurium LT2. Systematic comparisons of these typhoidal strains with S. typhimurium LT2 did not lead to the identification of genes common only to the human-adapted typhoidal strains. This raises two possibilities: (i) different Salmonella typhoid agents might have acquired different typhoid-causing traits, as suggested by the large number of genes common to S. typhi and S. paratyphi A [23] but not to S. paratyphi C, or SPI7 common to S. typhi and S. paratyphi C but not to S. paratyphi A; and (ii) many Salmonella serovars might carry genes that would participate in typhoid pathogenesis only in a small number of serovars due to the acquisition (or activation) or loss (or inactivation) of other genes. Both scenarios favor the convergence evolution model of the typhoid agents, implicating that the immediate ancestors of the extant human-adapted Salmonella lineages acquired the typhoid-causing traits independently and then converged under the same host pressure to become clinically similar pathogens.

On the other hand, we found that a total of 24 genes were either absent or inactivated in the sequenced S. typhi, S. paratyphi A and S. paratyphi C strains relative to S. typhimurium LT2 (Table 3), which suggests that these functions are not required for human infection. Of special interest are genes encoding fimbrins, as fimbrines have long been known to constitute a “signature” for Salmonella serovars [31,32]. More importantly, Salmonella fimbrines are known to be involved in infections and may play a role in host determination [33]. Although human-adapted typhoid agents possess special repertories of fimbrial genes that are involved in the bacterial infection process in humans [32], the inability of these bacteria to infect other host may be accounted for by loss of certain fimbral genes. We found that three fimbral genes, safC, hecC and sifD, are pseudogenes in the sequenced S. typhi, S. paratyphi A and S. paratyphi C strains, and one fimbral gene, stj, is entirely absent in the sequenced S. typhi, S. paratyphi A and S. paratyphi C strains; these four fimbral genes are present and intact in S. typhimurium LT2.

**Chromosomal rearrangement mediated by Gifsy sequences**

Previously, we reported that physical balance of the bacterial chromosome between replication origin, oriC, and terminus, terC, affects growth rate in S. typhi and therefore may influence the competition capability of the bacteria in nature [34]. Unlike S. typhimurium and S. paratyphi A, which have well balanced and very stable genome structures [20,35], S. paratyphi C and S. typhi both have less optimally balanced genomes and so have undergone frequent rearrangements [18,19,21]. Most often, chromosomal rearrangements occur through recombination between homologous sites such as rm genes [18] or IS290 [36]. However, as S. paratyphi C does not have IS290 (See Table S1), most genomic rearrangements among wild type strains of S. paratyphi C are mediated by rm genes [21], with an important exception as detailed below.

In RKS4594, oriC is located at 4016 kb and terC is around 2256 kb from thrL. As the genome size is 4833 kb, the balance is 249° clockwise and 131° counterclockwise between oriC and terC, which is far off the 180° balanced status. Through comparison with S. typhimurium LT2, an inversion of about 1600 kb was found between homologous regions of prophages Gifsy-1 and Gifsy-2 (Figure 3), which was confirmed by physical mapping [21]. To our knowledge, it is the first report of prophage mediated chromosomal rearrangement in Salmonella.

**Virulence plasmid**

S. paratyphi C RKS4594 contains a plasmid, pSPCV, with very high sequence identity with the virulence plasmids pSLT (S. typhimurium LT2) [10] and pKDSC50 (S. choleraesuis SC-B67) [15]. The three plasmids have decreasing sizes from pSLT (93.9 kb) to pSPCV (55.4 kb) and to pKDSC50 (49.6 kb), suggesting a gradual degradation process to shed unnecessary genes during evolution. All three Salmonella plasmids contain operons pso, pef and tra. The pso operon is conserved among all Salmonella virulence plasmids that have been characterized to date [37] and is proven to be required for the systemic phase of the infection in their host [38].

