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Comparative Genome Analysis of an Extensively Drug-Resistant Isolate of Avian Sequence Type 167 *Escherichia coli* Strain Sanji with Novel *In Silico* Serotype O89b:H9

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ABSTRACT Extensive drug resistance (XDR) is an escalating global problem. *Escherichia coli* strain Sanji was isolated from an outbreak of pheasant colibacillosis in Fujian province, China, in 2011. This strain has XDR properties, exhibiting sensitivity to carbapenems but no other classes of known antibiotics. Whole-genome sequencing revealed a total of 32 known antibiotic resistance genes, many associated with insertion sequence 26 (IS*26*) elements. These were found on the Sanji chromosome and 2 of its 6 plasmids, pSJ_255 and pSJ_82. The Sanji chromosome also harbors a type 2 secretion system (T2SS), a type 3 secretion system (T3SS), a type 6 secretion system (T6SS), and several putative prophages. Sanji and other ST167 strains have a previously uncharacterized O-antigen (O89b) that is most closely related to serotype O89 as determined on the basis of analysis of the *wzm-wzt* genes and *in silico* serotyping. This O89b-antigen gene cluster was also found in the genomes of a few other pathogenic sequence type 617 (ST617) and ST10 complex strains. A time-scaled phylogeny inferred from comparative single nucleotide variant analysis indicated that development of these O89b-containing lineages emerged about 30 years ago. Comparative sequence analysis revealed that the core genome of Sanji is nearly identical to that of several recently sequenced strains of pathogenic XDR *E. coli* belonging to the ST167 group. Comparison of the mobile elements among the different ST167 genomes revealed that each genome carries a distinct set of multidrug resistance genes on different types of plasmids, indicating that there are multiple paths toward the emergence of XDR in *E. coli*.

IMPORTANCE *E. coli* strain Sanji is the first sequenced and analyzed genome of the recently emerged pathogenic XDR strains with sequence type ST167 and novel *in silico* serotype O89b:H9. Comparison of the genomes of Sanji with other ST167 strains revealed distinct sets of different plasmids, mobile IS elements, and antibiotic resistance genes in each genome, indicating that there exist multiple paths toward achieving XDR. The emergence of these pathogenic ST167 *E. coli* strains with diverse XDR capabilities highlights the difficulty of preventing or mitigating the development of XDR properties in bacteria and points to the importance of better understanding of the shared underlying virulence mechanisms and physiology of pathogenic bacteria.

KEYWORDS O-antigen, antibiotic resistance, capsular polysaccharide, extensively drug resistant, genome comparison, insertion sequence, pathogen evolution, plasmid-mediated resistance, prophage, secretion systems

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Comparative genome analysis of XDR E. coli strain Sanji with other recently emerged XDR pathogenic ST167 strains reveals novel in silico serotype O89b:H9 and multiple paths toward achieving XDR and highlights difficulty in preventing development of XDR.

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The alarming increase in multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacterial strains is a global health crisis (1–3). Many currently circulating intestinal pathogenic *Escherichia coli* strains, such as the well-known O157:H7 strain (4, 5), are still susceptible to antibiotics. However, the threat of pathogenic *E. coli* acquiring antibiotic resistance genes from environmental reservoirs is of escalating concern (6, 7), and more-recent O104:H4 clonal lineages have acquired not only Shiga toxin-encoding phage but also extended-spectrum-β-lactamase (ESBL) resistance (8, 9). To tackle this problem, it is important to understand not only how multiple antibiotic resistances are acquired but also how they can be accumulated within a commensal or pathogenic bacterium.

Certain traits of genomes in transition toward niche or host adaptation include an increase in mobile genetic elements that imbue the bacteria with the potential to acquire additional traits that might enhance virulence in the host (10). Mobile genetic elements, such as plasmids, bacteriophages, insertion sequence (IS) elements, and transposons, are well-established players in the acquisition of virulence traits leading to the emergence and evolution of bacterial pathogens. Despite the critical role that plasmids and other mobile genetic elements play in antibiotic resistance spread (11, 12), we still cannot predict which resistance genes or plasmids will be acquired by a bacterial pathogen to cause the next XDR superbug to emerge.

Comparative whole-genome sequence analysis of MDR/XDR strains has enabled phylogenetic studies into the evolutionary mechanisms involved in acquisition and accumulation of antibiotic resistance genes (12), including studies exploring evolutionary trade-offs between virulence and resistance (13–16); tracking the spread of resistant pathogens (17–19), or monitoring within-host evolution of pathogens (20, 21). One comparative genomics study revealed the stepwise evolutionary process by which a highly infectious clone of extraintestinal pathogenic *E. coli* (ExPEC) of sequence type 131 (ST131) gained multiple virulence and antibiotic resistance gene clusters over a period of about 60 years (22), ultimately leading to its current global dominance as an XDR pathogen (23). A similar pattern of sequential emergence of increasing virulence potential and antibiotic resistances over a period of 30 years has been documented for another pathogenic *E. coli* clonal group, ST393 (24).

We report the comparative genome characterization of pathogenic *E. coli* strain Sanji, which was isolated from pheasants during a 2011 outbreak of colibacillosis and was refractory to clinical application of commonly used veterinary antibiotics. Antibiotic susceptibility testing confirmed that the isolate was XDR. Whole-genome sequencing of the bacterial genome, including its six plasmids, and comparative multilocus sequence typing (MLST) revealed that the core genome of Sanji is nearly identical to the genomes of a number of recently sequenced pathogenic XDR *E. coli* strains belonging to sequence type ST167. *In silico* serotyping revealed that Sanji, like other ST167 strains, has a unique capsular polysaccharide gene cluster and a previously unidentified *in silico* serotype, O89b. The presence of numerous antibiotic resistance gene clusters and IS*26* elements accounts for the observed XDR phenotype. Comparison of Sanji to other members of the ST167 lineage further revealed the extent and diversity of the paths used by these bacteria to achieve XDR. This group of ST167 strains represents another emerging pathogenic clonal lineage with XDR.

RESULTS AND DISCUSSION

Antibiotic susceptibility profile of *E. coli* Sanji. The antibiotic susceptibility profile of *E. coli* Sanji was compared directly to that of two reference strains: *E. coli* ATCC 25922, a standard strain used by the CLSI, and *E. coli* MG1655, a prototype K-12 strain chosen for its genetic similarity to *E. coli* Sanji. As shown in Fig. 1, Sanji has resistance to most antibiotics, exhibiting sensitivity only to carbapenem (meropenem) and partial sensitivity to a few others (e.g., amikacin, spectinomycin, furazolidone, and nitrofurantoin). Sanji also exhibits resistance to a β-lactam combination with β-lactamase inhibitor (piperacillin-tazobactam). All three *E. coli* strains, Sanji, MG1655, and ATCC 25922, displayed apparent resistance in the Kirby-Bauer assay to polymyxin B, even though

