The evolutionary history of *Shigella flexneri* serotype 6 in Asia

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Abstract

*Shigella flexneri* serotype 6 is an understudied cause of diarrhoeal diseases in developing countries, and has been proposed as one of the major targets for vaccine development against shigellosis. Despite being named as *S. flexneri*, *Shigella flexneri* serotype 6 is phylogenetically distinct from other *S. flexneri* serotypes and more closely related to *S. boydii*. This unique phylogenetic relationship and its low sampling frequency have hampered genomic research on this pathogen. Herein, by utilizing whole genome sequencing (WGS) and analyses of *Shigella flexneri* serotype 6 collected from epidemiological studies (1987–2013) in four Asian countries, we revealed its population structure and evolutionary history in the region. Phylogenetic analyses supported the delineation of Asian *Shigella flexneri* serotype 6 into two phylogenetic groups (PG-1 and −2). Notably, temporal phylogenetic approaches showed that extant Asian *S. flexneri* serotype 6 could be traced back to an inferred common ancestor arising in the 18th century. The dominant lineage PG-1 likely emerged in the 1970s, which coincided with the times to most recent common ancestors (tMRCA) inferred from other major Southeast Asian *S. flexneri* serotypes. Similar to other *S. flexneri* serotypes in the same period in Asia, genomic analyses showed that resistance to first-generation antimicrobials was widespread, while resistance to more recent first-line antimicrobials was rare. These data also showed a number of gene inactivation and gene loss events, particularly on genes related to metabolism and synthesis of cellular appendages, emphasizing the continuing role of reductive evolution in the adaptation of the pathogen to an intracellular lifestyle. Together, our findings reveal insights into the genomic evolution of the understudied *Shigella flexneri* serotype 6, providing a new piece in the puzzle of *Shigella* epidemiology and evolution.

DATA SUMMARY

Raw sequence data used in this publication are available in the NCBI Sequence Read Archive (project PRJEB5281: Phylogeny of *Shigella* spp. in Southeast Asia and PRJEB2508: Temporal and geographical of the *Shigella* genus diversity in Southern Vietnam). All supporting data and protocols have been provided within the article or through supplementary data files.

INTRODUCTION

*Shigella*, a member of the Gram-negative Enterobacteriaceae, is among the leading aetiologies responsible for diarrhoeal diseases. It has been estimated that shigellosis accounts for >160000 deaths annually worldwide, among which one-third were children under 5-years-old residing in developing countries [1, 2]. The *Shigella* genus is categorized into four species (or subgroups) based on the lipopolysaccharide O-antigen composition on the...
bacterial cell surface, including: *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup B), *S. boydii* (subgroup C), and *S. sonnei* (subgroup D). Each subgroup is further classified into various serotypes according to type-specific antigens. The numbers of serotypes also vary between different subgroups (*S. boydii*: 20 serotypes, *S. dysenteriae*: 15 serotypes, *S. flexneri*: 14 serotypes, and *S. sonnei*: one serotype).

Despite a comprehensive serotyping scheme, it has been demonstrated that serotyping is a poor predictor of phylogenetic relatedness for *S. flexneri*, regardless of whether it is phenotypically or in silico determined [3]. Although *S. flexneri* serotype 6 (Sf6) is serologically classified as a *S. flexneri* serotype [4–7], multiple phylogenetic studies (based on multilocus sequence typing (MLST) and whole genome sequencing (WGS) data) have demonstrated that Sf6 is distantly related to all other *S. flexneri* serotypes, and instead has closest phylogenetic relatedness to *S. boydii* [8–11]. Such distinct phylogenetic relationships with other *Shigella* species, however, have hampered research on Sf6. Species-wide studies on *S. flexneri* genomic and evolution, at both regional and global scale, have excluded Sf6 from in-depth analyses [12, 13]. On the other hand, research on *S. boydii* is currently limited due to its much lower contribution to disease burden over the past decade, compared to *S. flexneri* and *S. sonnei* [14, 15]. However, unlike *S. boydii*, Sf6 is prevalent in shigellosis-endemic regions in Africa and Asia, accounting for ~11% of documented shigellosis cases in a large scale surveillance study [15, 16]. Thus, Sf6 was proposed as one of the four essential targets (in conjunction with *S. sonnei*, *S. flexneri* 2a, and *S. flexneri* 3a) for developing a quadrivalent vaccine with broad coverage against diverse *Shigella* serotypes [15].

In spite of its epidemiological significance, there have been no large scale genomic studies focusing on the evolutionary history of Sf6, especially in Asia – a contemporary focus of endemic shigellosis. Herein, we performed WGS on a collection of representative Sf6 (n=96) isolated in South and Southeast Asia (collected between 1987 and 2013), and explored their phylogenetic structure and evolutionary history in the region. We also inspected their genetic repertoires and antimicrobial resistance profiles (resistomes). Finally, to further contextualize Sf6 evolution, our results were discussed in relation to previous findings in other *Shigella* species, as well as other enteric pathogens.

**METHODS**

**Bacterial isolates and whole genome sequencing**

In order to investigate the evolutionary history of Sf6 in Asia, we compiled a collection of 96 Sf6 isolates (Table S1, available in the online version of this article) from four countries: Thailand, Vietnam, Cambodia, and Bhutan. The isolates originated from multiple collaborative institutes, including: Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand (n=53); Oxford University Clinical Research Unit (OUCRU) in Ho Chi Minh City, Vietnam (n=30); Cambodia-Oxford Medical Research Unit (COMRU) in Siem Reap, Cambodia (n=6); Jigme Dorji Wangchuk National Referral Hospital (JDWNRH) in Thimphu, Bhutan (n=7). Serotyping was performed on all retrieved isolates at the collaborating institutes using the commercial antisera (Denka Seiken, Japan), and confirmed that they belong to Sf6. The bacterial isolates and data used in this study originated from several local diarrhoeal surveillance studies, which received ethical approvals from the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, all other participating hospitals, the Institutional Review Board of the Walter Reed Army Institute of Research (for Thailand data), the Research Ethics Board of Health in Bhutan, and the Oxford Tropical Research Ethics Committee (OxTREC) in the United Kingdom. The study also included characterization of bacterial isolates submitted for routine diagnostic activities (for Cambodian data). All examined Sf6 were isolated from patients with diarrhoea, predominantly young children, during the diarrhoea surveillance studies. Written informed consent from study participants or their parents/guardians was obtained prior to the collection of stool samples.

