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Yersinia pestis pFra Shows Biovar-Specific Differences and Recent Common Ancestry with a *Salmonella enterica* Serovar Typhi Plasmid

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Population genetic studies suggest that *Yersinia pestis*, the cause of plague, is a clonal pathogen that has recently emerged from *Yersinia pseudotuberculosis*. Plasmid acquisition is likely to have been a key element in this evolutionary leap from an enteric to a flea-transmitted systemic pathogen. However, the origin of *Y. pestis*-specific plasmids remains obscure. We demonstrate specific plasmid rearrangements in different *Y. pestis* strains which distinguish *Y. pestis* bv. Orientalis strains from other biovars. We also present evidence for plasmid-associated DNA exchange between *Y. pestis* and the exclusively human pathogen *Salmonella enterica* serovar Typhi.

Yersinia pestis is primarily a rodent pathogen and is usually transmitted subcutaneously by the bite of an infected flea (13). The microorganism can spread to the regional lymph nodes of infected rodents and humans, where local replication can cause a swelling, or bubo (55). Subsequent bacteremia may sometimes result in pulmonary infection (secondary pneumonic plague) and direct respiratory spread (55). There are historical data suggesting that epidemic plague has on occasion been the leading cause of death in humans—a third of the population of Europe may have died in the 14th century during the second pandemic of plague known as the Black Death (79). The third pandemic resulting from the Hong Kong outbreak of 1894 killed millions of people (mainly in Asia) in the early years of the 20th century (55). Currently, most plague cases are reported from developing countries in Africa and Asia such as Madagascar, Tanzania, and Vietnam (75). Plague continues to have a high mortality rate: 20% of the 200 annual confirmed or presumptive cases (95% bubonic) in Madagascar (16) and 6 out of 19 cases in a recent series from the United States (18) were fatal infections. However, most of the countries reporting plague currently have much higher disease burdens associated with other infectious agents, including the exclusively human pathogen *Salmonella enterica* serovar Typhi (53).

The factors influencing the rise and fall of plague epidemics remain obscure (34), and it is vital to understand the genetic basis of the origins and evolution of such a potentially devas-

tating pathogen as *Y. pestis*. The enteric pathogen *Yersinia pseudotuberculosis* is so closely related to *Y. pestis* in terms of chromosomal DNA hybridization that they could be classified as subspecies (5). On the basis of sequence analysis of multiple housekeeping genes, it has been proposed (1) that *Y. pestis* is a clone which has recently evolved from *Y. pseudotuberculosis*. Within the *Y. pestis* clone, distinct biovars (Antiqua, Medievalis, and Orientalis) have been associated with the three historically recognized pandemics (20). These biovars have been shown to be phylogenetically distinct by analysis of DNA restriction fragment polymorphisms (1) and ribotyping (29). It has been suggested that *Y. pestis*. bv. Orientalis has emerged most recently (1).

A role for key *Y. pestis*-specific gene mutations in evolution from *Y. pseudotuberculosis* was suggested by demonstration of increased virulence of *Y. pseudotuberculosis* for mice to a level comparable with that of *Y. pestis* when mutated in the chromosomal *inv* and plasmid-located *yadA* loci (61). These genes are intact open reading frames (ORFs) in *Y. pseudotuberculosis* but not in *Y. pestis* strains (61, 67). Conversely, introduction of a functional *Y. pseudotuberculosis yadA* gene into *Y. pestis* caused a significant decrease in virulence (61). However, the hypervirulent *Y. pseudotuberculosis* mutants were later found not to be isogenic with the wild-type strains (31), suggesting that the two single gene mutations were not the only factors involved.