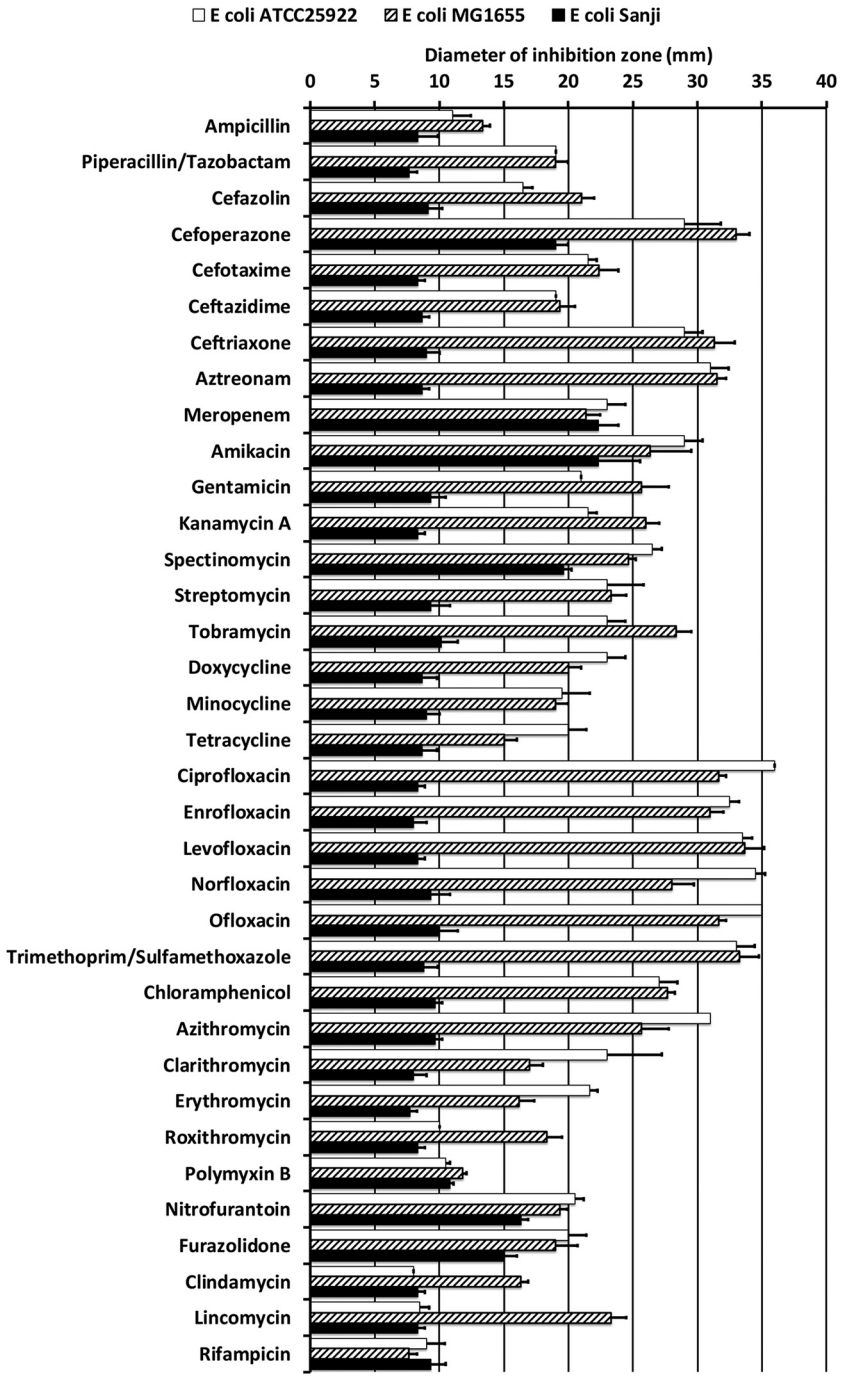

FIG 1 Antibiotic susceptibility profiles for *E. coli* strains Sanji, MG1655, and ATCC 25922. Shown are the mean zones of inhibition (in millimeters) recorded for Kirby-Bauer disc diffusion assays (6.5-mm to 7.0-mm disc diameter) for the indicated antibiotics. Open bars, *E. coli* ATCC 25922; hatched bars, *E. coli* MG1655; black bars, *E. coli* Sanji. Error bars represent means ± standard deviations of results from three independent experiments. Direct comparison of Sanji with ATCC 25922 and MG1655 showed little or no susceptibility of Sanji to most of the antibiotics listed (black bars), as evidenced by the lack of a zone of inhibition beyond the disk diameter. Note that Sanji and MG1655 were found to be susceptible to polymyxin B and colistin by the broth microdilution method.

they do not possess the *mcr-1* gene. Sanji does possess a phosphoethanolamine transferase (*eptA*) gene with homology to all *mcr* genes, notably, 41% identity with *mcr-3* and 43% identity with *mcr-8*. However, this *eptA* gene is also present in MG1655 and many other *E. coli* strains. When tested against polymyxin B and colistin using the

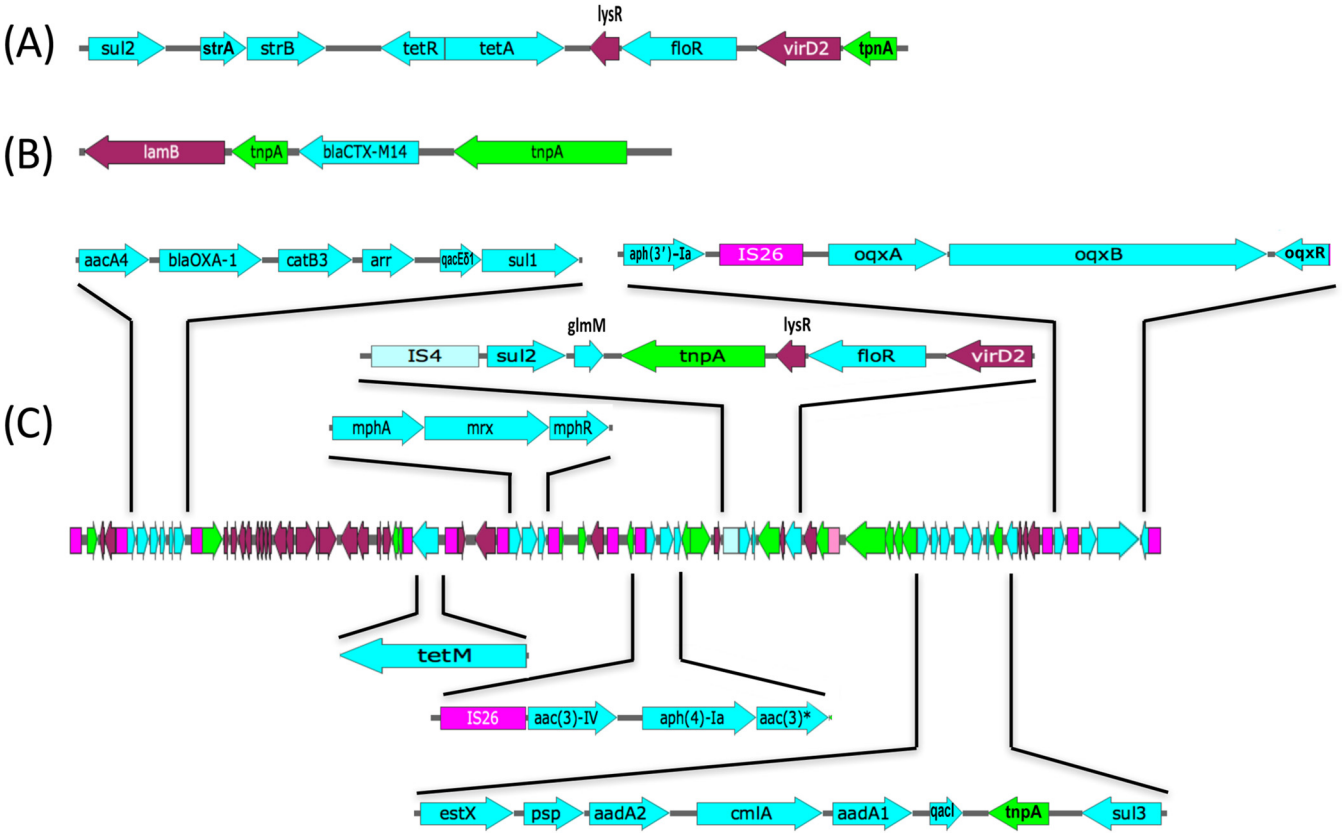

FIG 2 Antibiotic resistance gene clusters in *E. coli* Sanji. (A) An 8.9-kb resistance gene cluster was found in the 69.2-kb insertion on the chromosome. (B) *blaCTX-M14* gene plus flanking genes found on plasmid pSJ_82. (C) An 80-kb resistance gene cluster was found on plasmid pSJ_255. In panels A to C, known antibiotic resistance genes are indicated in cyan; IS*26* elements in magenta; IS*4* elements in pale blue; IS*1006* elements in pink; transposase genes in green; and other genes in maroon. The asterisk denotes a gene with a GNAT domain that overlaps a transposase gene.

broth microdilution method, the observed MICs for Sanji (0.3 μg/ml each for colistin and polymyxin B) were only 2-fold higher than that of MG1655 and not 10-fold to 100-fold higher (MIC of 3 to 32 μg/ml) such as would be expected for *mcr-1*-mediated resistance (25).

Antibiotic resistance genes in *E. coli* Sanji. PacBio whole-genome sequencing revealed that Sanji consists of a 4.9-Mb chromosome and 6 plasmids: pSJ_255 (255.4-kb), pSJ_98 (98.4-kb), pSJ_94 (94.7-kb), pSJ_82 (82.3-kb), pSJ_3 (3.4-kb), and pSJ_2 (2.6-kb). Sanji has all of the known drug efflux pump genes belonging to all five classes of drug transporters found in MG1655 (26). The Sanji chromosome harbors an 8.9-kb cluster of genes associated with known drug resistance to sulfonamides (*sul2*), aminoglycosides (*strAB*), tetracycline (*tetRA*), and chloramphenicol (*floR*) (Fig. 2A). In addition to this locus, we identified a total of 32 distinct antibiotic resistance genes in Sanji within identifiable mobile elements (Table 1), including 6 genes within the chromosome, 1 gene on plasmid pSJ_82, and 27 genes on the large plasmid, pSJ_255, with two of the genes appearing in both the chromosome and a plasmid. The resistance gene identified on pSJ_82 encodes a class A extended-spectrum β-lactamase (ESBL), *blaCTX-M-14* (Fig. 2B). CTX-M ESBLs have been implicated in resistance to third-generation β-lactams in multiple *Enterobacteriaceae* species (27). All 27 of the antibiotic resistance genes on pSJ_255 were localized to an 80-kb region (Fig. 2C). The genes carried on pSJ_255 included those conferring resistance to β-lactams (*blaOXA-1*), tetracyclines (*tetM*), aminoglycosides [*aac(6′)-Ib*, *aac(3)-IVa*, *aac(4)-Ia*, *aadA2*, *aadA1*, *aph(3′)-Ia*, *aph(4)-Ia*, and *aac(3)*], chloramphenicol (*catB3*, *floR*, and *cmlA1*), rifampin (*arr*), quaternary ammonium compounds (*qacEδ1* and *qacI*), sulfonamides (*sul1*, *sul2*, and *sul3*), and macrolides (*mphA*, *mrx*, *mphR*, and *glmM*), as well as a known RND multidrug efflux pump (*oqxABR*).