The genes pefABCD in the pef operon (plasmid-encoded fimbrines) are conserved among the three plasmids. However, the downstream region, i.e., between pefD and the repA loci, shows remarkable variability between pSLT and pSPCV, and is entirely absent from pKDSC50 (see Fig. 1b). Within this region of pSLT, two genes, safA (PST011, encoding thiol-disulphide oxidoreduce-tase) and safB (PST010, encoding a putative outer membrane protein), have significant homology to two genes in SPI-1 of both S. typhi and S. paratyphi A. As most S. typhi and S. paratyphi A strains do not have virulence plasmids, it was once speculated that the two genes might partly complement the functions of the virulence plasmids [39]. However, because the safA counterparts in S. typhi
Table 3. Deletion and pseudogene formation in the four human-adapted typhoidal strains.

| Locus_tag   | Symbol | Product                                | Category |
|-------------|--------|----------------------------------------|----------|
| SPC_0797    | slrP   | leucine-rich repeat protein             | 1        |
| SPC_1513    | mglA   | galactose (methyl-galactoside) transport protein | 1        |
| SPC_1647    | sopA   | secreted effector protein              | 1        |
| SPC_1757    | flbB   | N-methylation of lysine residues in flagellin | 1        |
| SPC_2542    | hnuE   | outer membrane receptor for ferric iron uptake | 1        |
| SPC_4172    | -      | putative permease of the Na\textsuperscript{+}-galactoside symporter family | 1        |
| SPC_0675    | ybeS   | putative molecular chaperone, DnaJ family | 2        |
| SPC_0675    | ybeV   | putative molecular chaperone, DnaJ family | 2        |
| SPC_0670    | -      | transcriptional regulator, lysR family  | 2        |
| SPC_0859    | -      | putative inner membrane protein        | 2        |
| SPC_1396    | yfbK   | putative von Willebrand factor, vWF type A domain | 2        |
| SPC_1703    | -      | putative endoprotease                  | 2        |
| SPC_2105    | -      | putative inner membrane protein        | 2        |
| SPC_2378    | ydsS   | flavoprotein                           | 2        |
| SPC_2702    | -      | Gifsy-2 prophage host specificity protein J | 2        |
| SPC_3146    | -      | putative mannitol dehydrogenase        | 2        |
| SPC_3591    | rtcR   | sigma N-dependent regulator of rtcBA expression | 2        |
| SPC_2077    | -      | putative methyl-accepting chemotaxis protein | 3        |
| SPC_2458    | -      | putative Methyl-accepting chemotaxis protein | 3        |
| SPC_2232    | dmsB   | anaerobic dimethyl sulfoxide reductase chain B | 3        |
| SPC_0311    | safC   | fimbrial operon protein                | 3        |
| SPC_0025    | bcfC   | fimbrial operon protein                | 3        |
| SPC_0213    | stfD   | fimbrial operon protein                | 3        |
| putative deletion | stj | fimbrial operon protein                | 3        |

Note: we divide the presumably lost genes, relative to S. typhimurium LT2, into three categories: 1, they are all pseudogenes in the four typhoidal strains; 2, they are pseudogenes in S. paratyphi C but entirely absent in S. typhi and S. paratyphi A; and 3, other genes in the same or a similar pathway are either pseudogenes or absent.

doi:10.1371/journal.pone.0004510.t003

Figure 3. Chromosomal rearrangement mediated by Gifsy-1 and Gifsy-2. (A) Alignment of Gifsy-1 and Gifsy-2 in S. paratyphi C. Common genes in Gifsy-1 and Gifsy-2 are colored in yellow, with the remaining genes of Gifsy-1 being colored in red and those of Gifsy-2 in green; conserved genes adjacent to Gifsy-1 and 2 are in black. The blue shade indicates identity $\geq$90\% between Gifsy-1 and Gifsy-2. (B) Chromosomal comparison showing the relative inversion between S. paratyphi C and S. choleraesuis. The scheme is color-coded as above and shows that the broad ranges of homologous regions between Gifsy-1 and Gifsy-2 instead of integrases mediate the chromosomal rearrangement.