The most obvious additional genetic complement present in *Y. pestis* compared with *Y. pseudotuberculosis* is in the form of plasmids. Both *Y. pestis* and *Y. pseudotuberculosis* possess a 70-kb plasmid (pCD1 in *Y. pestis* and pIB1 in *Y. pseudotuberculosis*) which encodes the Yop virulon, a type III secretion

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TABLE 1. Bacterial strains used in this study[a]

Strain	Properties	Source or reference
Y. pestis		
A1122	bv. Orientalis pFra	M. C. Chu, NCID[b]
PEXU2	bv. Orientalis pFra	M. C. Chu, NCID
PB6	bv. Orientalis	M. C. Chu, NCID
F361/66	bv. Orientalis pFra	M. C. Chu, NCID
15–91	bv. Orientalis pFra	M. C. Chu, NCID
16–34	bv. Orientalis pFra	M. C. Chu, NCID
AZ94-0666	bv. Orientalis pFra	M. C. Chu, NCID
ZE94-2122	bv. Orientalis pFra	M. C. Chu, NCID
TWS	bv. Orientalis pFra	P. Oyston, CBDE[c]
CO-92	bv. Orientalis pFra	P. Oyston, CBDE (21)
GB	bv. Orientalis pFra	P. Oyston, CBDE (51)
JAVA 9	bv. Orientalis	P. Oyston, CBDE
195P	bv. Orientalis pFra	P. Oyston, CBDE
KIM	bv. Medievalis pMT1	M. C. Chu, NCID
Harbin 35	bv. Medievalis pFra	M. C. Chu, NCID
Nepal 516	bv. Antiqua pFra	M. C. Chu, NCID
S. enterica serovar Typhi CT18	pHCM1 pHCM2	J. Wain, Centre for Tropical Diseases[d]

[a] The presence of pFra/pMT1 was demonstrated by *caf*1 PCR (52).
[b] NCID, National Center for Infectious Diseases, Fort Collins, Colo.
[c] CBDE, Chemical & Biological Defence Establishment, Porton Down, United Kingdom.
[d] The Centre for Tropical Diseases is in Ho Chi Minh City, Vietnam.

system essential for virulence in many hosts (9, 55). In addition, *Y. pestis* usually possesses two further plasmids which encode a variety of potentially virulence-associated determinants. A 9.5-kb plasmid called pPCP1 in *Y. pestis* KIM strains (alternatively known as pPst) encodes the plasminogen activator Pla (55). A 100- to 110-kb plasmid called pMT1 in KIM strains (alternatively known as pFra) encodes the murine toxin Ymt and the F1 capsular protein (55).

The key difference between *Y. pseudotuberculosis* and *Y. pestis* is the latter's capacity to colonize the flea and be transmitted to mammalian hosts by a subcutaneous route of infection. The chromosomal *hms* locus that is required (33) for blockage of the flea midgut by *Y. pestis* to maximize onward transmission is also present in *Y. pseudotuberculosis* (11). However, the *Y. pestis*-specific pMT1 or pFra plasmid encodes a murine toxin, which is apparently essential for flea colonization (35), and the F1 capsular antigen, which is an important protective immunogen although it is not essential for virulence in mammalian hosts (25). The Pla protease encoded by the pPCP1 or pPst plasmid is required (70) by some strains of *Y. pestis* for systemic spread after subcutaneous injection into a mammalian host. It seems likely that acquisition of these two plasmids was an essential step in the evolution of *Y. pestis* from *Y. pseudotuberculosis*. However, the limited sequence similarity between the *Y. pestis* plasmids (37, 42) and other bacterial plasmids gives few clues about their origin and capacity for mobility. In an effort to understand the pathogenicity and evolution of *Y. pestis*, we have undertaken the genome sequencing of this devastating pathogen, and plasmid DNA sequence comparison is an important application of these data. Genetic diversity of plasmids exceeds that of host chromosomes, largely due to a higher rate of recombination (6), and genetic variation associated with biovar emergence may be more easily revealed in plasmids than in the homogeneous chromosomal background (1).