TABLE 1 Antibiotic resistance genes found in ST167 *Escherichia coli* strains[a]

Parameter	Result for *E. coli* strain:														
	Sanji	ECONIH6	AR_0011	AR_0014	AR_0149	AR_0150	AR_0151	AR_0162	WCHEC005237	FDAARGOS_434	CRE1493	CREC-532	CREC-629	Y5	SCEC020007
No. of plasmids	6	2	3	2	2	3	2	4	8	1	5	3	3	3	2
Total no. of AR genes[b]	32 (34)	21 (22)	12 (13)	8	3	14	4	15	21 (28)	15 (24)	30 (36)	19 (25)	19 (23)	25 (43)	16 (24)
Chromosome-carried AR genes	6	0	1	0	0	0	0	0	0	0	0	6	6	12 (20)	0
Plasmid-carried AR genes	28	21 (22)	12 (13)	8	3	14	4	15	21 (28)	15 (24)	30 (36)	17 (19)	17	21 (23)	16 (24)
No. of IS*26* elements on plasmids	12	8	6	6	1	3	1	9	10	5	12	10	8	10	6
No. of AR genes near IS*26*[c]	24	16	12	8	0	12	2	13	8	24	25	15	8	21	22
Presence of gene:															
aac(3)-IIa	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	N
aac(3)-IId	N	N	N	N	N	N	N	N	N	N	Y	Y	Y	Y	N
aac(3)-IVa	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
*aac(3)**[d]	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
aac(6′)Ib-cr	Y	N	Y	Y	N	N	N	N	Y	N	Y	N	N	Y	N
aadA1	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
aadA2	Y	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	N
aadA5	N	N	N	N	N	Y	N	N	N	Y	Y	Y	Y	Y	Y
aadA16	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	Y
aph(4)-Ia	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
aph(3′)-Ia	Y	N	N	N	N	N	N	N	N	N	N	N	N	Y	N
arr-3	Y	N	N	N	N	N	N	N	Y	N	N	N	N	Y	N
blaCMY-42	N	N	N	N	Y	Y	Y	N	N	N	N	N	N	Y	N
blaCTX-M-14	Y	N	N	N	N	N	N	N	N	N	N	Y	Y	Y	N
blaCTX-M-15	N	Y	Y	Y	N	N	N	Y	N	Y	Y	N	N	Y	N
blaCTX-M-55	N	N	N	N	N	N	N	N	Y	N	N	Y	Y	N	N
blaNDM-5	N	Y	N	N	N	Y	Y	N	Y	Y	Y	N	N	N	Y
blaNDM-7	N	N	N	N	Y	N	N	Y	N	N	N	Y	Y	Y	N
blaOXA-1	Y	Y	Y	Y	N	N	N	N	N	N	Y	N	N	Y	N
blaTEM-1A	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N
blaTEM-1B	N	N	N	N	N	Y	Y	Y	Y	N	N	Y	Y	N	Y
ble	N	Y	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
catB3	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
cmlA1	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
dfrA12	N	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	Y
dfrA14	N	Y	N	N	N	N	N	N	Y	N	N	N	N	N	N
dfrA17	N	N	N	N	N	Y	N	N	N	Y	Y	Y	Y	Y	Y
dfrA27	N	N	N	N	N	N	N	N	Y	N	N	N	N	Y	N
eamA	N	Y	Y	Y	N	Y	N	Y	Y	Y	Y	N	N	Y	Y
erm(B)	N	Y	N	N	N	N	N	Y	N	N	N	Y	Y	N	N
estX	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
floR_2	Y	N	N	N	N	N	N	N	Y	N	Y	N	N	Y	N
fosA_14	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N
glmM	Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	N
mcr-1	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N
mph(A)	Y	Y	N	N	N	Y	N	Y	N	Y	Y	Y	Y	Y	Y
mphR	Y	Y	N	N	N	Y	N	Y	N	Y	Y	Y	Y	Y	Y
mrx	Y	Y	N	N	N	Y	N	Y	N	Y	Y	Y	Y	Y	Y
nimC/nimA	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N
oqxA	Y	N	N	N	N	N	N	N	N	N	Y	N	N	N	N
oqxB	Y	N	N	N	N	N	N	N	N	N	Y	N	N	N	N
oqxR	Y	N	N	N	N	N	N	N	N	N	Y	N	N	N	N
psp	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N

(Continued on next page)

TABLE 1 (Continued)

Parameter	Result for *E. coli* strain:														
	Sanji	ECONIH6	AR_0011	AR_0014	AR_0149	AR_0150	AR_0151	AR_0162	WCHEC005237	FDAARGOS_434	CRE1493	CREC-532	CREC-629	Y5	SCEC020007
qacEd1	Y	Y	N	N	N	Y	N	N	Y	Y	Y	Y	Y	Y	Y
qacI	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
qnrS1	N	Y	N	N	N	N	N	Y	Y	N	N	N	N	N	N
rmtB	N	Y	N	N	N	N	N	N	Y	N	N	N	N	N	Y
strA	Y	Y	Y	N	N	N	N	Y	Y	N	Y	N	N	Y	N
strB	Y	Y	Y	N	N	N	N	Y	Y	N	Y	N	N	Y	N
sul1	Y	Y	N	N	N	Y	N	N	Y	Y	Y	Y	Y	Y	Y
sul2	Y	Y	Y	N	N	N	N	Y	Y	N	Y	N	N	Y	N
sul3	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
tet(A)	Y	Y	Y	Y	N	Y	N	Y	Y	Y	Y	N	N	Y	Y
tet(B)	N	N	N	N	N	N	N	N	N	N	N	Y	Y	N	N
tetC	N	N	N	N	N	N	N	N	N	N	N	Y	Y	N	N
tetD	N	N	N	N	N	N	N	N	N	N	N	Y	Y	N	N
tet(M)	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
tetR	Y	Y	Y	Y	N	Y	N	Y	Y	Y	Y	N	N	Y	Y
tetR(B)	N	N	N	N	N	N	N	N	N	N	N	Y	Y	N	N
tmrB	N	N	Y	Y	N	N	N	N	N	N	Y	Y	Y	N	N

[a]Y, yes (present); N, no (not present).

[b]AR genes, antibiotic resistance genes, including resistance genes carried on both plasmids and chromosomes and their associated transcriptional regulators. Multiple copies of the same genes were counted only once each. Numbers in parentheses represent all copies of genes.

[c]No. of AR genes near IS*26*, number of antibiotic resistance genes, including multiple copies of same gene, in a cluster within 20 kb of an IS*26* element. Antibiotic genes found within 10 kb of each other were considered to be part of the same gene cluster.

[d]A gene with a GNAT domain and overlapping with a transposase.

Comparative genome sequence analysis of the Sanji chromosome. At the time of Sanji genome completion, the closest genome available was that of prototypic *E. coli* K-12 strain MG1655. Genome alignment of Sanji chromosome to MG1655 revealed that 77% of the open reading frames in Sanji are shared with MG1655. A synteny plot generated based on the genome alignment between Sanji and MG1655 showed high collinearity with 10 major insertions (Fig. 3A). Since then, many additional genomes within the K-12 clade showing close relationships with Sanji have become available. Comparison of Sanji with two closely related strains, WCHEC005237 and CRE1493, revealed even greater collinearity (Fig. 3B).

Multilocus sequence typing (MLST) analysis using seven housekeeping genes (*purA*, *adk*, *icd*, *fumC*, *recA*, *mdh*, and *gyrB*) (28) classified Sanji into the sequence type ST167 group. Genome BLAST searches, using the unique insertions identified in comparisons with MG1655 as the query, revealed additional genomes that share some of these unique features, including strains with sequence types ST10, ST167, and ST617. An MLST-based phylogenetic tree of these strains revealed that these sequence types are indeed related to each other and fall within the K-12 clade (Fig. S1). Comparative genome sequence analysis of the entire chromosome of Sanji with the other 14 ST167 strains (Fig. 4) further revealed that the ST167 genomes are highly similar beyond the seven genes used for MLST. Some of these strains contain up to 12 distinct resistance genes on the chromosome (see Table 1).

