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Scrub typhus is caused by the obligate intracellular rickettsia *Orientia tsutsugamushi* (previously called *Rickettsia tsutsugamushi*). The bacterium is maternally inherited in trombicuid mites and transmitted to humans by feeding larvae. We report here the 2,127,051-bp genome of the Boryong strain, which represents the most highly repeated bacterial genome sequenced to date. The repeat density of the scrub typhus pathogen is 200-fold higher than that of its close relative *Rickettsia prowazekii*, the agent of epidemic typhus. A total of 359 *tra* genes for components of conjugative type IV secretion systems were identified at 79 sites in the genome. Associated with these are >200 genes for signaling and host–cell interaction proteins, such as histidine kinases, ankyrin-repeat proteins, and tetratricopeptide-repeat proteins. Additionally, the *O. tsutsugamushi* genome contains >400 transposases, 60 phage integrases, and 70 reverse transcriptases. Deletions and rearrangements have yielded unique gene combinations as well as frequent pseudogenization in the *tra* clusters. A comparative analysis of the *tra* clusters within the genome and across strains indicates sequence homogenization by gene conversion, whereas complexity, diversity, and pseudogenization are acquired by duplications, deletions, and transposon integrations into the amplified segments. The results suggest intragenomic duplications or multiple integrations of a massively proliferating conjugative transfer system. Diversifying selection on host–cell interaction genes along with repeated population bottlenecks may drive rare genome variants to fixation, thereby short-circuiting selection for low complexity in bacterial genomes.

bacterial genome | duplication | repeats

The obligate intracellular bacterium *Orientia tsutsugamushi* is the causative agent of scrub typhus (1). This disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation, which, if left untreated, can lead to severe multiple organ failure (2). Mortality rates from scrub typhus in untreated patients range from 1% to 40%, depending on endemic area and strain of *O. tsutsugamushi* encountered (3). During World War II, Allied forces suffered more fatalities due to scrub typhus than as a direct consequence of the fighting in South-East Asia (4). Scrub typhus is restricted to a well defined geographic region that extends from Eastern Russia and Northern Japan in the north and Northern Australia in the south to Pakistan and Afghanistan in the west (3). This distribution distinguishes scrub typhus from other enzootic rickettsiosis and results from the ecology of the trombicuid mite vector and their vertebrate hosts (3). *O. tsutsugamushi* is harbored in the salivary glands of mites and is transmitted to humans and other hosts during larval feeding (5).

Scrub typhus cases can effectively be treated by using broad-range antibiotics such as doxycycline or chloramphenicol. However, treatment and disease control are complicated by frequent reinfection and relapses, which may be explained by the high antigenic diversity of this bacterium (6). Recent increases in the number of scrub typhus cases, coupled with observations of reduced effectiveness of antibiotic treatments (7–10), have highlighted the need for effective vaccines. However, vaccine development has been hampered by a limited host immune response (11) and *O. tsutsugamushi*'s ability to immunosuppress the host (12).

We report here the complete genomic sequence of the *O. tsutsugamushi* Boryong strain, isolated from a Korean patient. The genome has an extraordinary structure, with 37% identical repeats in the form of mobile genetic elements and accessory genes, putatively involved in host–parasite interactions. These striking features provide insights into the evolutionary aspects of intracellular parasitism as well as the pathogenic properties of the scrub typhus agent.

Results and Discussion

Overview of the *O. tsutsugamushi* Genome. The complete genome of *O. tsutsugamushi* strain Boryong is a single circular chromosome consisting of 2,127,051 bp with an average G+C content of 30.5% [supporting information (SI) Table 1]. The genome size and estimated number of genes are the largest among the currently sequenced genomes in the order *Rickettsiales*. We identified 2,179 potential protein-coding sequences, of which an estimated 963 sequences represents fragmented genes (Fig. 1; red first and second circles), as a result of which the overall coding capacity of the genome is estimated to be as low as 49.6%. Despite the larger genome size, there is a core of 512 genes (Fig. 1; yellow third circle) shared with seven other *Rickettsia* species. The majority of fragmented genes coincided with repeated DNA regions, which are

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Abbreviations: TFSS, type IV secretion system; HK, histidine kinase.