Our data provide supporting evidence for the emergence of

Y. pestis bv. Orientalis after biovars Medievalis and Antiqua. We present data demonstrating the common ancestry of more than 50% of the *Y. pestis* pMT1/pFra plasmid and a cryptic plasmid from a recent clinical isolate of *S. enterica* serovar Typhi, another species of *Enterobacteriaceae* which is an exclusively human pathogen.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

DNA library construction and sequencing. A single colony of *Y. pestis* CO-92 was picked from Congo red agar and grown overnight at 28°C in BAB broth (50). A single colony of *Salmonella enterica* serovar Typhi CT18 was picked from Luria agar and grown overnight at 37°C in Luria broth. Total DNA was isolated as described previously (51). Random shotgun libraries of each genome were constructed and sequenced as previously described (54).

Sequence annotation and analysis. The DNA was compared with sequences in the EMBL database using BLASTN and BLASTX (2). Transfer RNAs were predicted with tRNAscan-SE (43). Potential coding sequences (CDSs) were predicted using ORPHEUS (26) and GLIMMER (64) and also stop-to-stop prediction; the results were combined. The predicted protein sequences were searched against a nonredundant protein database using WUBLASTP and FASTA. The complete six-frame translation was used to search PROSITE, and the predicted proteins were compared against the PFAM (4) database of protein domain hidden Markov models. The results of all these analyses were assembled using the Artemis sequence viewer (63). Plasmids were defined as separate contiguous sequences from analysis of shotgun sequence data with no plasmid manipulations (separation or specific cloning). Statistical analysis of the frequency of occurrence of Chi sequences was performed using the RMES package (Unité de Biométrie, INRA, Jouy-en-Josas, France [http://www-bia.inra.fr/J/AB/genome/RMES/welcome.html]) to rank the number of Chi and Chi complement sequences against the frequency of occurrence of all octamers and their complements on one plasmid sequence strand.

PCR. Reaction mixtures (20 μl) were prepared with 1 U of *Taq* polymerase (Roche Molecular Biochemicals, Lewes, United Kingdom), 1× *Taq* polymerase buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl$_2$), 0.2 mM (each) deoxynucleoside triphosphate and 0.2 μM (each) primer. DNA was obtained by boiling a single colony in 200 μl of distilled water for 5 min, using 1 μl of supernatant for the PCR template. For the 1,083-bp amplicon, primer P121 (5′-CCGTTCTACATCATCCATA-3′) and primer P221 (5′-TTATGGCTGGC AAATCTGA-3′) were used. PCR conditions were 94°C for 1 min and 25 cycles of 94°C for 30 s, 57.5°C for 30 s, and 72°C for 1 min, and then 72°C for 10 min. For the 1,865-bp amplicon, primer P126 (5′-GTTGAGCATTAGCGAGACC-
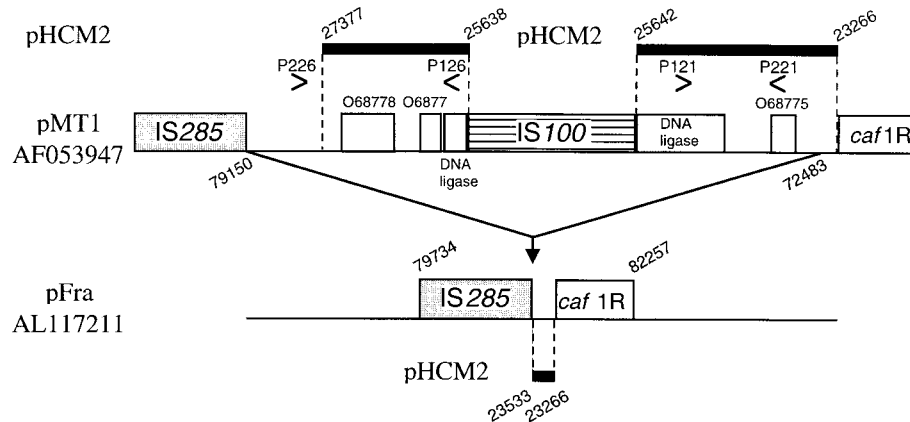
FIG. 1. IS*100*-associated deletion in pFra of *Y. pestis* CO-92 compared with *Y. pestis* KIM5 pMT1 (GenBank accession no. AF053947) and *S. enterica* serovar Typhi CT18 plasmid pHCM2. A region of 6,668 bp present in KIM5 is shown above the corresponding region in CO-92, which lacks this insertion. Thick black lines above and below the two *Y. pestis* plasmids and linked to them by dotted lines indicate the regions of pHCM2 showing more than 95% identity to the adjacent *Y. pestis* sequence. Locations of PCR primers for the reactions shown in Fig. 2 are indicated by arrowheads. Numbers shown at an angle to the horizontal correspond to base-pair locations in the labeled plasmid. In one KIM5 sequence (GenBank accession no. AF074611), the regions shown on either side of this IS*100* copy are separated by 24 kb due to IS*100* recombination.