In comparison to MG1655, four of the chromosomal insertions in Sanji appear to be prophages (see Fig. 3A). Three insertions also found in other ST167 strains harbor specialized secretion systems (SS), namely, a 19.8-kb insertion containing a type 3 secretion system (T3SS), a 30.6-kb insertion containing a T6SS, and a 75.1-kb insertion containing a T2SS, although in some strains this insertion is truncated. Each of these insertions contains additional uncharacterized genes.

A 17.5-kb insertion containing an O-antigen biosynthesis cluster, flanked by a pair of insertion sequence 26 (IS*26*) elements, is shared with other ST167 strains, suggesting horizontal acquisition. Initial immunoserotyping analysis of the O-antigen gave positive results for type O6 but was unable to determine the H-type. PCR analysis failed to confirm the O6 serotype but gave positive results for H9 antigen. *In silico* serotyping based on the whole-genome sequence assigned the Sanji strain as serotype H9 based on the presence of the *fliC* gene sequence (98.9%). For the O-antigen, the closest match was related to serotype O89, based on the presence of *wzm* (94.1%) and *wzt* (93.5%). This newly determined 17.5-kb O-antigen gene cluster (≥99% sequence identity) was found to be present in all ST167 and ST617 strains examined, as well as in some strains within the ST10 clonal complex, including ST744, ST44, ST4981, ST1284, and ST10 (Fig. S1) (Table S1). We propose to designate this *in silico* serotype "O89b." A few of the O89b-containing strains have additional genes encoding other O-antigen types, including O9 (based on genes *wzm* and *wzt*) or O8 (based on a truncated *wzt* gene). With the exception of a few strains, all of the ST167, ST617, and ST10 complex strains examined are predominantly H9 or H10 (Table S1).

Maximum likelihood phylogenetic analysis of these O89b-containing strains was performed using MEGA7 for 6,890 core single nucleotide variants (SNV) across 39 Sanji-related genomes plus 19 ST167 assemblies and MG1655 (Fig. S2). Here, Sanji clustered with the early isolates of ST167, while later ST167 isolates showed more diversity. The ST617 isolates examined were less tightly clustered. The ST744, ST44, and ST10 isolates were more distant than the ST167 and ST617 groups. Using the same core SNV data set, the molecular evolution of these O89b-containing strains was also determined by a time-scaled Bayesian phylogenetic analysis in BEAST2 (29). From this analysis, it was estimated that development of these O89b-containing lineages took place about 30 years ago (Fig. 5). However, there is no clear geographical location associated with this emergence since members of this group appear to be dispersed globally. There also has been no clear time-dependent shifting of these lineages,

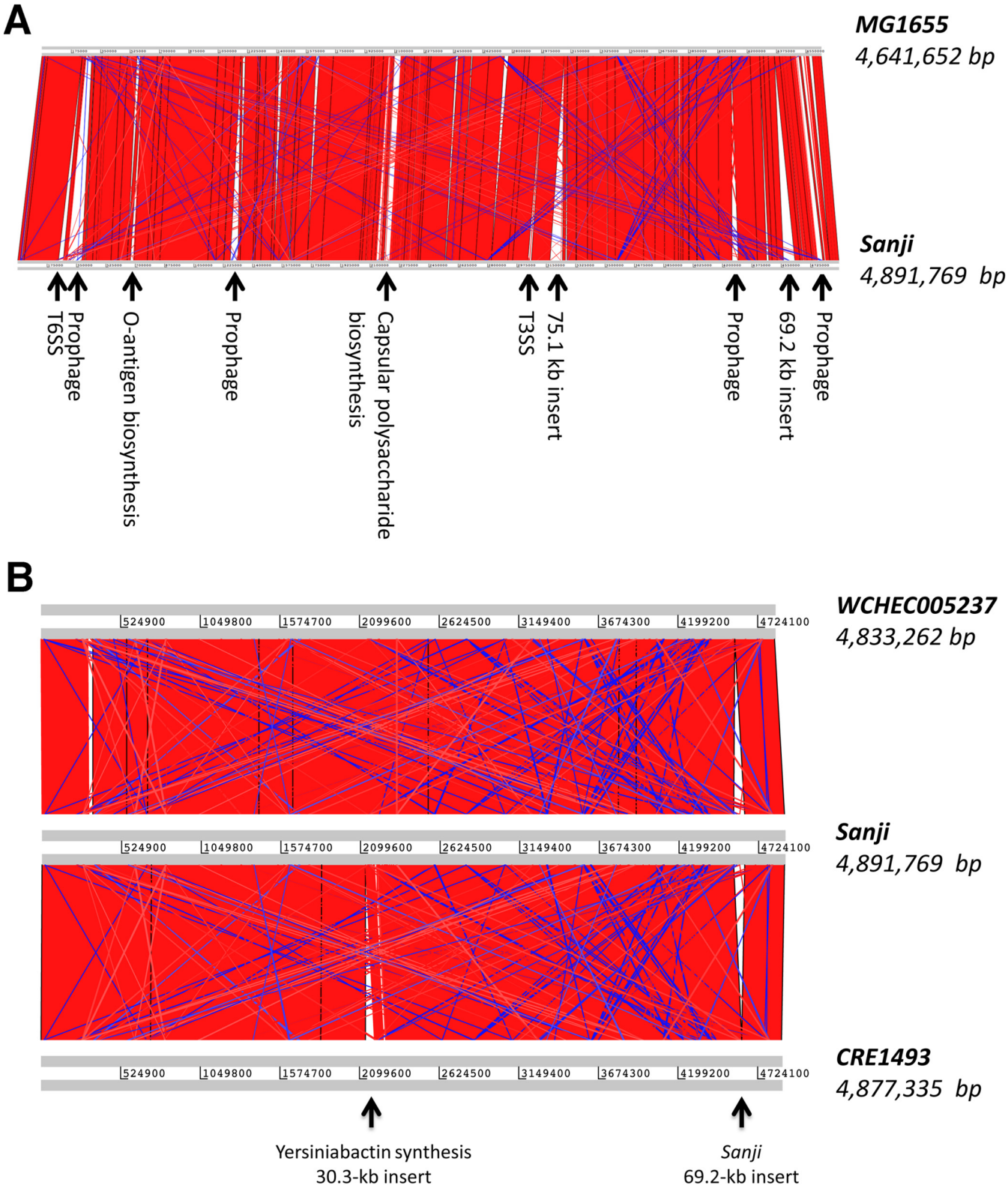

FIG 3 Synteny between the genomes of Sanji and related *E. coli* strains. (A) A pairwise genome comparison plot showing collinearity of genes between Sanji and MG1655. The 10 major insertions in the Sanji genome are labeled. The location of the capsular polysaccharide biosynthesis gene cluster in the Sanji corresponds to that of a lipopolysaccharide biosynthesis gene cluster in MG1655. All other insertions in MG1655 appear to be prophage-related genes. (B) A synteny plot comparing Sanji with two of the ST167 strains, CRE1493 and WCHEC005237. The 69.2-kb insertion is present only in Sanji, while the 30.3-kb insertion conferring yersiniabactin biosynthesis is present only in CRE1493. Each of the strains also has a few unique prophage insertions. The red and blue bands represent the forward and reverse matches, respectively.