Data deposition: The sequence reported in this paper has been deposited in the European Molecular Biology database (accession no. AM494475).

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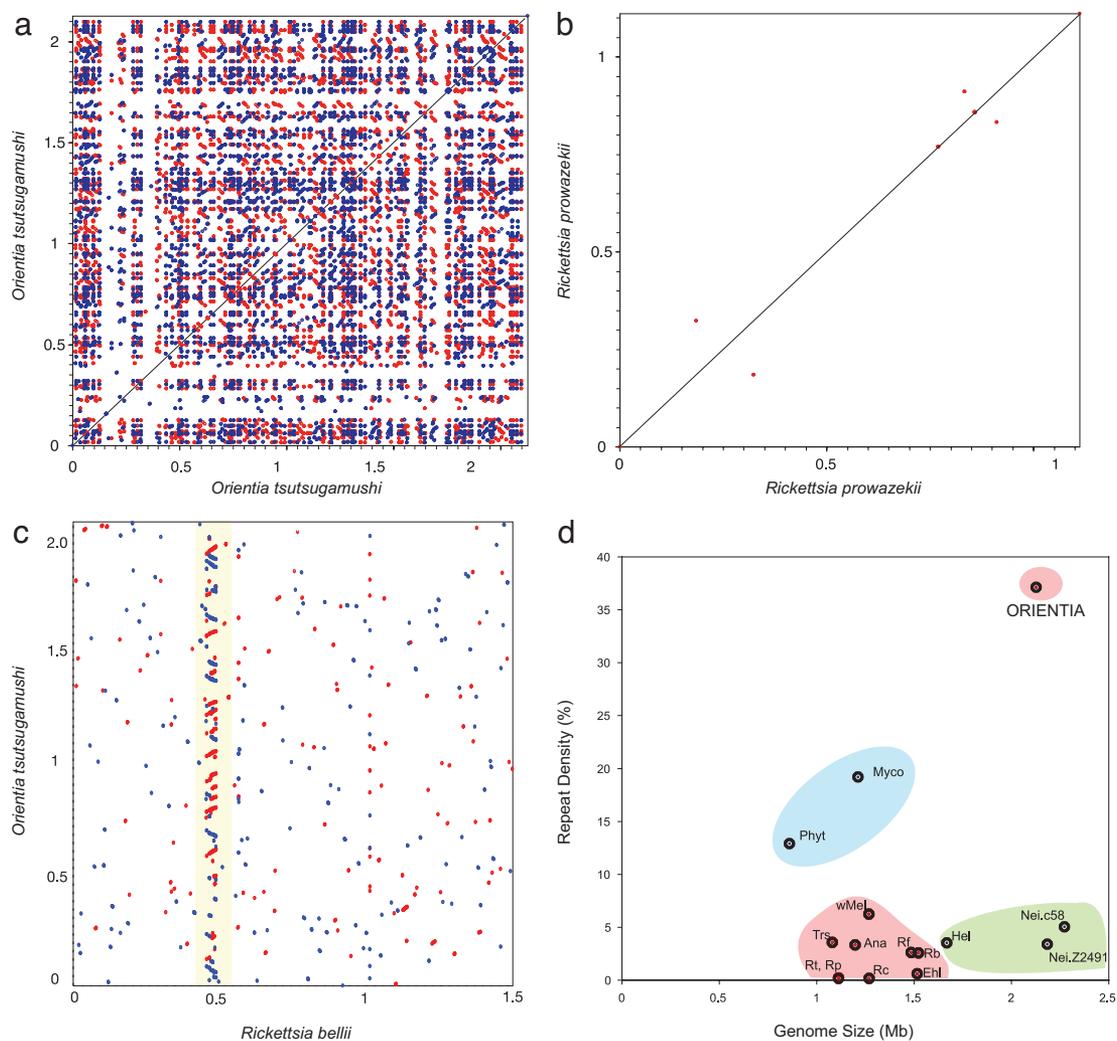