We extracted genomic DNA for all bacterial isolates using Wizard Genomic DNA extraction kits (Promega, Wisconsin, USA), and DNA was stored at ~20°C until shipment to the Wellcome Trust Sanger Institute for whole genome sequencing on an Illumina HiSeq2000 platform. This produced a library of paired-end reads of 125 bp in length for each bacterial isolate.
Short read mapping and phylogenetic reconstruction

The chromosome sequence of *S. boydii* serotype 4 Sb227 (accession number: CP000036.1) was used as the reference genome for mapping of all Sf6 since it was the most closely related complete genome at the time of analysis [9, 11]. The BWA-MEM algorithm (v0.7.12) [17] was used for short read mapping. Duplicate reads were removed by PICARD (v2.18.5) (https://github.com/broadinstitute/picard), and GATK (v3.7) [18] was deployed for indel realignment. Using SAMtools and bcftools (v1.8) [19], high-quality SNPs were called and filtered. Unqualified single nucleotide polymorphism (SNP) sites were discarded if they matched any of the following criteria: consensus quality <50, mapping quality <30, ratio of SNPs to reads <75%, and read depth <4.

Following the read mapping process, we created an alignment of pseudogenome sequences of the same length. For phylogenetic inference, recombination and prophage regions were removed from the alignment, resulting in an alignment of 3794 recombination-free bp. Prophage regions were detected on the reference chromosome sequence using the PHASTER web server [20], and recombination elements were predicted by Gubbins (v1.4.5) [21]. The best-scoring maximum-likelihood phylogenetic tree was inferred using RAxML (v8.2.4) under the GTRGAMMA substitution model, with 100 rapid bootstrap searches and 20 maximum-likelihood searches [22]. The resulting phylogeny was comprised of two clusters, separated by significant genetic distance. We herein refer to them as the PG-1 (*n*=82) and PG-2 (*n*=14). The maximum-likelihood phylogenetic tree was midpoint rooted in FigTree (v1.4.3) (http://tree.bio.ed.ac.uk/software/figtree/) for the purpose of visualization. Metadata were annotated to the phylogenetic tree and visualized using the ggtree package (v1.14.6) [23] in the R programming platform [24].

Temporal structure analysis and Bayesian phylogenetic inference

In order to analyse potential temporal structure in the phylogeny, we subsampled the Sf6 collection to include 82 representative sequences. In the subsampling process, we included one representative isolate (and excluded the other(s)) from each terminal phylogenetic groups with bootstrap support less than 70. The intention of this subsampling procedure was to yield a phylogeny with high confidence support for the temporal structure analysis and subsequent Bayesian phylogenetic inference. A maximum-likelihood phylogeny was reconstructed for this subsampled dataset, using the approach described above. The most appropriate substitution model was determined by the ModelFinder algorithm of IQTree (v1.6.7) [25]. TempEst (v1.5.3) [26] was utilized to assess the linear relationship between root-to-tip divergence of the inferred maximum-likelihood phylogeny and year-of-sampling, which yielded a signal of high temporal structure (R²=0.864). We deployed BEAST (v1.8.3) [27] to co-infer the phylogeny and divergence times, which estimated the substitution rate and time to the most recent common ancestor (tMRCA) by Bayesian phylogenetic inference.

To select the best-fit model, analyses were performed independently on six combinations of different models of molecular clock (strict, uncorrelated relaxed, and random local clock) and demographic models (constant model and Bayesian Skyline). Analyses on each model combination were performed in triplicate on an ensemble of 400 million continuous Markov Chain Monte Carlo (MCMC) chains, with samples taken every 40 000 chain generations. Convergence of estimated parameters were visually inspected in Tracer (v1.7.1) [28], ensuring the effective sample size (ESS) values of estimated parameters were ≥200 for a successful run. Among the tested models, parameters estimated on the combination of a random local clock and Bayesian Skyline did not successfully converge in three separate runs. Thereby, it was not considered in downstream analyses. We applied path sampling and stepping-stone sampling approaches in every BEAST run to approximate the marginal likelihood for model selection [29, 30], in order to identify the best-fit model. Parameters inferred from triplicate runs of the best-fit model were combined using LogCombiner, with 10 % burn-in removal, and a Maximum Clade Credibility tree was generated from the combined estimates using TreeAnnotator.

The model incorporating the GTR + Γ4 substitution model, random local clock, and constant population size was chosen as best fitting to the data.

In addition, Bayesian inference was performed separately for PG-1 and PG-2 isolates, following the procedure as described above but without subsampling. For PG-1 analyses, two sequences were discarded in the input alignment since they showed high proportions of gaps and ambiguous nucleotides. BEAST runs on PG-1 and PG-2 were conducted on 100 million and 30 million MCMC chains, with sampling period every 10 000 and 3000 chains, respectively. For PG-1, the best-fit model was GTR + Γ4 in combination with a random local clock and Bayesian Skyline demographic model. On the other hand, the combination of a TVM + Γ4 substitution model, strict molecular clock, and constant population size was best-fitting for PG-2.

Accessory genome profiling

For each isolate, the paired-end sequencing reads were trimmed using Trimmomatic (v0.38) [31], and input into SPAdes (v3.12.0; k-mer sizes of 21, 33, and 55 bp; error correction option) [32] to produce a de novo assembly. Only contigs of more than 500 bp were included in subsequent analyses. For each isolate, antimicrobial resistance (AMR) genes were detected directly from short reads using ARIBA (v2.12.0) [33], based on the curated ResFinder database [34], with a minimum alignment length of 50% and minimum nucleotide identity of 95% to consider a hit. The output results were then manually curated for confident hits.