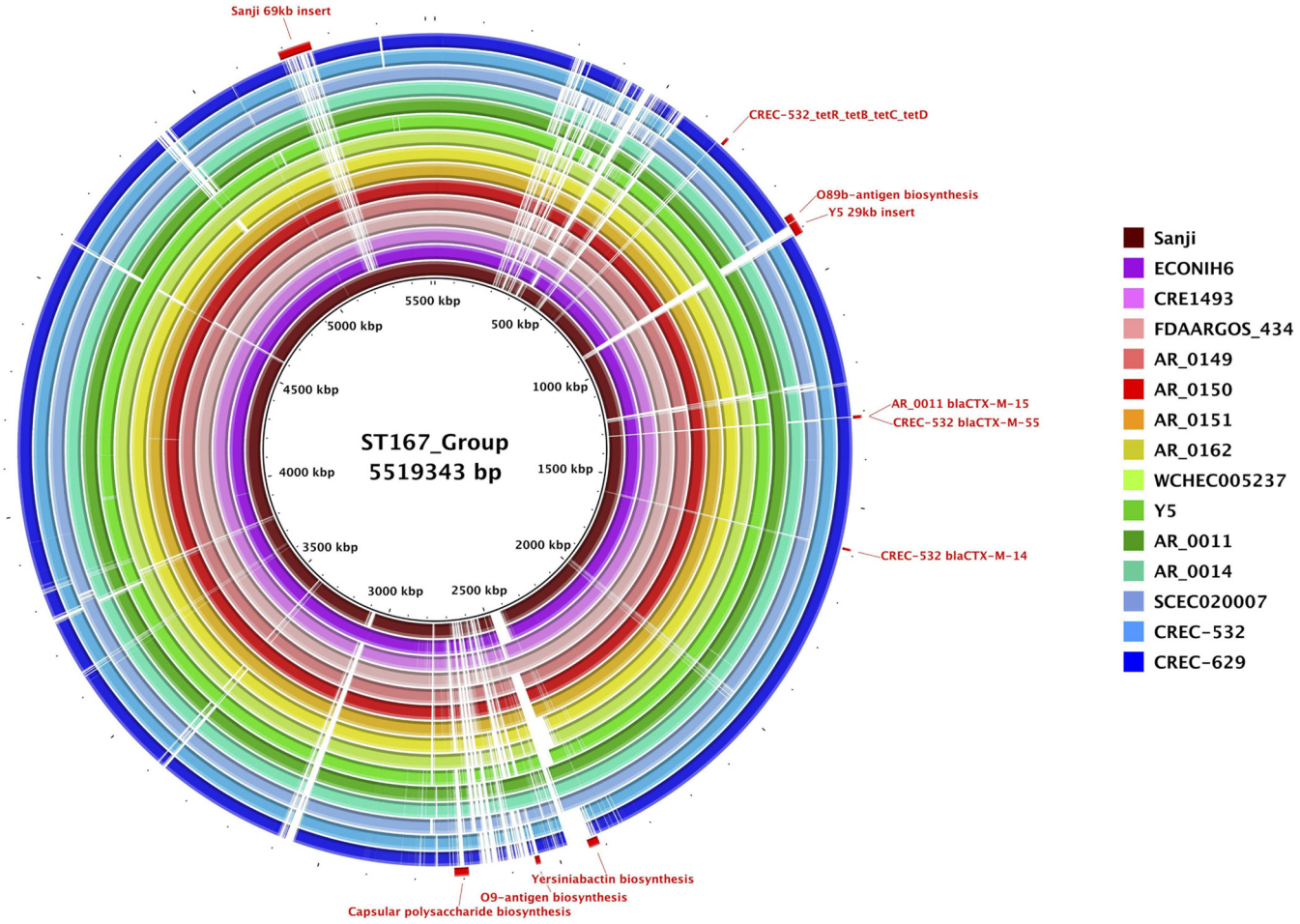

FIG 4 Comparisons of ST167 chromosomes. Shown is a BRIG circular genome plot for BLASTN comparisons of Sanji and 14 other ST167 strains. The reference sequence was a composite generated by inserting DNA segments into the Sanji chromosome that were absent from Sanji. Selected gene clusters involved in antibiotic resistance, O-antigen biosynthesis, capsular polysaccharide biosynthesis, and virulence are labeled.

though it appears that ST167 and ST617 are the dominant O89b-containing strains. ST617 strains are also known to carry many antibiotic resistance genes (30–33).

A 32.7-kb insertion in Sanji contains a capsular polysaccharide biosynthesis (*cps*) gene cluster at a location that corresponds to a lipopolysaccharide biosynthesis gene cluster in MG1655. This *cps* gene cluster, flanked by IS elements, is also present in *E. coli* strains 127 and WCHEC005237 and has sequence homology with several K30 *Klebsiella pneumoniae* strains (28) but is truncated in several other ST167 strains (see Fig. 4).

A 69.2-kb insertion, unique to Sanji among the ST167 strains, contains the 8.9-kb antibiotic resistance gene cluster (see Fig. 2A), a raffinose utilization operon (*rafRABDY*), two toxin-antitoxin systems (*relE*/*parE* and *yeeV*/*yeeU*), and a number of unidentified genes. This insertion was also found in the chromosome of six other non-ST167 *E. coli* genomes (strains HB-Coli0, CRE1540, H8, MRY15-117, 14EC017, and WCHEC4533) (Table S2).

In addition to these major insertions, there are smaller insertions containing metabolic and nutrient acquisition genes, such as a 5.5-kb sucrose utilization operon (*cscBKAR*) shared with other ST167 strains. There were no other obvious toxins or other unique virulence factors that distinguished Sanji from the other ST167 strains. However, Sanji did exhibit *in vitro* growth inhibition against a laboratory strain of *E. coli* TOP10 expressing green fluorescence protein (GFP) (Fig. S3).

Comparative sequence analysis of the Sanji plasmids. For most ST167 genomes, including Sanji, the majority of their antibiotic resistance genes were located on various

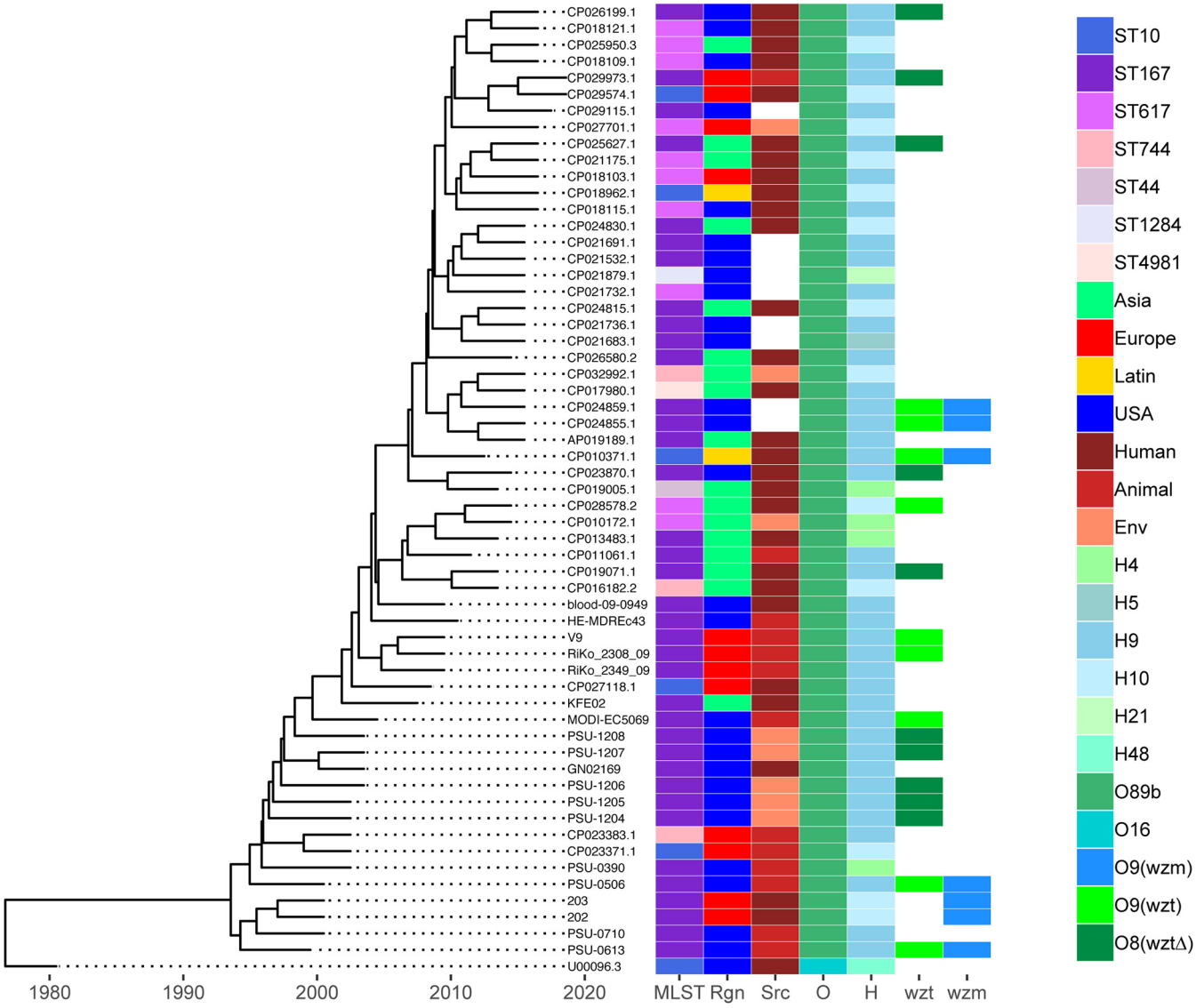

FIG 5 Time-scaled phylogeny of O89b-containing *E. coli* strains. Molecular phylogeny based on the HKY85 model was calculated using a BEAST2 MultiTypeTree module of 6,890 core SNVs from 39 Sanji-related complete genomes plus 19 ST167 assemblies isolated between 1999 and 2010, as well as MG1655. The tree shown was generated by using R package ggtree, with associated 7-gene MLST, isolation region, source, and *in silico* O-antigen and H-antigen serotypes indicated.