Fig. 2. Repeat density in obligate intracellular bacteria. The frequency of repeats in the genomes of (a) *O. tsutsugamushi* and (b) *R. prowazekii*, with each dot representing a repeat >95% identical and >200 bp in size; blue, direct repeats; red, reverse repeats. (c) Scrambled gene order structures in *O. tsutsugamushi* and *R. bellii*. The yellow box indicates the location of the single *tra* cluster for TFSSs in the *R. bellii* genome and the homologous amplified *tra* clusters in the *O. tsutsugamushi* genomes. (d) Repeat density is plotted against genome size (% identical repeats >200 bp). Bacterial groupings are indicated by circles; red, obligate intracellular members of the Rickettsiales including *Orientia*, *O. tsutsugamushi*; Ana, *Anaplasma marginale*; Ehl, *Ehrlichia ruminantium*; Rb, *R. bellii*; Rc, *Rickettsia conorii*; Rf, *R. felis*; Rp, *R. prowazekii*; Rt, *Rickettsia typhi*; Trs, *Wolbachia endosymbiont*; TRS, wMel, *Wolbachia wMel*; green, Hel, *Helicobacter pylori*; Nei.c58, *Neisseria meningitidis* C58; Nei.z2491, *N. meningitidis* z2491; blue, the most highly repeated bacterial genomes until the time of this study are those of Myco, *Mycoplasma mycoides*; and Phyt, *Phytoplasma* strain OY M.

The application of phylogenetic methods to the analysis indicated that the amplified *tra* genes in *O. tsutsugamushi* were in all cases more similar to each other than either was to its homolog in *R. bellii* (SI Fig. 7). The tree topologies suggested groups of nearly identical genes, with different genes supporting different clustering schemes. A plot of the Shannon–Weiner diversity index in sliding windows along *tra* gene alignments revealed extensive sequence similarity across the gene copies, with some of the centrally located *tra* genes, such as *traU*, *traW*, and *trbC* having no polymorphic sites over most of the alignments (SI Fig. 8). Such a patched similarity pattern across and within the repeated *tra* genes is consistent with ongoing sequence homogenization by gene conversion and also with multiple insertions in a short time span.

Gene Family Expansion of *tra*-Associated Genes. Located within or in the immediate vicinity of the *tra* clusters in *O. tsutsugamushi* are >200 genes encoding paralogous proteins putatively involved in signaling and host–cell interaction processes (Fig. 3b). For a list of the proteins in each family, see SI Tables 4–9, and for a represen-

tation of their domain structures, see SI Figs. 9–14. These include 27 TPR and 50 ankyrin-repeat proteins (23) and fragments thereof, some of which may mediate DNA and protein interactions with the eukaryotic host cell. Phylogenetic analysis of the TPR-repeat protein family (SI Fig. 15) indicates three groups that share a defined gene order and consists of one full-length “master” gene and several additional nearly identical gene or pseudogene copies. Based on phylogeny (SI Fig. 16), gene order, and domain structure (SI Fig. 10), the 40 ankyrin-repeat proteins formed three broad groups, and the remaining 10 single-copy genes showed a loose association with these groups. All ankyrin-repeat genes were flanked on one or both sides by *tra* genes, *tra*-associated genes, transposases, and/or reverse transcriptases.

Additionally, we identified 50 SpoT global regulators. *O. tsutsugamushi* is the only sequenced species in the Rickettsiales to have a full-length *spotT/reLA* gene that contains both the synthase and hydrolase catalytic residues, and the total number of *spotT/reLA*-like genes is five times that described in *R. bellii*. The other 49 *spotT/reLA*-like ORFs identified contained either the synthase ($n = 1$) or