Pan-genome profiles were constructed by Roary (v3.12.0) [35] for all 96 sequenced Sf6, using the default 95% BLASTP identity. The inputs for this analysis were assembled contigs annotated by Prokka (v1.13) [36]. To identify accessory genomes encompassing detected genes and their associated
contigs, the assembly of each isolate was ordered against the chromosome sequence of Sb227 and the virulence plasmid pCP301 of S. flexneri 2a (accession number: NC_004851.1) using ABACAS (v1.3.1) [37]. The pCP301 was used instead of the virulence plasmid pSB4_227 of S. boydii 4, since the latter lacks several virulence determinants such as mxi-spa, icsA/virG and virA [38, 39]. The remaining contigs were then sequentially ordered against a set of plasmids and the Shigella resistance locus pathogenicity island of S. flexneri 2a (SRL-PAI, accession number: AF326777.3). The list of plasmids used in the aforementioned procedure is provided in Table 1. The plasmids were selected based on the results of plasmid typing analysis, which was performed using ARIBA on the highly curated PlasmidFinder database [33, 40]. BLASTN [41] was utilized to compare ordered contigs with the reference sequences. Artemis and Artemis Comparison Tool (ACT) [42] were used to visually inspect the presence of specific genetic elements in the isolates.

**RESULTS**

The population structure and evolutionary history of *Shigella flexneri* 6 in Asia

Our study included 96 Sf6 isolates, which were collected in previous diarrhoeal surveillance studies in Vietnam (1995–2010) [43, 44], Thailand (1987–2005) [45], Cambodia (2005–2007), and Bhutan (2011–2013) [46]. In *silico* MLST, as implemented in ARIBA using the *Escherichia coli*’s seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) confirmed that all Sf6 belonged to ST145, resembling previous findings [8]. Previous phylogenetic investigations on pan-*Shigella* species showed that Sf6 was phylogenetically clustered with *S. boydii* serotypes 2, 4, and 14 [9–11]. Read mapping using the *S. boydii* reference genome (Sh227) produced good mapping quality, with almost all unmapped regions pertaining to repetitive sequences.

The constructed maximum-likelihood phylogeny delineated our 96 Sf6 into two phylogenetic groups (PG-1 and –2), both of which were supported by bootstrap values of 100 (Fig. 1). The evolutionary distance separating the two PGs was estimated to be ~2442 SNPs. PG-1 included 82 isolates from the four sampled countries while PG-2 only contained 14 isolates originating from Thailand and Vietnam. As depicted in Fig. 1, PG-2 appeared to harbour greater genetic diversity, with isolates spanning a broad timeframe (1987–2010), and was comprised of three subgroups: I, II, and III. The distances from subgroup III to subgroups I and II were estimated to be 719 and 730 SNPs, respectively. Subgroups I and II, on the other hand, were separated from each other by 621 SNPs. Geographical clustering was notable for isolates belonging to PG-1 (Fig. 1). Specifically, isolates from Southeast Asian nations and Bhutan belonged to two separate subgroups. Within the Southeast Asia subgroup, the Vietnamese Sf6 were interspersed with those of neighbouring Cambodia, and were separated from the paraphyletic Thai isolates (Fig. 1). Further inspection showed that several Thai strains (=9) were basal to the Vietnamese and Cambodian counterparts. These observations may reflect the local establishment of Sf6 in Asia and their cross-border propagation between neighbouring countries.

In order to estimate the time of divergence for the Asian Sf6, we utilized Bayesian phylogenetic inference (BEAST v1.8.3 [27] on a subset of 82 representative strains (with attached sampling date) selected across the entire phylogeny. The mean nucleotide substitution rate was calculated to be 1.04×10⁻⁶ substitutions per site per year (95% highest posterior density (HPD): 8.84×10⁻⁷ to 1.21×10⁻⁵). This rate is consistent with previous estimates from global evolutionary history analyses for *S. flexneri* (6.46–9.54×10⁻⁷, species-wide, excluding Sf6 [12]), *S. dysenteriae* serotype 1 (8.70×10⁻⁷ [47]), and *S. sonnei* (6.0×10⁻⁷ [48]). Our Bayesian analyses also estimated that the examined Sf6 population likely arose in the 18th century (95% HPD: 1715.6–1814.6) (Fig. 2). The Bayesian phylogenetic topology matched that inferred from the maximum-likelihood method. With PG-1’s estimated most recent common ancestor (MRCA) dating back to ~1972 (95% HPD: 1967.0–1977.0), it was younger than PG-2, which was estimated to descend from an MRCA circa 1910 (95% HPD: 1901.3–1930.6) (Fig. 2). The calculated substitution rates of the two PGs were comparable (PG-1: mean 1.09×10⁻⁶ substitutions per site per year, 95%
HPD: 7.92×10⁻⁷ to 1.21×10⁻⁶; PG-2: mean 9.88×10⁻⁷, 95% HPD: 8.14×10⁻⁷ to 1.17×10⁻⁶). For PG-2, all subgroups (I, II, and III) diverged during the late 20th century (95% HPD of the MRCA of subgroups I: 1971.7–1979.0; II: 1982.9–1986.0; and III: 1991.3–1995.2). Additionally, both PG-1 and PG-2 were independently assessed using similar BEAST analyses. The resulting tMRCAs and substitution rates for the two PGs were consistent with those inferred from the entire phylogeny, demonstrating the robustness of our analyses.

**Antimicrobial resistance profile in Shigella flexneri 6**

In order to investigate the AMR profiles of Sf6, we used a genotyping approach, supported by the consistency between AMR genotyping and phenotyping results in *Shigella* as reported previously [49]. The resistome of Sf6 revealed a similar trend as observed in other *Shigella* species, in which resistance to first-generation antimicrobials for shigellosis treatment is commonplace [12, 47, 48, 50, 51]. In particular, genetic elements conferring resistance to sulphonamide (*sul1* or *sul2*, *n*=89/96), trimethoprim (*dfrA* variants, *n*=89/96), and tetracycline (*tetA* or *tetB*, *n*=92/96) were prevalent across the phylogeny (Fig. 2). However, the elements carrying these AMR genes were variable across and within the PGs. In PG-2, the multidrug resistance (MDR) element (carrying *sul2*, *tetA*, and *strAB*) was detected in all except two isolates (*n*=12/14), but this element was co-transferred in two different plasmid backbones. These include the spA-like plasmid (recovered previously in *S. sonnei* [38]) in subgroup III, and the IncQ1
pNUC-like plasmid in most isolates of subgroups I and II. On the other hand, a single acquisition of plasmid RCS87_p (~6 kb, carrying sul2) in the MRCA of PG-1 explains the widespread resistance to sulphonamide in this PG. This, coupled with the acquisition of dfrA1 (carried on a class II integron) in most PG-1 isolates, subsequently rendered prevalent resistance to co-trimoxazole in the 1970s.