doi:10.1371/journal.pone.0004510.g003
Analysis of the S. paratyphi C genome has revealed to us new facts about the genetic divergence of Salmonella pathogens and helped clarify the phylogenetic relationships among the human-adapted typhoid agents and other Salmonella lineages. This work will also significantly facilitate the studies of pathogenic divergence of Salmonella as a whole and, especially, the Group C Salmonella lineages bearing the common antigenic formula 6,7:z:1,5, including S. paratyphi C, S. choleraesuis and S. typhimurium [41]. The highly similar genomic constructions between S. paratyphi C and S. choleraesuis [13] and their distinct pathogenic features [5,42] make them excellent models for studies of Salmonella host adaptation and pathogenic divergence. Our results strongly suggest that the two lineages had a common immediate ancestor and that they diverged fairly recently and provide further evidence about the closer relatedness between S. paratyphi C and S. choleraesuis than either to S. typhi or S. paratyphi A [3]. Perhaps an occasional invasion of and gradual adaptation to human body caused a branch of the ancestor to become settled in the new niche. During this process, favorable changes of nucleotides/amino acids may have been quickly selected and accumulated to facilitate the host shift, as reflected by the greater dN than dS substitutions between S. paratyphi C and S. choleraesuis.

Usually, when a bacterial lineage begins to diverge from the ancestor, dN may transiently be greater than dS among members of the same diverging lineage due to the nature of genetic codons (changes in the first two of the three nucleotides in a codon tends to cause dN). For example, S. typhi may have diverged from its ancestor for no more than fifty thousand years [43] and individual strains still have relatively high dN/dS values as seen between CT18 and Ty2 (See Table 2). Then as deleterious mutations (i.e., many dNs) are eventually purged [44], dS would gradually exceed dN. Therefore, the ratio of dN/dS may in a way reflect evolutionary distances among a certain range of closely related bacteria living in the same kind of niche, e.g., independent isolates of S. typhi that dwell only in the human body; in such cases, more closely related strains may have greater dN/dS values than more distantly related ones and the dN/dS ratio may then decrease with time. We speculate that the scenario may be different, however, in bacteria that are closely related but do not dwell in the same niche, such as S. paratyphi C and S. choleraesuis, in which the dN/dS ratio may remain relatively high for long evolutionary times due to the potential benefits brought to the bacteria by the non-synonymous nucleotide substitutions.

Although several lines of evidence, especially those presented in this paper, support the convergence evolution model of the human-adapted typhoid agents [11,23], genes directly contributing to the typhoid phenotypes remain to be identified. In this study, we compared the genomes of the human-adapted typhoidal strains (S. typhi CT18 and Ty2, S. paratyphi A ATCC9150 and S. paratyphi C RKS4594) with those of S. typhimurium LT2 and S. choleraesuis SC-B67 to attempt identifying typhoid-associated genes. However, we did not obtain a significant list of genes present in the former but absent in the latter. This might be because S. typhimurium, though causing gastroenteritis in humans, does have genes to cause typhoid-like disease in mice and some of the genes might be related to those in the human-adapted typhoid agents. Additionally, S. choleraesuis, having a narrow host range and causing invasive infections in humans [24–27], can cause swine-paratyphoid [45]. Therefore, all six sequenced Salmonella strains compared in this study have the potential of causing typhoid-like diseases in humans or animals, so none of them could be used as a real “typhoid-free” reference for comparison to identify typhoid-associated genes. As a result, the genomic sequence of a Salmonella lineage that does not cause typhoid-like disease in any host, such as S. pullorum, is desired in studies for further narrowing down the typhoid determinants. On the other hand, the large number of degraded genes (pseudogenes) and the distinct set of selected amino acids (dN) identified in the S. paratyphi C genome through this study will provide a guide in studies for the elucidation of the genetic basis for host adaptation of this pathogen to humans.