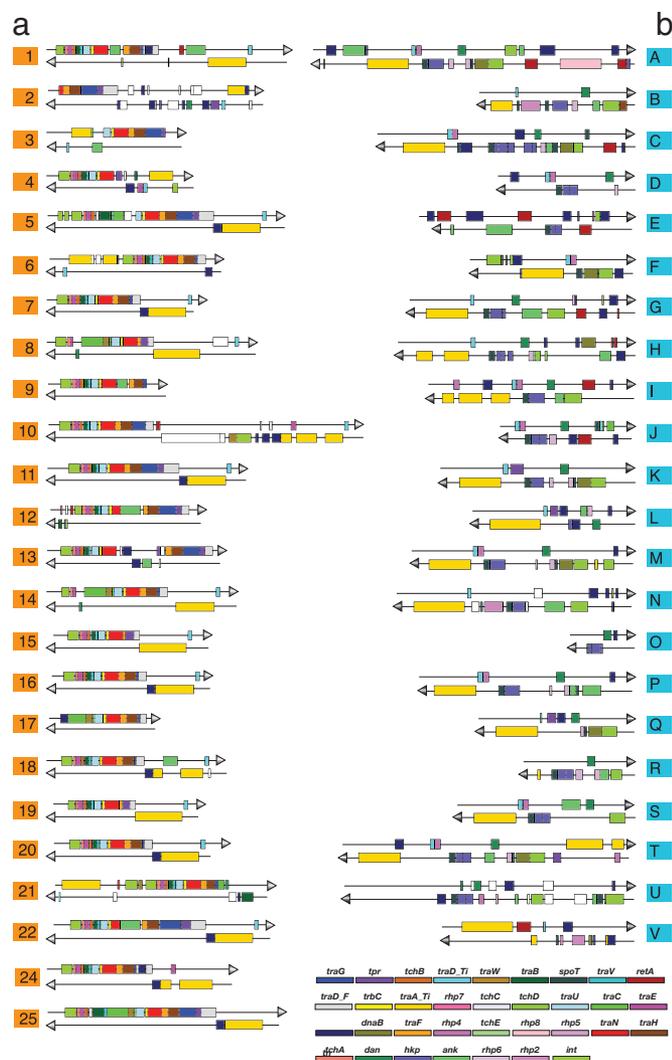


Fig. 3. Gene order structure of (a) 24 long repeated *tra* gene clusters and (b) the flanking *tra*-associated gene clusters encoding putative effector proteins. The position of the clusters in the genome is indicated in Fig. 1.

hydrolase ($n = 48$) catalytic residue. Also identified were 56 histidine kinase (HK) and response regulator domains, 10 of which are typical HKs (24) and include *pleCD*, *ntrYX*, *czcT*, and *phoB*-like genes, most with homologs in *Rickettsia* and *Wolbachia*. The remaining 46 HKs were associated with the *tra* clusters, grouped by phylogenetic analysis with RBE.415 and were organized in the motif *spoT*-HK-*dam*. Also located in the vicinity of the *tra* clusters were gene fragments for 33 DnaB helicases and 34 Dam proteins.

Transposon-Mediated Diversity. The 414 transposases identified in total were classified into five different families, IS5, IS630, IS982, IS110, and ISNCY, with 27–195 copies per family, with IS982 being the most abundant (SI Table 10 and SI Figs. 17 and 18). Approximately 86% are pseudogenes, with the most-abundant families containing the highest fraction of pseudogenes. Within each family, all copies >600 bp formed a single clade. Located within or in the immediate vicinity of the 24 *tra* clusters were ≈ 250 gene fragments for transposases and 51 *retA* genes for reverse transcriptases, which are probable constituents of retrotransposons. The reverse transcriptases were classified into two different types. The major type has 48 copies in the genome, whereas the minor type contains only three members. Additionally, we identified 61 phage-related ORFs,

a majority of which were phage integrases associated with the *tra* clusters.

To study the mutational mechanisms of decay and expansion, we have compared the sequences of three regions with transposons in the sequenced Boryong strain with the strains Gilliam, Karp, and Kato. One of the selected regions contains a transposase and the short 5'-terminal fragment of the *gltA* gene in the Boryong genome. Although conserved in size across all four strains, a notable difference is that the *gltA* gene fragment in the Gilliam strain shows none of the frameshifts or premature stop codons observed in the other three strains (SI Fig. 19). This suggests that the *gltA* gene has been inactivated by a transposon insertion and is gradually eroding by deletion and nucleotide substitutions, with none of the strains encoding a functional citrate synthase.