Another notable MDR element in our Sf6 collection was the *Shigella* resistance locus pathogenicity island (SRL-PAI) [52], which confers resistance to tetracycline (*tetB*), chloramphenicol (*catA1*), ampicillin (*bla_{OXA-1}*), and aminoglycosides (*aadA1*). This SRL-PAI was likely introduced into the MRCA of PG-1, but not in PG-2, at least 50 years ago, and has been persistently maintained (Fig. 2). However, we observed the loss of the *catA1* and *bla_{OXA-1}* in all Bhutanese and one Cambodian isolate, showing that the SRL was subject to modification. Resistance to macrolides or quinolones was identified in one Sf6 isolate each. Both of these were isolated in Vietnam, lacked the chromosomal SRL-PAI, carried *bla_{TEM-1B}* and harboured large multidrug resistance plasmids (~75–87.7 kb). Specifically, one isolate (PG-1) carried an IncFII plasmid (pCTXM3_020032-like) co-transferring five AMR genes (*mphA*, *ermB*, *sul1*, *aadA5*, and *dfrA17*), conferring additional resistance to macrolides. The other isolate (PG-2) harboured a pRC960-1-like plasmid co-transferring seven AMR genes (*tetA*, *sul2*, *strAB*, *dfrA14*, *bla_{TEM-1B}* and *qnrS*), predictively conferring additional resistance to quinolones. This isolate’s closest relative was also likely to have acquired a similar plasmid, but the MDR region (with the exception of *tetA*) had been lost (Fig. 2).

**Virulence determinants**

Pan-genome analysis indicated the presence of 5894 genes in 96 examined Sf6, which consisted of 3468 core genes and 2426 accessory genes. Various virulence determinants were ubiquitous across the collection. These include the virulence plasmid and the aerobactin biosynthesis cluster (*iutA, iucABCD*), the deletions of which are known to attenuate the

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**Fig. 2.** The temporal phylogenetic reconstruction of *Shigella flexneri* serotype 6 in Asia. The figure shows a maximum clade credibility phylogenetic reconstruction of 82 *S. flexneri* serotype 6 in four Asian countries (Thailand, *n*=45; Vietnam, *n*=25; Cambodia, *n*=6; and Bhutan, *n*=6). The phylogeny comprises two principal phylogenetic groups, which are herein referred as the major group (PG-1, *n*=68) and the minor group (PG-2, *n*=14). PG-2 includes three subgroups: I, II, and III. Red circles indicate posterior probability support ≥90 % on internal nodes. Right-hand columns correspond to the countries of origin, the presence of several antimicrobial resistance (AMR) genes (blue), the presence of the *Shigella* resistance locus pathogenicity island (SRL-PAI) (brick-red), and the presence of different plasmid backbones (pink), respectively (see key). Gene inactivation and gene loss events (purple) are overlaid on their corresponding branches.
virulence of S. flexneri [53, 54]. The cell-entry region (mxi-spa type III secretion system) on the Shigella virulence plasmid is a prominent player in their pathogenesis [55]. In our Sf6 collection, the mxi-spa region was detected in 94/96 isolates. In each of these genomes, we detected the presence of a single large contig (33472–37893 bp) with 90% nucleotide identity to the cell-entry region of S. flexneri 2a virulence plasmid pCP301. Likewise, the virulence genes icsA/virG and virA were identified in almost all isolates (n=94). Deletions of the cell-entry region, icsA/virG, and virA were scattered across the two principle PGs, and appeared to occur stochastically, likely as a result of culturing and/or storage conditions of the isolates. This was seen previously for the cell-entry region in the virulence plasmid pSB4_227 of S. boydii Sb227 [38]. In comparison to pCP301, certain loci were consistently missing in the virulence plasmid of S. flexneri. These include sepA (serine protease autotransporter for tissue invasion [56]), phoN1 (periplasmic non-specific acid phosphatase), stbAB (type II partitioning system), rfuU (UDP-sugar hydrolase), and ipgH (sugar phosphate transport protein) [38, 57]. These are also absent from the virulence plasmid pSB4_227 (S. boydii) as well as PSS_046 (S. sonnei), showing that their presence is probably a distinguishing feature of the S. flexneri 2a virulence plasmid.

For chromosomally encoded virulence factors, the SHI-1 pathogenicity island of examined Sf6 was more similar to that of S. boydii serotype 4 (Sb227) than of the S. flexneri 2a, since only sigA was detected while the pic/set1AB region (of S. flexneri 2a) was absent. As aforementioned, the iut/iuc operon, which encodes an aerobactin system (for iron acquisition), was present in all isolates. This suggested the existence of the Shigella pathogenicity island SHI-3. Besides this operon, Sf6 also harboured many other iron-uptake systems and associative regulators, which are commonly found in Shigella spp. These include the sit locus (sitABCD), fec locus (fecABC), fhu locus (fhuABC), and the regulators fur, fnr, and arcAB. We also detected the presence of the complete enterobactin biosynthesis operon (entABCDDEF – fecABCDE – fes) in all Sf6 isolates. This siderophore production system has only been found in some S. boydii strains, and its function in S. flexneri is currently disputed [12, 54, 58]. In S. flexneri, the system has been reported to be rarely utilized, but global phylogenetic analyses suggest that it is ancestral in the species and has been retained in several lineages [12, 58]. Closer inspection on the virulence gene repertoire of Sf6 highlighted the disparity between PG-1 and PG-2. One notable difference was the SRL-PAI-mediated fec locus (fecIRABCD) encoding for ferric-dicitrate uptake [52], which was only present in PG-1 (Fig. 2). This locus has been proposed to confer selective advantages to Shigella by broadening the availability of nutrient iron [12, 59, 60].