3′) and primer P226 (5′-CTACCGCATTACTCCACTC-3′) were used. PCR conditions were 94°C for 1 min and 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then 72°C for 10 min.

Nucleotide sequence accession numbers. The annotated *Y. pestis* plasmid sequences have been deposited in EMBL under accession numbers AL117211 (pFra), AL117189 (pYV), and AL109969 (pPst). The sequences of *S. enterica* serovar Typhi CT18 and the plasmid pHCM2 are available from the Sanger Centre (http://www.sanger.ac.uk/Projects/S_typhi).

RESULTS

***Y. pestis* interbiovar comparisons.** The plasmid sequences obtained for *Y. pestis* CO-92 were compared with those previously reported for *Y. pestis* KIM5. *Y. pestis* CO-92 is a recent clinical isolate of *Y. pestis* bv. Orientalis from a fatal case of primary plague pneumonia in the United States (21). *Y. pestis* KIM is a laboratory-passaged bv. Medievalis strain originally isolated in the Middle East (Kurdistan Iran man) (55). Two of the *Y. pestis* CO-92 plasmids, pPst and pCD1, show minor differences from their counterparts in *Y. pestis* KIM5. More extensive rearrangements and indels were seen for pMT1/pFra (Fig. 1; see Fig. 3).

pPst. The 9,612-bp pesticin plasmid pPst showed two single-base-pair differences (one insertion and one deletion) and a single 2-bp insertion compared with the previously reported 9,610-bp KIM5 plasmid pPCP1 (GenBank accession no. AF053945) (37). All changes involved intergenic homopolymeric base-pair tracts. Only the single-base-pair differences were adjacent to a coding region: an insertion and a deletion 20 and 40 bp, respectively, upstream of the *pst* gene encoding pesticin. Neither involved the promoter or any proposed regulatory site (59). One 5-bp direct repeat flanking the IS*100* insertion site in both the pPst and pPCP1 plasmids was also found, together with the next 5 bp of downstream sequence adjacent to one side of an IS*100* insertion in *Y. pestis* bv. Medievalis KIM pMT1 and *Y. pestis* bv. Orientalis pFra (data not shown), suggesting that IS*100* interplasmid recombination had occurred before divergence of these biovars.

pCD1. Three of the four clusters of insertion elements identified in the 70,305-bp *Y. pestis* CO-92 pCD1 plasmid showed deletions or insertions compared with the two previously reported *Y. pestis* KIM5 sequences. These were 70,504 (37) and 70,509 (56) bp, respectively (the 5-bp indel accounting for the size difference between the two KIM sequences is not deleted from the CO-92 sequence). The major difference between the two KIM sequences and the CO-92 sequence was the location of the IS*100* insertion element. In both biovars it was nested within another insertion element in a cluster of mobile genetic elements, but in the case of CO-92 it was inserted into a degenerate IS*21*-like element between *sycE* and *sycH* instead of a degenerate IS*D1*-like element between *yscM* and *yopH* as in *Y. pestis* KIM5. In both cases, the IS*100* insertion site was flanked by a direct repeat of 5 bp. The absence of ambiguities on sequence assembly suggests that all copies of pCD1 in the cells of the colony used to make the sequence library had the same IS*100* insertion site, distinct from that in KIM5 strains. However, PCR with primers from IS*100* and both sets of flanking sequences gave products with the *Y. pestis* bv. Orientalis strains listed in Table 1 (data not shown), and we were unable to assign strain or biovar specificity to the pCD1 IS*100* insertion site. Another insertion site for IS*100* in pCD1 from *Y. pestis* bv. Orientalis strain EV76 has previously been demonstrated (GenBank accession no. X78302) (data not shown).