The other two regions cover multiple transposase pseudogenes and show a more extensive size variation among the various strains. The first region (at nucleotide position 172,980–176,345) shows a 48% size difference between strains Gilliam and Boryong, which is due to one vs. four segments with transposon pseudogenes, respectively. Two of the transposon sites (Fig. 4a, regions 2 and 3) are uniquely present in the Boryong strain, perhaps indicative of recent insertions in this strain. All transposons appear to have been rapidly deactivated, with insertion region 3 (Fig. 4a) representing a partially deleted transposon in the Boryong genome. The sole transposon identified in the Gilliam strain is located in segment 4, which is inverted in this strain relative to the corresponding segments in the other strains.

The second region (at nucleotide position 1,316,813–1,322,637) shows a unique transposon insertion in the Gilliam strain that is not present in any of the other strains (Fig. 4b, region 5). Additional differences are due to strain-specific deletions in genes such as *int*, *traE*, *traV*, and *traC*. Taken together, the in-depth analyses of these regions show the influence of two opposing forces, expansions due to the insertions of transposons and contractions due to short deletions. Both of these processes are disruptive and occur at high frequencies.

Outer Membrane Proteins. Selection for antigenic variability is a possible evolutionary driving force behind the multiplication of the *tra*-associated genes. The 56-kDa OmpA strain-specific antigen (1) is uniquely present in *O. tsutsugamushi*, whereas the 47-kDa OmpA group-specific protein has homologues all of the members of the Rickettsiales. Three additional OmpA-like membrane proteins were identified, two of which are located immediately downstream of the genes for the 47- and 56-kDa proteins, respectively, suggesting they may be coordinately expressed. A multigene family with 18 members also encode OmpA-like motifs and signal peptides, all of which are located within the regions of the integrated conjugative elements and may have been amplified together with the conjugative transfer genes. Additionally, *O. tsutsugamushi* has multiple proteins in the Sca family; these are known antigenic determinants in *Rickettsia* that contain autotransporter domains (25) and may play a role in adhesion to host cells (26). Another six genes also encode proteins with autotransporter domains, all of which have the transmembrane domains in the N-terminal region. Two of these are identical and may have been amplified through the action of transposons.

***O. tsutsugamushi* and the Genome Complexity Hypothesis.** The genome complexity hypothesis suggests that the enormous long-term effective population size of bacteria acts as an efficient barrier to the expansion of mobile elements and repeated sequences (27). This could explain why the content of repeated sequences in prokaryotes is normally much lower than in eukaryotes (27). With a repeat content (>200 bp) approaching 40%, i.e., similar to that of the human genome, the *O. tsutsugamushi* genome is exceptional among bacterial genomes. We have shown here that the high-repeat density is due to an extreme proliferation of transposons, conju-

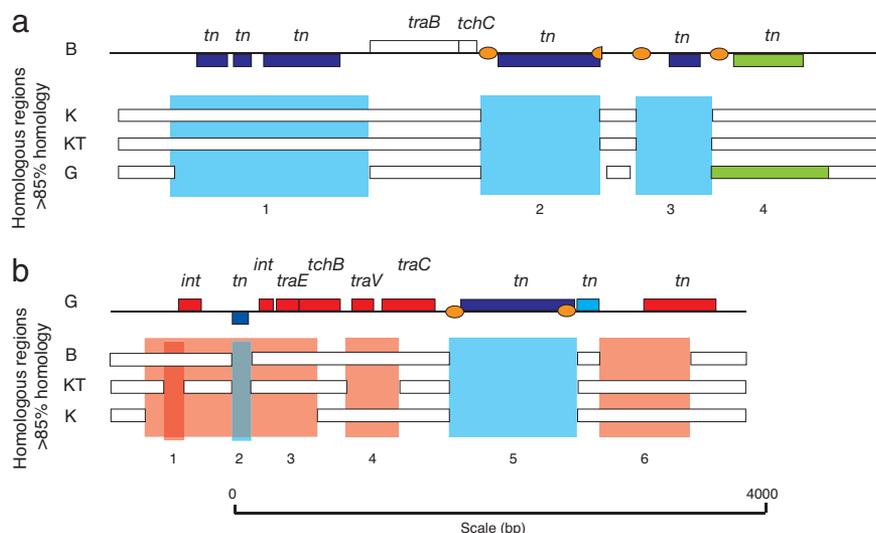


Fig. 4. A sequence comparison of selected *O. tsutsugamushi* repeated regions from different clinical isolates. (a) Expansion of region due to transposon insertion. (b) Reduction of the region due to deletion. The top line shows the gene order structure in the Boryong strain (B), and the bars underneath represent homologous regions found in strains Karp (K), Kato (KT), and Gilliam (G). Blue indicates inferred transposon insertions and red color sequence deletions. Green indicates the insertion of a transposon in strain G.