**Reductive evolution**

Substantial gene inactivation (pseudogenization) and gene loss are hallmarks in the genomic evolution of the Shigella spp. as they evolved into human-restricted pathogens. In this study, we identified several such instances in our Sf6 collection. The pseudogenized genes were those involved in metabolic functions and biosynthesis of cellular appendages (Table 2). Moreover, most of these targets exhibited differentiation between the two principal PGs. For instance, the mtdADR operon encoding for d-mannitol catabolism was only absent in the Bhutanese isolates of PG-1 (n=4). Genes phnD and ulaD, involved in phosphanate transport and l-ascorbate catabolism, respectively, were each inactivated by a nonsense mutation in all PG-1 descendants. On the other hand, the same genetic operon could undergo differing pseudogenization mechanisms in PG-1 and PG-2, eventually leading to the same predicted phenotypic consequences. The operon xylRHPF, responsible for D-xylose metabolism, was disrupted in different manners among the two PGs. In all PG-1 isolates, xylH was inactivated by a frameshift mutation, whereas in PG-2, the operon activator xylR was truncated and xylH remained intact. These predicted that D-xylose utilization was dysfunctional in both PGs and served as an exemplar of pseudogenization-mediated convergent evolution in Sf6.

We found that several operons, which had undergone some degrees of pseudogenization in the ancestral Sb227, continued to be disrupted in Sf6. For example, the operons encoding for curli structure (csgBAC), assembly, and secretion (csgGFED) were subjected to degradation in the Sb227 genome [61, 62], leaving only csgA and csgD intact. Detailed genetic characterization revealed that all PG-2 genomes harboured additional disruptive mutations in csgB and csgD, as compared to the Sb227 genome, while PG-1 likely lost nearly all genes belonging to these two operons. Likewise, several genes involved in L-fucose catabolism, including fucO, fucP, and fucK, were further disrupted or lost in PG-2. These genes were inactivated in the Sb227 genome, and genome-based metabolic models predicted that most Shigella species could not utilize L-fucose [63]. Therefore, the separate pseudogenization events in the fuc operon signify an ongoing evolutionary pathway towards exclusion of L-fucose metabolism in Shigella.

**DISCUSSION**

By constructing the phylogeny of Sf6 isolated from four Asian countries at whole genome level, we have identified two principal phylogenetic groups (PG-1 and –2) circulating in the region. The phylogenetic distance between the two PGs was considerable, and the MRCA of extant Asian Sf6 dated back to the 18th century. The two PGs were uneven in both size and TMRCA, with PG-1 being more frequently detected and emerging more recently than PG-2. The most distinguishing difference in their genetic repertoire was the exclusive presence of SRL-PAI in PG-1, which encodes multiple AMR genes and an iron uptake fec locus [12, 52, 60]. This island has been acquired independently and stably maintained on several occasions across multiple lineages of other S. flexneri serotypes [12] and of S. dysenteriae type 1 [47]. In the latter, the SRL-PAI was also the major genetic element associated with an MDR phenotype, and it was most prevalent in the recently emerging lineage [47]. Therefore, the SRL-PAI may grant a competitive advantage for PG-1, thus leading to
its dominance in our collection. While our isolates mostly originated from diarrhoeal surveillance studies and should reflect the epidemiological trend of Sf6, the uneven distribution of samples across the two PGs might also be attributed to sampling bias in our data. The absence of Cambodian and Bhutanese isolates in PG-2 could be due to the narrower sampling windows in these two countries compared to those of Thailand (1987–2005) and Vietnam (1995–2010). Sf6 isolated in 1994 in Thailand constituted the largest proportion of our Thai isolates \( n=19/53 \), and all these 1994 isolates belonged to PG-1. This spike reflected the occurrence of an Sf6 outbreak in 1994 in Thailand, which has been captured by a previous shigellosis surveillance study in the country (1993–2006) [64].

The major lineage PG-1, as well as all subgroups (I, II, III) of PG-2, were estimated to have emerged in the 1970s, with PG-1 undergoing clonal expansion in both Bhutan and Southeast Asia. However, the absence of non-Asian Sf6 genomes in our analysis did not allow us to conclude whether the two PG’s MRCA emerged within or were introduced into Asia. Our previous study on other S. flexneri serotypes and S. sonnei in Southeast Asia has demonstrated that the extant progenies of these Shigella species likely got introduced into the region in the same time frame (1970s-90s) [13]. These findings together point to the intensity of multiple Shigella introduction events into Asia post-1970s, and subsequent propagation may have been facilitated by expanding population size and heightened human migration in Asia. Most of the examined Sf6 harboured several AMR genes, mostly conferring resistance to first-generation antimicrobials used to treat shigellosis (sulphonamide, trimethoprim, tetracycline, and chloramphenicol). On the other hand, resistance to more recent first-line antimicrobials (quinolone and macrolides) was rare (2/96 isolates). This finding mirrors the resistomes inferred from other S. flexneri serotypes in the same period in Southeast Asia, but is different from that of S. sonnei. More specifically, for other S. flexneri serotypes, the emergence of macrolide resistance was sporadic and showed no fixation, while S. sonnei isolated post-2010s showed an increase in macrolide resistance [13]. Likewise, resistances to third-generation cephalosporins and quinolone were both more prevalent in S. sonnei, with higher occurrence of bla\textsubscript{CTX-M} variants and gyrA/parC mutations [13, 44, 51].

Detailed genetic investigation allowed us to catalogue a number of gene inactivation events in Sf6, and we focused on genes involved in metabolism and biosynthesis of cellular appendages.