plasmids (Table 1). Interestingly, all of the strains carried distinct sets of plasmids with different backbones and sizes (Table 2). The IncHI2 plasmid, pSJ_255, is unique to Sanji among ST167 strains and carries 27 of the 32 distinct antibiotic resistance genes found in Sanji (Fig. 2C). This plasmid belongs to a family of plasmids whose prototypical member is *Serratia marcescens* plasmid R478 (34) (Fig. 6). This family of plasmids contains the *ter* gene cluster, which has been shown to confer resistance to tellurite, some bacteriophages, and pore-forming colicins (35, 36). MDR plasmids in this family differ in the number of antibiotic resistance genes (Table S3). For example, R478 and a few others carry 4 to 8 antibiotic resistance genes, while others, including pSJ_255, carry 23 to 30. Additionally, each of these plasmids carries a different but overlapping set of antibiotic resistance genes.

One explanation for this high variability in the number and types of antibiotic resistance genes in SJ_255 is the presence of several IS*26* elements (Fig. 2C). IS*26* elements are known to facilitate the horizontal movement and accumulation of antibiotic resistance genes at a relatively high frequency (37, 38). Although pSJ_255 does

TABLE 2 Plasmid MLST types associated with ST167 *E. coli* strains

Parameter	Value(s) for *E. coli* strain:														
	Sanji	ECONIH6	AR_0011	AR_0014	AR_0149	AR_0150	AR_0151	AR_0162	WCHEC005237	FDAARGOS_434	CRE1493	CREC-532	CREC-629	Y5	SCEC020007
No. of plasmids (PubMLST)	6	2	3	2	2	3	2	4	8	1	5	3	3	3	2
Plasmid size (bp)[a]															
FIA_4; FIA_20; FII_36	94,712 (0)					117,703 (10)				149,485 (24)	127,772 (15)			124,378 (13)	144,225 (24)
FIA_1; FIA_6; FII_22; FII_36												216,181 (17)	176,274 (15)		
FIA_4; FIA_20; FIB_1; FII_31; FII_36			181,436 (8)	172,588 (8)											
FIB_24											73,992 (18)				
FII_2	82,288 (0)	100,989 (12)						84,929 (5)							
FII_33									70,691 (2)						
FII_47									100,229 (12)						
HCM1 178ac_2 IncHI1				33,548 (0)											
IncHI2 DLST ST 3	255,368 (27)														
smr0018 IncHI2	2,640 (0)														
ardA_4; repI1_1; trbA_15; IncI1														61,695 (2)	
ardA_4; repI1_3; trbA_15; IncI1						50,235 (2)	50,228 (2)								
ardA_5; repI1_4; trbA_15; IncI1					48,528 (1)										
ardA_19 IncI1															84,952 (0)
ardA_24 IncI1									6,200 (3)						
trbA_43 IncI1			57,991 (0)												
repN_6 IncN			76,680 (4)												
IncA/C ST 3														136,243 (8)	
A009_11 IncA/C								23,332 (0)			33,858 (1)				
A157: 6 IncA/C		97,800 (10)						95,850 (8)							
A165_4 IncA/C	98,436 (1)										96,986 (0)	96,987 (0)	96,990 (0)		
A165_6 IncA/C	3,373 (0)								3,684 (0)						
A175_5 IncA/C						46,159 (2)	46,161 (2)	49828 (2)	46,161 (2)		46145 (2)				
parB_9 IncA/C					46,161 (2)							46137 (2)	46,161 (2)		
repA_4 IncA/C									121,908 (9)						
None[b]									2,959 (0)						
None									2,444 (0)						
No. of chromosome-borne resistance genes	6	0	1	0	0	0	0	0	0	0	0	6	6	20[c]	0

[a]Numbers indicate plasmid sizes (bp). Numbers of resistance genes, including replicates and associated transcriptional regulators, are shown in parentheses.

[b]None, no match in PubMLST database.

[c]Value includes 3 copies of a 4-gene cluster.

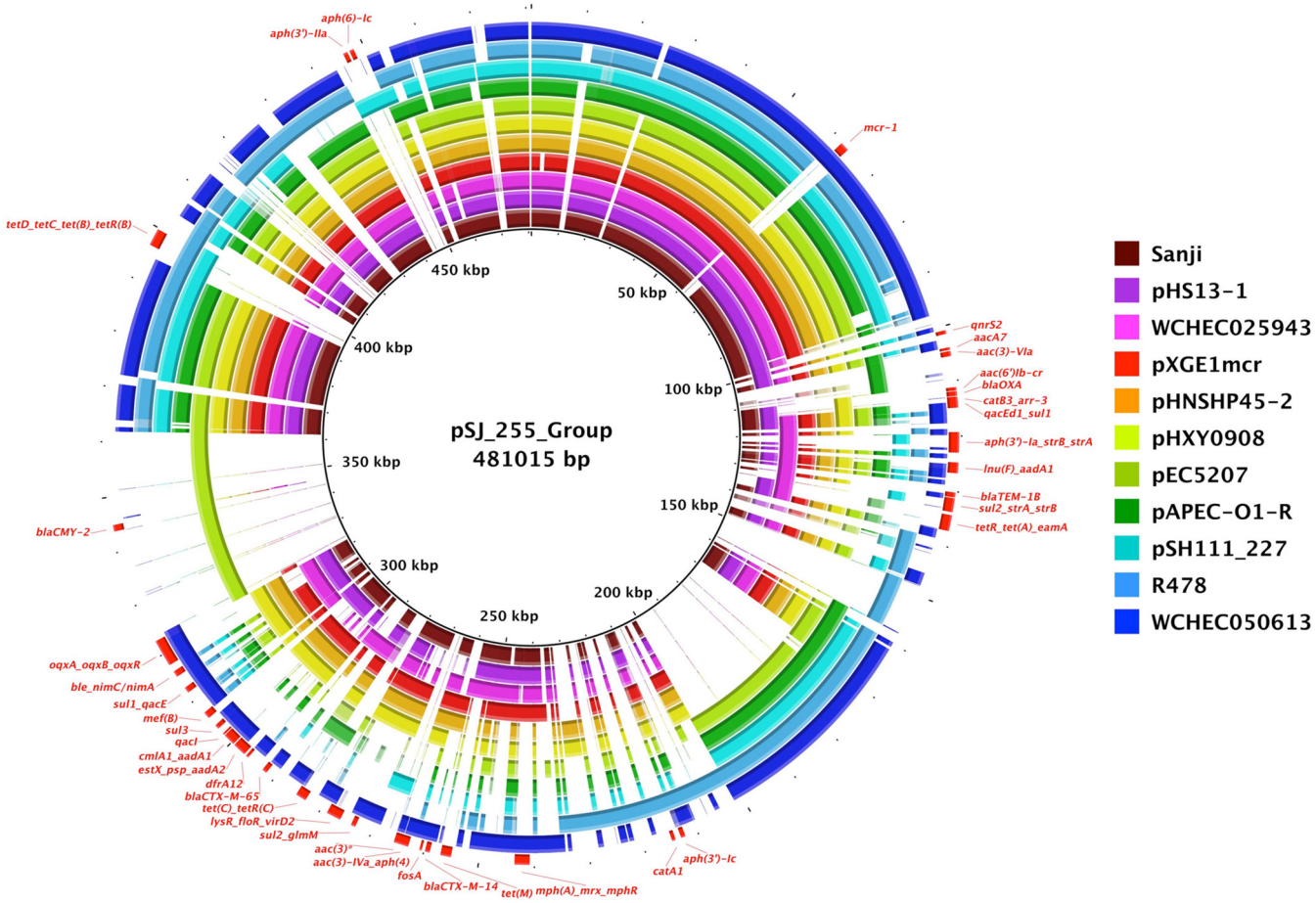

FIG 6 Comparisons of plasmids related to pSJ_255. Shown is a BRIG circular plot for BLASTN comparisons of pSJ_255 and related plasmids. The reference sequence is a composite generated by inserting sequences into the pSJ_255 sequence that were absent from pSJ_255. Each ring corresponds to a different plasmid, as follows from inner to outer ring: pSJ_255 represents plasmid pSJ_255 from *E. coli* Sanji; pHS13-1 represents plasmid pHS13-1 from *E. coli* HS13-1; WCHEC025943 represents plasmid pMCR1_025943 from *E. coli* WCHEC025943; pXGE1mcr represents plasmid pXGE1mcr from *E. coli* XG-E1; pHNSHP45-2 represents plasmid pHNSHP45-2 from *E. coli* SHP45; pHXY0908 represents plasmid pHXY0908 from *Salmonella enterica* serovar Typhimurium strain GDS147; pEC5207 represents plasmid pEC5207 from *E. coli* EC5207; pAPEC-O1-R represents plasmid pAPEC-O1-R from *E. coli* APEC O1; pSH111_227 represents plasmid pSH111_227 from *Salmonella* Heidelberg; R478 represents plasmid R478 from *Serratia marcescens*; and WCHEC050613 represents plasmid pMCR_WCHEC050613 from *E. coli* WCHEC050613. All identifiable antibiotic resistance genes are labeled in red on the outer ring.

not carry genes with resistance to the current "antibiotics of last resort" (e.g., *blaNDM* or *blaKPC*, conferring resistance to carbapenems, or *mcr-1*, conferring hyperresistance to colistin), several plasmids in the IncHI2 family have acquired the *mcr-1* gene (Fig. 6; see also Table S3). Moreover, a recent report identified an IncHI2 plasmid that carries both *blaNDM-4* and *mcr-1* (39).