gative elements, and a suite of associated genes coding for ankyrin-repeat proteins, TPR-repeat proteins, HK, and SpoT regulators. Although transposons may amplify trivially by continuous transposition events, we can think of two explanations for the presence of >20 fragmented operons for conjugation systems, namely massive duplications within the genome or repetitive invasions by conjugative integrative plasmids.

In bacterial genomes, gene duplications have so far been considered rare (28), and innovation has mostly been attributed to horizontal gene transfer (29–31). Hence, it is tempting to favor explanations based on multiple integrations of a similar type of plasmid. However, recent genomic studies point toward an important role of gene duplications (32–34). For example, the diversity of HK is driven by gene family expansion, rather than horizontal gene transfers (35). Tandem duplication is a mechanism that could generate many identical sequence copies. Indeed, the duplication-loss model suggests that the background frequency of tandem duplications is high, but that the copy number is normally kept in balance by an equally rapid collapse unless there is selection for more than one copy (36, 37). Speaking against such a scenario is that the *tra* clusters are not situated in tandem, that there are few tandem gene repeats inside the clusters, and that the sizes of the repeated clusters is >10-fold longer than normally found for tandem repeats, such as for example in *E. ruminantium* (38, 39). Furthermore, subsequent intragenomic recombination events must be invoked to explain the current distribution of the *tra* clusters across the genome. Although the presence of genes for recombination processes, such as *recA* and *ruvABC*, and sequence homogenization of the *tra* genes by gene conversion support active recombination processes in the genome, the mere existence of homologous recombination elements does not necessarily imply high recombination frequencies (40) nor does it rule out multiple plasmid invasions as the main source of genome complexity.

Another important issue that deserves discussion is whether the amplifications and rearrangements have contributed to the functional and antigenic diversity of the *O. tsutsugamushi* population. Previous studies of TFSS have shown that horizontal gene transfers are often associated with functional shifts (41). Likewise, detailed analyses of HK (35) suggest that gene family expansion is linked with domain shuffling and thereby with potentially modified gene functions. However, because many genes in the *tra* clusters are pseudogenized, their functional status has to await experimental verification; this is an interesting avenue for future research.

Before this study, only sporadic discoveries of mobile elements had been made in obligate intracellular lineages, such as of phages

in *Wolbachia pipientis* (42, 43) and of a plasmid in *R. felis* (21). Other host-adapted bacteria, such the vertically inherited aphid and psyllid endosymbionts with genomes in the 180- to 600-kb range (44–48) are completely devoid of viral sequences, conjugative transfer systems and repeated sequences above 25 bp. These are also extraordinarily stable genomes with few rearrangements over time periods that span millions of years (45). The loss of repeats and mobile elements has been attributed to the influence of Muller's ratchet on small bacterial populations that undergo frequent bottlenecks (49–51).

One hypothesis to explain the variability in repeat and mobile DNA content in intracellular bacteria is that the isolation of bacteria adapted to a single host acts as a barrier to horizontal gene transfer, whereas the more permissive lifestyle of host-switching bacteria facilitates the spread of mobile DNA (43). *O. tsutsugamushi* is a host-switching pathogen, which oscillates between two different types of inheritance patterns, maternal inheritance in its natural host, the mite, and horizontal transmission to small rodents and humans. Thus, conjugation among cells within the population or with other *Rickettsia* species during coinfections of the same host may facilitate the spread of plasmids and integrative conjugation systems. Different host adaptation strategies, such as the number of hosts and the density and duration of the infection, as well as the mode of transmission, may have a profound effect on bacterial population sizes and may go a long way toward explaining the different levels of genome complexity in bacteria with an obligate intracellular lifestyle.