### Table 2. Reductive evolution at genes for metabolism and biosynthesis of cellular appendages. The presence/absence and intactness/disruption of genes involved in metabolic pathways and cellular appendages synthesis in the Sb227 genome (S. boydii 4) and in two principal phylogenetic groups of S. flexneri 6 in Asia (PG-1 and -2) are described. Asterisks annotate that the whole operon for \( \Delta \)-xylose utilization was absent in one Thailand isolate within PG-1.

| Product / Biochemical reaction | Gene | Functions | Sb227 (CP000036.1) | S. flexneri 6 | Major group (PG-1) | Minor group (PG-2) |
|-------------------------------|------|-----------|-------------------|----------------|-------------------|-------------------|
| **Metabolic function**        |      |           |                   |                |                   |                   |
| \( \Delta \)-mannitol          | mtlADR | \( \Delta \)-mannitol permease and catabolism | Intact          | Whole operon loss in Bhutanese isolates \( n=4 \) | Intact            |                   |
| Phosphonate                    | phnD  | Phosphonate transport | Intact          | Stop codon in all PG-1 | Intact            |                   |
| \( \Delta \)-ascorbate         | ullaD | Anaerobic \( \Delta \)-ascorbate degradation | Intact          | Stop codon in all PG-1 | Intact            |                   |
| \( \Delta \)-xylose            | xylH  | \( \Delta \)-xylose ABC transporter | Intact          | Frameshift in all PG-1* | Intact            |                   |
|                               | xylR  | \( \Delta \)-xylose degradation regulator | Intact          | Intact*            | Truncated in subgroups I and II |
| \( \Delta \)-fucose            | fucO  | L-1,2-propanediol oxidoreductase | Frameshift      | Frameshift        | Additionally truncated |
|                               | fucP  | \( \Delta \)-fucose/proton symporter | Truncated       | Truncated         | Gene loss in subgroups I and II; additional deletion in subgroup III |
|                               | fucK  | \( \Delta \)-fuculokinase | Stop codon      | Intact            | Frameshift in subgroups I and II |
| **Cellular appendages**       |      |           |                   |                |                   |                   |
| Curli                         | csgA  | Major curlin subunit | Intact          | Gene loss in all PG-1 | Intact            |                   |
|                               | csgB  | Minor curlin subunit | Truncated       | Gene loss in all PG-1 | Additional truncation |                   |
|                               | csgD  | csgBAC transcriptional regulator | Intact          | Gene loss in all PG-1 | Disrupted by IS element |                   |
| Flagella                      | flhA  | Flagella biosynthesis | Intact          | Truncated in one Bhutanese isolate | Truncated in subgroup I |
appendages (flagella, fimbriae, curli, etc.). Genes involved in the utilization of D-mannitol, D-xylose, phosphonate and L-ascorbate, which are intact in S. boydii 4 genome Sb227, were found to be inactive in our examined Sf6 genomes. Components involved in these metabolisms have also been found to be pseudogenized or lost in other Shigella species (D-mannitol: lost in Sf301 (S. flexneri 2a) and pseudonized in Ss046 (S. sonnei); D-xylose: pseudonized and/or lost in Sd197 (S. dysenteriae 1), Sf301, and Ss046; phosphonate: pseudonized in Sd197 and lost in Sf301; L-ascorbate: pseudonized in Sd197 and Ss046) [38]. Additionally, evidence of convergent evolution between the two PGs was observed in differing pseudogenizations in D-xylose metabolism. Together, these results underscored the prominent role of reductive evolution affecting the metabolic flexibility of Sf6 and Shigella spp., which has been a hallmark of the pathogen’s adaptation for intracellular lifestyle [39, 65]. An exemplar for this is the contrast in L-fucose catabolism between Shigella and other extracellular enteric pathogens. Genes encoding L-fucose utilization have been inactivated in multiple Shigella species [38], and we also observed disruptions in such elements in our Sf6 collection. Similarly, genome-wide metabolic reconstruction indicated that most Shigella strains (7/8 tested, except S. boydii CDC 3083) could not sustain growth on L-fucose [63]. In contrast, other extracellular enteric pathogens (including Campylobacter jejuni [66], enterohemorrhagic Escherichia coli [67], Salmonella enterica serovar Typhimurium, and Clostridium difficile [68]) have been found to rely on L-fucose for their survival and pathogenesis, as it is abundant (incorporated in mucin) in the intestinal milieu or could be foraged from the host microbiota. Shedding of cellular appendages has been proposed as pathoadaptive in Shigella spp., differentiating them from many other Enterobacteriaceae [62]. Although curli are important in biofilm formation and host cell adhesion and invasion of E. coli and Salmonella spp [61, 62], the operon responsible for curli synthesis (csgA-G) has been independently inactivated across all Shigella species [38, 61]. In Sf6, it was noted that the operon carried additional reductions in PG-2 and many further gene losses in PG-1. These demonstrated the momentum of gene degradation in Sf6 when the bioprocess is not functional, possibly in order to reduce the energy expenditure on superfluous genetic elements that might have conferred a fitness cost to the bacteria [69].

Our research sheds light into the evolutionary history and genomic evolution of the understudied Sf6 in four Asian countries. Our findings, however, were constrained by certain limitations. Firstly, due to limited data, global isolates from other continents were not incorporated for phylogenetic context. Thus, our findings could not be extended to Sf6 circulating in other endemic regions such as Africa or South America. In addition, our samples were unevenly distributed among countries and years, and were over-represented by urban paediatric populations. These may bias interpretations, such as inducing a false impression of the country of origin, when there is much more data collected from a single country (Thailand in our case). Our investigations on the presence/absence and intactness/disruption of genetic elements were not exhaustive. Therefore, other features of genomic evolution events still remain unexplored.

Notwithstanding these limitations, our study builds a framework for future investigations into Sf6, which is the fourth most common Shigella serotype and a target for pan-Shigella vaccine development. Insights into Sf6 will help portray a more thorough representation of Shigella epidemiology and evolution, as well as informing the optimal development of therapeutic and public health interventions.

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Author contributions
Conceptualization: S.N.M.T., S.B., H.C.T. Data curation: P.V.V., T.H.T., S.N.M.T., H.C.T. Formal analysis: S.N.M.T., H.C.T. Investigation: S.N.M.T., H.C.T. Methodology: S.N.M.T., H.C.T. Writing – original draft: S.N.M.T., H.C.T. Writing – review and editing: D.T.P., S.B., M.A.R., G.E.T., N.R.T., H.C.T. Resources: L.B., P.T., S.W., D.T.P., N.R.T.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study received ethical approvals from the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, all other participating hospitals, the Institutional Review Board of the Walter Reed Army Institute of Research (for Thailand data), the Research Ethics Board of Health in Bhutan (Bhutan data), and the Oxford Tropical Research Ethics Committee (OxTREC) in the United Kingdom. Written informed consent from study participants or their parents/guardians was obtained prior to the collection of stool samples.