Sanji plasmid pSJ_82 belongs to the IncFII family of plasmids, which includes prototypical member pHK01 (40). Members of this family carry the ESBL-encoding *blaCTX-M-14* gene (41). Sanji, ECONIH6, and AR_0162 all have a plasmid with an FII_2 backbone (SJ_82, tig00008015, and pNDM-d2e9, respectively), carrying 0, 12, and 5 distinct antibiotic resistance genes, respectively (Fig. S4). Sanji plasmid pSJ_94 carries both IncFIA and IncFII replicons but no identifiable antibiotic resistance genes (Fig. S5). However, it does contain an IS*26* element. In fact, close relatives of both pSJ_94 and pSJ_82 carry IS*26* elements and a large number of associated antibiotic resistance genes (see Fig. S4 and S5).

Among the ST167 strains analyzed, the majority of their antibiotic resistance genes are associated with IS*26* elements (see Table 1). Sanji pSJ_255 carries 24 IS*26*-associated antibiotic resistance genes. ST167 strain FDAARGOS_434 carries a plasmid related to pSJ_94 with 24 IS*26*-associated resistance genes, and strain ECONIH6 carries a plasmid

related to pSJ_82 that has 12 IS*26*-associated resistance genes. In Sanji, the ESBL encoded by *blaCTX-M-14* appears to be mobilized by ISEcp1 and is not associated with IS*26* elements. However, there has been a report of a *blaCTX-M-14_ISEcp1* gene cluster that inserted into an IS*26* element in a strain of *Proteus* (42), while there have been multiple reports of the *blaCTX-M-15* gene being associated with IS*26* elements in *E. coli* (43, 44). Several of the ST167 strain plasmids also contained Tn*3*-mediated insertions of the *blaCTX-M-15* gene into IS*26* elements (Fig. S5). The existence of these related plasmids containing IS*26* elements suggests that pSJ_94 and pSJ_82 in Sanji have the potential to also accumulate multiple antibiotic resistance genes in a manner similar to that observed for pSJ_255.

Sanji plasmid pSJ_98 appears to be a P1-like enterobacteriophage. Closely related plasmids can be found in many bacteria, including some ST167 strains, CRE1493, CREC-532, and CREC-629 (Fig. S7). In rare cases, these plasmids can carry an antibiotic resistance gene, but there is no evidence for accumulation of multiple resistance gene clusters such as was observed with the other large Sanji plasmids.

Multiple paths to achieve XDR. ESBL-producing MDR and XDR *E. coli* isolates with sequence types ST167 and ST617 and others in the ST10 clonal complex have emerged over the past 5 years as common isolates from nosocomial sources as well as wild animal and domestic animal sources, including dairy and livestock sources, in Germany (45–47), Taiwan (48, 49), Tunisia (50, 51), and the Americas (52). Indeed, ST167 strains carrying carbapenemase activity have become the second most prevalent sequence type, behind only ExPEC ST131, among human clinical ESBL-producing *E. coli* isolates reported in China (27, 53–62), Spain (63, 64), France (65), India (66), Italy (67), Iran (68), Romania (69), and Tunisia (50).

Because IS*26* elements can be readily exchanged between different DNA molecules (transposons, phages, conjugated plasmids, transformed DNA chromosomes, etc.), bacteria that can acquire multiple IS*26*-containing plasmids can facilitate the generation of expanded gene clusters with multiple antibiotic resistance genes. This process is accelerated under conditions conducive to the coalescence of diverse bacterial strains that are also amenable to horizontal gene transfer. The animal gut has been shown to be particularly conducive to high rates of conjugal transfer between bacteria under conditions of inflammation or disease (70–72). These disease conditions frequently coincide with administration of antibiotics, creating a strong selective pressure for accumulation of genes that confer antibiotic resistance.

The observed diversity in the number and type of antibiotic resistance genes and the diverse mechanisms for their spreading among Sanji and related O89b-containing *E. coli* strains indicate that acquisition of XDR properties can occur through multiple evolutionary paths. One implication of this observation is that targeted elimination of any existing XDR strain is unlikely to prevent the emergence of new strains with similar XDR properties. A second implication is that even the best antibiotic stewardship is unlikely to be sufficient to prevent or mitigate the development of XDR pathogens. These potential consequences underscore the urgency of the quest for better understanding the shared physiology and virulence mechanisms of pathogenic bacteria, such as the group identified here with O89b-antigens.

MATERIALS AND METHODS

Isolation and serotyping of *E. coli* strain Sanji. *E. coli* strain Sanji was isolated from the duodenum of a pheasant during a 2011 outbreak of fowl colibacillosis on a farm in Fujian province, China, that had about 400 pheasants. Symptoms included drooping, anorexia, diarrhea, soft feet, and inability to flutter or fly. The disease was refractory to common veterinary antibiotics, including amikacin, which was administered after drug sensitivity testing during the second week of the outbreak. Within 1 month, all of the pheasants became severely ill and died or had to be culled. Serotyping of *E. coli* Sanji, which formed mucoid colonies on LB agar plates, was performed by the Tianjin Biochip Corporation.

Antibiotic susceptibility profiling of *E. coli* strain Sanji. Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method with test discs (6.5-mm to 7.0-mm diameter), according to Clinical & Laboratory Standards Institute (CLSI) M100 guidelines (https://clsi.org). Antibiotic susceptibility to colistin (Arcos) and polymyxin B (Sigma) was assayed using the broth microdilution method, according to EUCAST guidelines (www.eucast.org). Reference *E. coli* strain ATCC 25922 and

Kirby-Bauer test discs were obtained from Hangzhou Tianhe Microorganism Reagent Co., and reference *E. coli* strain MG1655 was obtained from Miao Ling Bio (Wuhan, China).

Genome sequencing, assembly, and annotation of the *E. coli* Sanji genome. Total genomic DNA was prepared using a Qiagen Genomic-tip kit, according to the manufacturer's protocol. Illumina sequencing was performed at the Beijing Genomics Institute (BGI; Shenzhen, People's Republic of China) using a HiSeq 2000 platform with insertions of 484 bp and 6,354 bp. Assembly of the 815 Mb of 90-bp read-length paired-end sequencing data generated from the Illumina platform was unable to close the genome, so we applied a PacBio SMRT sequencing and *de novo* assembly platform. For PacBio sequencing, library construction, sequencing, assembly, and annotation were performed by Pacific Biosciences (Menlo Park, CA), using a PacBio RS II system. Totals of 518,559,882 and 306,969,330 postfilter bases from the size-selected and non-size-selected libraries were obtained with mean subread lengths of 6,292 and 1,590, respectively. The size-selected library assay was performed using a BluePippin system (SageScience) to remove shorter DNA insertions with a size cutoff of ≤15 kb. The non-size-selected library was also included to capture and sequence the smaller 3.4-kb and 2.6-kb plasmids. A total of 839,222,725 bases were assembled using the HGAP (v. 2.3) long-read assembler (73) into 15 polished contigs (maximum contig length of 4,926,777) with mean coverage of 135×. The resulting genomes of the single circular chromosome (4,891,769 bp) and six circular plasmids (255,368 bp, 98,436 bp, 94,712 bp, 82,288 bp, 3,373 bp, and 2,640 bp) were annotated using the best-placed reference protein set (GeneMarkS+) in the NCBI Prokaryotic Genome Annotation Pipeline (ver. 3.3).