A 212-bp deletion of a partial IS*285* sequence between *yopD* and *yopM*, apparently following recombination between two 3-bp direct repeats (located at 18,852 bp in CO-92), accounted for most of the difference in size between the KIM5 sequences and those of CO-92. It was located within one of three repeated sequences (R1-3) (60) forming part of an IS element which have apparently recombined to invert the *yopM* region in *Y. enterocolitica* pYVe227 (38) with respect to that in *Y. pestis* and *Y. pseudotuberculosis*. The deletion extended the length of R1 in *Y. pestis* CO-92 from 189 to 264 bp.

There were four other minor differences in non-insertion sequence ORFs from the KIM5 pCD1 sequences: three single-base-pair substitutions changing a single amino acid and an 11-bp insertion extending the Syc-like ORF7 reported upstream of *ypkA* (56) and rendering this ORF in CO-92 (ORF

1.73c) identical to that upstream of *ypkA* in *Y. pseudotuberculosis* pIB1 (28). There were no differences in the gene order in pCD1 between KIM5 and CO-92 other than the insertion sequence transposition. The point mutation in *yadA* which distinguishes *Y. pestis* pCD1 from its homologues in *Y. enterocolitica* and *Y. pseudotuberculosis* (56) was present.

pFra. The 96,210-bp *Y. pestis* CO-92 pFra plasmid contained two IS*100* elements present as inverted repeats, as described for pMT1 in KIM5 (37, 42). Deletions of 2,109 and 2,606 bp in CO-92 pFra compared with the published KIM5 pMT1 sequences were also found, corresponding to regions adjacent to an IS*100* element (Fig. 1; see Fig. 3). In one of the published pMT1 sequences (GenBank accession no. AF053947) (37), these regions flanked a single IS*100* element with a 5-bp direct repeat at the site of insertion and together with that element formed a single insertion of 6,669 bp compared with the CO-92 sequence (Fig. 1). In the other published KIM5 sequence (GenBank accession no. AF074611) (42), each was adjacent to a different IS*100* copy, neither of which was flanked by a direct repeat. This reflected a previously reported inversion of the 24-kb region between the two IS*100* copies in one KIM5 sequence compared with the other (37, 42). One of the two IS*100* copies was inserted in a different location in CO-92 (bp 57267 to 59220) compared with the two KIM5 sequences (Fig. 1; see Fig. 3). Other differences in gene order between the two KIM5 sequences and CO-92 could be accounted for by insertion of this IS*100* copy into a DNA polymerase III gene followed by recombination with the other IS*100* sequence, inverting a 37-kb region in CO-92 (see Fig. 3).

In addition, a 63-bp deletion was detected in CO-92 pFra compared with the KIM5 pMT1 sequences, apparently following recombination between 12-bp imperfect direct repeats, resulting in the fusion of two unidentified KIM5 ORFs (O68739 and O68738 in one KIM5 sequence [37], Y1032 and Y1033 in the other [42]) to form ORF 1.22c. There were 12 sites showing differences with both KIM5 sequences. Four of these were conservative single-base-pair substitutions in coding regions, four were 1- or 2-bp indels in noncoding sequence, and four were single-base-pair substitutions in coding regions changing one or two amino acids. There were 12 sites where the CO-92 sequence showed identity with one but not both KIM5 sequences (i.e., KIM5 polymorphisms were as common as interbiovar differences). These comprised three single-base-pair substitutions (one conservative, two in noncoding regions), eight single-base-pair indels (three in noncoding regions), and one 5-bp polymorphism in a noncoding region. There were no differences in any known virulence-related factors.