Conclusions

S. paratyphi C does not share a common ancestor with other human-adapted typhoid agents, supporting the convergent model of the evolution of the typhoid agents. S. paratyphi C has diverged from a common ancestor with S. choleraesuis by accumulating genomic novelty during adaptation to man.

Materials and Methods

Sequencing

The genome sequence of S. paratyphi C RKS4594 was determined by dye terminator chemistry on Megabace1000 and ABI3730 automated sequencers, with DNA clones from several pUC18 genomic shotgun libraries (insert sizes ranging from 1.5 to 4.0 kb). The Phred/Phrap/Consed package was used for quality assessment and sequence assembly. Gaps were filled by PCR amplification and primer walking methods. Ambiguous areas were re-sequenced and the assembly was verified by a physical map; the final sequence reached accuracy over 99.99%.

Annotation

Gene prediction was performed by use of GLIMMER3 and by comparison with the annotated genes from five available Salmonella genomes, i.e., S. typhimurium LT2, S. choleraesuis SC-B67, S. paratyphi A ATCC9150, and S. typhi CT18 and Ty2. Intergenic regions were searched against NCBI non-redundant libraries for potential genes. The function of all coding sequences was further investigated by searching against InterPro database. Genes that contains insertion, deletion or mutation to a stop codon compared with those known Salmonella genes were categorized as pseudo-genes. Transfer RNA genes were predicted with tRNAscan-SE, and ribosomal RNA genes were predicted by similarity to other Salmonella rRNA genes.

Comparative and phylogenetic analysis

Whole genome alignment was made by use of MAUVE and MUMmer program. Phylogenetic tree construction was done with PHYLIP 3.6 package. Nucleotide divergence in this article was
defined as the number of mismatch bases divided by that of comparable bases after pairwise alignment made by CLUSTALW. dN/dS values were calculated with Yit00 program in PAML 3.15 package.

**URLs**

The Phred/Phrap/Consed package is available at http://www.phrap.org/ phredphrapconsed.html. GLIMMER3 is available at http://www.clcb.umd.edu/software/glimmer/. NCBI non-redundant database is available at ftp://ftp.ncbi.nih.gov/blast/db/. InterPro database is available at http://www.ebi.ac.uk/interpro/. tRNAscan-SE is available at http://lowelab.ucsc.edu/tRNAscan-SE/. MAUVE program is available at http://gel.ababs.wisc.edu/mauve/. MUMmer program is available at http://mummer.sourceforge.net/. PHYLIP 3.6 package is available at http://evolution.genetics.washington.edu/phylip.html. CLUSTALW program is available at http://www.ebi.ac.uk/Tools/clustalw/. PAML 3.15 package is available at http://abacus.gene.ucl.ac.uk/software/paml.html.

**Accession numbers**

Genbank: S. typhimurium LT2 [NC_003197]; S. choleraesuis SC-67 [NC_006905]; S. paratyphi A ATCC9150 [NC_006511]; S. typhi CT18 [NC_003193]; S. typhi Ty2 [NC_004631]; S. paratyphi C RK54594 chromosome [CP000857]; S. paratyphi C RK54594 plasmid pSPCV [CP000858].

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**Supporting Information**

**Table S1** Genotype annotation

Found at: doi:10.1371/journal.pone.0004510.s001 (1.23 MB XLS)

**Tables S2 and S3**

Found at: doi:10.1371/journal.pone.0004510.s002 (0.32 MB DOC)

**Table S4**

Found at: doi:10.1371/journal.pone.0004510.s003 (0.02 MB XLS)

**Acknowledgments**

We thank Dr. R. K. Selander for the bacterial strain S. paratyphi C RKS4594, and the sequencing and informatics teams at the BGI LifeTech for their technical assistance.

**Author Contributions**

Conceived and designed the experiments: SLL. Performed the experiments: WQL YW QHZ FC JTG YHP YJ YGL SH GRL SLL. Analyzed the data: WQL YF SLL. Contributed reagents/materials/analysis tools: RJ. Wrote the paper: YF SLL.
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