***In silico* serotyping, antibiotic resistance gene profiling, and IS element analysis of *E. coli* strain Sanji.** Sequence-based bacterial serotyping was performed using SerotypeFinder (ver. 1.1) at https://cge.cbs.dtu.dk/services/SerotypeFinder/ (74). Antibiotic resistance genes were identified using blastn against a database generated from the Resfams database at www.dantaslab.org/resfams (75), the ResFinder database at www.genomicepidemiology.org (76), and information obtained from the review published previously by Roberts et al. (77). A shell script was used to extract the list of antibiotic resistance gene clusters from the blastn output. Insertion sequence (IS) elements were identified using ISfinder at http://www-is.biotoul.fr (78).

Comparative genome sequence analysis. Genome sequences of *E. coli* ST167 strains (ECONIH6 [GenBank accession no. CP026199.1], AR_0150 [GenBank accession no. CP021736.1], AR_0151 [GenBank accession no. CP021691.1], AR_0149 [GenBank accession no. CP021532.1], WCHEC005237 [GenBank accession no. CP026580.2], FDAARGOS_434 [GenBank accession no. CP023870.1], CRE1493 [GenBank accession no. CP019071.1], AR_0014 [GenBank accession no. CP024859.1], AR_0011 [GenBank accession no. CP024855.1], AR_0162 [GenBank accession no. CP021683.1], CREC-532 [GenBank accession no. CP024830.1], CREC-629 [GenBank accession no. CP024815.1], Y5 [GenBank accession no. CP013483.1], SCEC020007 [GenBank accession no. CP025627.1], 51008369SK1 [GenBank accession no. CP029973.1], AR435 [GenBank accession no. CP029115.1], M217 [GenBank accession no. AP019189.1), ST617 strains (AR_0114 [GenBank accession no. CP021732.1], MRSN346355 [GenBank accession no. CP018121.1], MRSN346638 [GenBank accession no. CP018115.1], MRSN346595 [GenBank accession no. CP018109.1], MRSN352231 [GenBank accession no. CP018103.1], 5CRE51 [GenBank accession no. CP021175.1], SCEC020023 [GenBank accession no. CP025950.3], H8 [GenBank accession no. CP010172.1], WCHEC005784 [GenBank accession no. CP028578.2], 675SK2 [GenBank accession no. CP027701.1), ST10 strains (1283 [GenBank accession no. CP023371.1], Ecol_422 [GenBank accession no. CP018962.1], 6409 [GenBank accession no. CP010371.1], DA33133 [GenBank accession no. CP029574.1], 26561 [GenBank accession no. CP027118.1), ST744 strains (1223 [GenBank accession no. CP023383.1], EC590 [GenBank accession no. CP016182.2], W5-6 [GenBank accession no. CP032992.1), and other strains (Ecol_AZ155 [GenBank accession no. CP019005.1], CH611_eco [GenBank accession no. CP017980.1], AR_0137 [GenBank accession no. CP021879.1], MG1655 [GenBank accession no. U00096.3), including their plasmids, were used for comparative genome sequence analysis. Multilocus sequence typing (MLST) of the Sanji strain and comparison with sequences of other related *E. coli* strains were based on the use of Achtman's seven housekeeping genes for *E. coli* (*purA*, *adk*, *icd*, *fumC*, *recA*, *mdh*, and *gyrB*) (28) and performed using a shell script based on the Enterobase database at https://enterobase.warwick.ac.uk/species/ecoli/download_7_gene. Molecular phylogenetic analysis was performed by the maximum likelihood method based on the Tamura-Nei model (79) using MEGA7 (80) with 1,000 bootstrap iterations. Synteny plots were generated using Artemis Comparison Tool (ACT) software (81) and blastn results based on genome alignments. Circular plots for genome comparison were produced using BLAST Ring Image Generator (BRIG) (82). Plasmids were analyzed and typed by plasmid multilocus sequence typing (pMLST) using the PubMLST database (http://pubmlst.org/plasmid) (83). IS*26*-associated antibiotic resistance genes were defined as resistance genes located within 10 kb of an IS*26*-like element. Gene graphics were generated with the aid of SnapGene Viewer software (GSL Biotech).

The 39 Sanji-related genomes plus 19 ST167 assemblies, from isolates obtained between 1999 and 2010, and MG1655 were used to generate core single nucleotide variants (SNVs). The sequences of the 19 ST167 assemblies were downloaded from the Enterobase database at https://enterobase.warwick.ac.uk and concatenated as a continuous fasta file. The recurring regions and unique regions of the 40 complete genome sequences were removed using a shell script. This method is based on genome-to-genome blastn at 99% coverage and 99% identity and subsequent removal of recurring and unique regions. The Sanji genome reference template was used as a query for blastn analysis against another genome. The resulting common regions shared by the two genomes were then joined (as a "stitched" sequence) and used for blastn analysis of another new genome sequence to generate a new stitched sequence until all 40 of the genomes, including Sanji, were compared. This entire process was then repeated 10 times. After 6 iterations, a convergent, consensus stitched sequence of 2,493,769 bp was

obtained. This consensus stitched sequence was then subjected to blast analysis against each individual genome, followed by the use of a shell script to remove all sites with gapped or identical sequences to generate a string of ungapped 6,890 core SNVs. The consensus core stitched sequence was used similarly to generate a string of 6,890 core SNVs for each of the 19 assemblies. This alignment of SNVs was used for modeling mutation rate estimates and time-scaled phylogeny using MEGA7 and BEAST2.5.1 packages.

The MultiTypeTree module of BEAST2 was used with the following parameters: (i) tip dates were set as the sample isolation dates (or as the database submission date for cases where no isolation date was provided); (ii) tip locations were set as three geographic regions (Americas, Asia, and Europe); (iii) the gamma site model was selected with the HKY85 nucleotide substitution model (84); and (iv) a strict clock model was used with an initial mutation rate set at 10^{-10} mutations per site per year. For the priors, a uniform distribution was selected for clockRate.c with an initial value of 10^{-10} and an upper limit of 10^{-7}; exponential distribution was selected for gammaShape.s with an initial value of 1; log normal was selected for kappa.s with an initial value of 2; 1/*X* distribution was selected for popSizes.t; exponential distribution was selected for rate Matrix.t; and equal population sizes and a symmetric migration rate matrix were assumed for the migration model. In trial runs sampling 10^6 Markov chain Monte Carlo (MCMC) steps, we explored HKY85, TN93, and generalized time-reversible (GTR) nucleotide substitution models with various parameters. The TN93 and GTR models could not accommodate mutation rates lower than 0.001, and even with the clock rate accepted by the module, runs were often terminated prematurely. For those runs that were completed, the models gave results comparable to those obtained with the HKY85 model. Using the HKY85 model, five runs with 10^8 MCMC steps were performed, with 10% discounted as representing burn-in and a tree logging frequency of 10^5. Tree files were combined using LogCombiner in the BEAST2 package, followed by the use of TreeAnnotator to annotate the combined trees. The annotated output trees file was used to generate the phylogenetic tree with associated metadata, including 7-gene MLST, isolation region (United States, Latin America, Europe, Asia), source (human, animal, environmental), and *in silico* O-antigen and H-antigen serotypes, using the R package ggtree.

Data availability. The complete genome and plasmid sequences of *E. coli* strain Sanji have been deposited in the NCBI under accession numbers CP011061.1 (circular chromosome; 4,891,769 bp), CP011062.1 (pSJ_255; 255,368 bp), CP011063.1 (pSJ_98; 98,436 bp), CP011064.1 (pSJ_94; 94,712 bp), CP011065.1 (pSJ_82; 82,288 bp), CP011066.1 (pSJ_3; 3,373 bp), and CP011067.1 (pSJ_2; 2,640 bp).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00242-18.

FIG S1, PDF file, 0.1 MB.
FIG S2, PDF file, 0.02 MB.
FIG S3, PDF file, 1.6 MB.
FIG S4, PDF file, 0.3 MB.
FIG S5, PDF file, 0.4 MB.
FIG S6, PDF file, 0.1 MB.
FIG S7, PDF file, 0.9 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.1 MB.
TABLE S3, PDF file, 0.1 MB.

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M.H. and X.Z. designed and conceived the experiments. X.Z., X.C., M.H., and D.M. performed the experiments. C.L., R.H., and P.B. performed the sequencing and assembly of the genomes. M.H. performed scripting and bioinformatic analysis. M.H., B.T.H., R.J.H., and B.A.W. analyzed and interpreted the data. S.W., P.B., and B.A.W. provided reagents, materials, analytical tools, and support. B.A.W., B.T.H., and M.H. wrote the paper. All of us read, edited, and approved the final version of the paper.

C.L., R.J.H., and P.B. are full-time employees and shareholders of Pacific Biosciences, a company developing and commercializing single-molecule DNA sequencing technologies.

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