References
1. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, et al. Burden and etiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 2013;382:209–222.
2. Troeger C, Foreman K, Rao P, Khatib I, Brown A, et al. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Infect Dis 2017;17:909–948.
3. Gentle A, Ashton PM, Dallman TJ, Jenkins C. Evaluation of molecular methods for serotyping Shigella flexneri. J Clin Microbiol 2016;54:1456–1461.
4. Dmitriev BA, Knirel YA, Sheremet OK, Shashkov AA, Kochetkov NK, et al. Somatic antigens of Shigella. The structure of the specific polysaccharide of Shigella enterica (Sh. flexneri type 6) lipopolysaccharide. Eur J Biochem 1979;98:309–316.
5. Cheah K-C, Beger DW, Manning PA. Molecular cloning and genetic analysis of the rfb region from Shigella flexneri type 6 in Escherichia coli K-12. FEMS Microbiol Lett 1991;83:213–218.
6. Allison GE, Verma NK. Serotype-converts converting bacteriophages and O-antigen modification in Shigella flexneri. Trends Microbiol 2000;8:17–23.
7. Liu B, Knirel YA, Feng L, Perepelov AV, Senchenkova SN, et al. Structure and genetics of Shigella 0 antigens. FEMS Microbiol Rev 2008;32:627–653.
8. Choi SY, Jeon Y-S, Lee JH, Choi B, Moon SH, et al. Multilocus sequence typing analysis of Shigella flexneri isolates collected in Asian countries. J Med Microbiol 2007;56:1460–1466.

9. Yang J, Nie H, Chen L, Zhang X, Yang F, et al. Revisiting the molecular evolutionary history of Shigella spp. J Mol Evol 2007;64:71–79.

10. Gorgé O, Lopez S, Hilaire V, Lisanti O, Ramisse V, et al. Selection and validation of a multilocus variable-number tandem-repeat analysis panel for typing Shigella spp. J Clin Microbiol 2008;46:1026–1036.

11. Sahl JW, Morris CR, Emberger J, Fraser CM, Ochiumg JB, et al. Defining the phylogenomics of Shigella species: A pathway to diagnostics. J Clin Microbiol 2015;53:951–960.

12. Connor TR, Barker CR, Barker KS, Weill F-X, Talukder KA, et al. Species-wide whole genome sequencing reveals historical global spread and recent local persistence in Shigella flexneri. Theor Biol 2015;25:2078–2079.

13. Chung The H, Bodhidatta L, Pham DT, Mason CJ, Ha Thanh T, et al. Evolutionary histories and antimicrobial resistance in Shigella flexneri and Shigella sonnei in Southeast Asia. Commun Biol 2014;1:353.

14. Gu B, Cao Y, Pan S, Zhuang L, Yu R, et al. Comparison of the prevalence and changing resistance to nalidixic acid and ciprofloxacin of Shigella between Europe-America and Asia-Africa from 1998 to 2009. Int J Antimicrob Agents 2011;29:33–39.

15. Livio S, Stockbine NA, Panchalagnam S, Tennant SM, Barry EM, et al. Shigella isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 2014;59:933–941.

16. von Seidlein L, Kim DR, Ali M, Lee H, Wang X, et al. A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. PLoS Med 2006;3:e353.

17. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:13033977v2 [q-bio.GN]; USA: Cornell University, 2013. http://arxiv.org/abs/1303.3977

18. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011;43:491–498.

19. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25:2078–2079.

20. Arndt D, Grant JR, Marcu A, Sajed T, Por A, et al. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res 2016;44:W16–21.

21. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res 2015;43:e15.

22. Stamatakis A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 2014;30:1312–1313.

23. Yu G, Smith DK, Zhu H, Guan Y, Lam TT, et al. ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol Evol 2016;8:28–36.

24. Core Team R: R: a language and environment for statistical computing. Vienna, Austria; 2018. https://www.r-project.org/

25. Kalyaanamorthy S, Minh BQ, Wong TFK, von Haeseler A, Jermiin LS. ModelFinder: Fast model selection for accurate phylogenetic estimates. Nat Methods 2017;14:587–589.

26. Rambaut A, Lam TT, Max Carvalho L, Pybus OG. Exploring the temporal structure of heterogeneous sequences using TempEst (formerly Path-O-Gen). Virus Evol 2016;2:vwe007.

27. Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 2012;29:1969–1973.

28. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Syst Biol 2018;67:901–904.

29. Baele G, Lemey P, Bedford T, Rambaut A, Suchard MA, et al. Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. Mol Biol Evol 2012;29:2157–2167.

30. Baele G, Li WLS, Drummond AJ, Suchard MA, Lemey P. Accurate model selection of relaxed molecular clocks in Bayesian phylogenetics. Mol Biol Evol 2013;30:239–243.

31. Bolger AM, Lahse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30:2114–2120.

32. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19:455–477.

33. Hunt M, Mather AE, Sánchez-Busó L, Page AJ, Parkhill J, et al. ARIBA: Rapid antimicrobial resistance genotyping directly from sequencing reads. Microb Genom 2017;3:e000131.

34. Jermiin LS, Hmasan H, Cosentino S, Vestergaard M, Rasmussen S, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 2012;67:2640–2644.

35. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, et al. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 2015;31:3691–3693.

36. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014;30:2068–2069.

37. Assøfa T, Keane TM, Otto TD, Newbold C, Berriman M, et al. ABACAS: Algorithm-based automatic contiguration of assembled sequences. Bioinformatics 2009;25:1968–1969.

38. Yang F, Yang J, Zhang X, Chen L, Jiang Y, et al. Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery. Nucleic Acids Res 2005;33:6445–6458.

39. The HC, Thanh DP, Holt KE, Thomson NR, Baker S. The genomic signatures of Shigella evolution, adaptation and geographical spread. Nat Rev Microbiol 2016;14:235–250.

40. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, et al. In silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014;58:3895–3903.

41. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–410.

42. Carver T, Berriman M, Tivey A, Patel C, Böhm U, et al. Artemis and ACT: Viewing, annotating and comparing sequences stored in a relational database. Bioinformatics 2008;24:2672–2676.