We hypothesize that the proliferation of the conjugative secretion systems and the associated genes at one stage provided a basis for adapted evolution, possibly driven by positive or diversifying selection on bacterial components that target host proteins and/or trigger the host immune response in incidental hosts. In large free-living bacterial populations, genes that are selected during one growth regime but that are less fit in another environment will be rapidly eliminated. However, the small population sizes of obligate host-associated bacteria may render selection against selfishly reproducing sequences inefficient and slow down the loss of protein family members that no longer confer a selective advantage. Thus, the retention of pseudogenized clusters of *tra* genes and associated genes in the *O. tsutsugamushi* genome suggests that genetic drift can be more powerful than selection in shaping bacterial genome complexity, leading to the uncontrolled spread of selfish genetic elements.

Materials and Methods

DNA Preparation. *O. tsutsugamushi* Boryong strain was propagated in L929 cells (DMEM, 5% FCS) and bacteria purified on a Percoll

gradient (52). Bacteria were washed twice in TS buffer (33 mM Tris-HCl, pH 7.4/250 mM sucrose) and treated with TNE buffer (10 mM Tris-HCl, pH 7.4/150 mM NaCl/10 mM EDTA) supplemented with lysozyme (2 mg/ml) for 3 h at 37°C. Lysis was performed overnight with the addition of 1% SDS, RNase I (25 µg/ml), and proteinase K (2 mg/ml). After three rounds of phenol-chloroform extractions and ethanol precipitation, genomic DNA was resuspended in TE buffer.

Genome Sequencing Strategy. Five genome shotgun libraries were generated by random shearing of genomic DNA. One fosmid library with mean insert size ≈40 kb (CopyControl Fosmid library production kit, Epicentre, Madison, WI) and four smaller insert libraries of 1.2–1.7, 1.8–2.6 (pBluescript, Stratagene, La Jolla, CA), 3, and 4 kb (pTrueblue, Genomics One, Quebec, ON, Canada). The following strategy was used to assemble a scaffold of 118 contigs (>1 kb) covering ≈2 Mb. First, all repetitive DNA sequences >300 bp that were represented more than three times were masked from the assembled contigs. Gaps between contigs (without repetitive sequences) were filled by using sequence data obtained from 51 sequenced fosmid clones or by primer walking. The final single contig had 14.5-fold coverage of high-quality DNA sequences (total 32 Mb) of the *O. tsutsugamushi* genome (≈2.2 Mb). Sequences were analyzed and assembled into contigs by using PhredPhrap and Consed software (53).

Because of the complexity of the genome, the integrity of the assembly was validated in three steps: (i) 100 fosmid clones (mean insert size ≈40 kb) that corresponds to a 2-fold coverage were analyzed by restriction mapping by HindIII or NspI and compared with an *in silico* digestion pattern of the assembled genome. (ii)

Genomic regions containing repetitive sequences were randomly selected for amplification by PCR and sequencing. (iii) Genomic DNA was analyzed by pulsed-field gel electrophoresis after digestion with restriction enzymes (ApaI, SmaI, and NotI) and compared with an *in silico* digestion pattern of the consensus sequence. The whole genomic sequencing process was performed according to the Bermuda standard (error rate of 0.42 per 10 kb and the depth with at least two subclones for all nucleotide positions).

Informatics. Putative protein-coding regions were identified with Glimmer (54) and Critica software (55). The tRNA genes were detected by tRNA-scan (56). Critica, Psi-Phi (57), BLAST (58), and substitution frequency estimates were used to identify putative pseudogene regions. Repeated genes and regions were detected and clustered by using the REPuter (59) and MUMmer (60) programs. Shared orthologs in seven *Rickettsia* species were identified by first clustering homologs into Tribe-MCL families and sorting these into true orthologs with the aid of conserved gene order structures, by using a software developed in-house (H.-H.F. and S.G.E.A., unpublished data). Homologs in *O. tsutsugamushi* were added to these clusters with the aid of Tribe-MCL. KEGG (61), COG (62), InterProScan (63), Pfam (64), and NCBI databases were used to predict putative functions of genes and domains. All predictions were confirmed, corrected, and verified manually.

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