43. Vinh H, Anh VTC, Anh ND, Campbell JI, Hoang NV, et al. A multi-center randomized trial to assess the efficacy of ciprofloxacin versus ciprofloxacin for the treatment of shigellosis in Vietnamese children. PLoS Negl Trop Dis 2011;5:e1264.

44. Holt KE, Thieu Ngay TD, Thanh DP, Vinh H, Kim DW, et al. Tracking the establishment of local endemic populations of an emergent enteric pathogen. Proc Natl Acad Sci U S A 2013;110:17522–17527.

45. Bodhidatta L, Vithayasai N, Eimpokalarp P, Bitarangsi C, Serichantalergs O, et al. Bacterial enteric pathogens in children with acute dysentery in Thailand: increasing importance of quinolone-resistant Campylobacter. Southeast Asian J Trop Med Public Health 2002;33:752–757.

46. Ruekit S, Wangchuk S, Dori T, Tshering KP, Pootong P, et al. Molecular characterization and PCR-based replicon typing of multidrug resistant Shigella sonnei isolates from an outbreak in Thimphu, Bhutan. BMC Res Notes 2014;7:95.1:9–.

47. Njamkepo E, Fawal N, Tran-Dien A, Hawkey J, Stockbne N, et al. Global phylogeography and evolutionary history of Shigella dysenteriae type 1. Nat Microbiol 2016;1:16027.

48. Holt KE, Baker S, Weil F-X, Holmes EC, Kitchen A, et al. Shigella sonnei genome sequencing and phylogenetic analysis indicate recent global dissemination from Europe. Nat Genet 2012;44:1056–1059.

49. Sadouki Z, Day MR, Doumith M, Chattaway MA, Dallman TJ, et al. Comparison of phenotypic and WGS-derived antimicrobial resistance profiles of Shigella sonnei isolated from cases of diarrhoeal
YSH6000 is Shigella flexneri dicitrate transport system (Fec) of Luck SN, Schmitt MP, Gene sequences encoding bacterial sugar phosphate transport proteins. flexneri invasion plasmid gene, ipgH, with homology to IS629 and integrity via cofilin activation. Gut Microbes Shigella depends on SepA to destabilize the intestinal epithelial Shigella flexneri genes in J Bacteriol, 2019;10:4828. Shigella sonnei Nat Commun Dissecting the molecular evolution of fluoroquinolone-resistant Chung The H, 2017;72:2496–2502. J Antimicrob Chemother 2017;72:2496–2502.

50. Chung The H, Boinett C, Pham Thanh D, Jenkins C, Weill F-X, et al. Dissecting the molecular evolution of fluoroquinolone-resistant Shigella sonnei. Nat Commun 2019;10:4828.

51. Thanh Duy P, Thi Nguyen TN, Vu Thuy D, Chung The H, Alcock F, et al. Commensal Escherichia coli are a reservoir for the transfer of XDR plasmids into epidemic fluoroquinolone-resistant Shigella sonnei. Nat Microbiol 2020;5:256–264.

52. Luck SN, Turner SA, Rajakumar K, Sakellaris H, Adler B. Ferric dicitrate transport system (Fec) of Shigella flexneri 2a YSH6000 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. Infect Immun 2001;69:6012–6021.

53. Sansonetti PJ, Kopecko DJ, Formal SB. Involvement of a plasmid in the invasive ability of Shigella flexneri. Infect Immun 1982;35:852–860.

54. Wyckoff EE, Boulette ML, Payne SM. Genetics and environmental regulation of Shigella iron transport systems. BioMetals 2009;22:43–51.

55. Schroeder GN, Hilbi H. Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. Clin Microbiol Rev 2008;21:134–156.

56. Maldonado-Contreras A, Birtley JR, Boll E, Zhao Y, Mummy KL, et al. Shigella depends on SepA to destabilize the intestinal epithelial integrity via cofilin activation. Gut Microbes 2017;8:544–560.

57. Venkatesan MM, Alexander WA, Fernandez-Prada C, A Shigella flexneri invasion plasmid gene, ipgH, with homology to IS629 and sequences encoding bacterial sugar phosphate transport proteins. Gene 1996;175:23–27.

58. Schmitt MP, Payne SM. Genetics and regulation of enterobactin genes in Shigella flexneri. J Bacteriol 1988;170:5579–5587.

59. Luck SN, Turner SA, Rajakumar K, Sakellaris H, Adler B. Ferric dicitrate transport system (Fec) of Shigella flexneri 2a YSH6000 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. Infect Immun 2001;69:6012–6021.

60. Turner SA, Luck SN, Sakellaris H, Rajakumar K, Adler B. Molecular epidemiology of the SRL pathogenicity island. Antimicrob Agents Chemother 2003;47:727–734.

61. Sakellaris H, Hannink NK, Rajakumar K, Bulach D, Hunt M, et al. Curli loci of Shigella spp. Infect Immun 2000;68:3780–3783.

62. Barnhart MM, Chapman MR. Curli biogenesis and function. Annu Rev Microbiol 2006;60:131–147.

63. Monk JM, Charusanti P, Aziz RK, Lerman JA, Premyodhin N, et al. Genome-scale metabolic reconstructions of multiple Escherichia coli strains highlight strain-specific adaptations to nutritional environments. Proc Natl Acad Sci U S A 2013;110:20338–20343.

64. Bangtrakulnonth A, Vieira AR, Lo Fo Wong DMA, Porroneongwong S, Pulsrikkarn C, et al. Shigella from humans in Thailand during 1993 to 2006: Spatial-time trends in species and serotype distribution. Foodborne Pathog Dis 2008;5:773–784.

65. Bliven KA, Maurelli AT. Antivirulence genes: Insights into pathogen evolution through gene loss. Infect Immun 2012;80:4061–4070.

66. Garber JM, Nothaft H, Pluvinage B, Stahl M, Bian X, et al. The gastrointestinal pathogen Campylobacter jejuni metabolizes sugars with potential help from commensal Bacteroides vulgatus. Commun Biol 2020;3:2.

67. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, et al. Fucose sensing regulates bacterial intestinal colonization. Nature 2012;492:113–117.

68. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature 2013;502:96–99.

69. Koskiniemi S, Sun S, Berg OG, Andersson DI. Selection-driven gene loss in bacteria. PLoS Genet 2012;8:1002787.

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