PCR with primers internal to the two large deletions yielded no specific amplicons with a variety of *Y. pestis* bv. Orientalis strains, but they were present (Fig. 2) in a *Y. pestis* bv. Antiqua strain and a *Y. pestis* bv. Medievalis strain unrelated to KIM, suggesting that this deletion could be biovar specific. In addition to previously reported homologies, BLASTN searching showed that most of this deleted region (apart from the IS*100* sequence) was present in pHCM2, one of two plasmids present in a multidrug-resistant strain of *S. enterica* serovar Typhi CT18 which originated in Vietnam (http://www.sanger.ac.uk/Projects/S_typhi). This plasmid also harbored 60 bp of the 63-bp deletion.
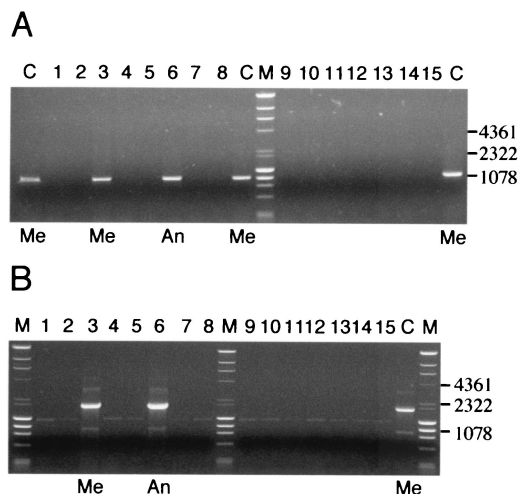

FIG. 2. PCR for IS*100*-flanking regions from *Y. pestis* KIM5 pMT1 (bv. Medievalis) showing absence in *Y. pestis* bv. Orientalis strains and presence in other biovar Medievalis and Antiqua strains. DNA fragment sizes are given in base pairs. (A) PCR with primers P121 and P221. (B) PCR with primers P226 and P126. Lanes: 1, strain A1122; 2, PEXU2; 3, Harbin 35; 4, PB6; 5, F361/66; 6, Nepal 516; 7, 15–91; 8, 16–34; 9, AZ94-0666; 10, ZE94-2122; 11, TWS; 12, CO-92; 13, GB; 14, JAVA 9; 15, 195P; C, KIM (control); M, molecular weight marker. Additional text labels indicate lanes containing DNA templates from *Y. pestis* bv. Antiqua (An) and bv. Medievalis (Me) strains. All other numbered lanes contain DNA from *Y. pestis* bv. Orientalis strains. A 0.9% agarose gel was used. The digital image was obtained with Gel-Doc (Bio-Rad) and was cropped and resized in Adobe Photoshop.

pFra and pHCM2 comparison. More than 52% of pFra from *Y. pestis* CO-92 was highly similar to pHCM2 (Table 2). Because some of the shared DNA sequence was in regions absent in *Y. pestis* bv. Orientalis strains, the percentage of KIM5 pMT1 resembling pHCM2 was higher than that of CO-92 pFra at 56.2%, with 57.6% of pHCM2 showing similarity with pMT1. More than 90% of the similar regions showed more than 96% DNA identity. The G+C content of the regions in common (51.9%) was closer to the overall *S. enterica* serovar Typhi G+C content of 52.05% than the *Yersinia* background level of 47.6%, while the unique regions of both plasmids showed a lower G+C content.

Sixty-two ORFs in *Y. pestis* CO-92 pFra had their closest homologue outside *Y. pestis* in predicted ORFs in pHCM2. For a further four predicted ORFs in pHCM2, homologues in the region of *Y. pestis* KIM5 pMT1 were absent in CO-92 (Fig. 1). The DNA sequences sharing significant homology between pFra and pHCM2 included those encoding the *repA* gene (42) and the surrounding iteron-based replication control system in the *ori* region (Fig. 3). pFra encodes a *parABS*-like system (ORFs 1.68c and 1.67c) (Fig. 3) resembling that of bacteriophages P1 and P7 (42) which is functional in *Escherichia coli* (78), but this region is absent in pHCM2. The partial repeats of fragments of the *S. enterica* serovar Typhimurium *parAB* loci previously noted (42) adjacent to the murine toxin gene in *Y. pestis* KIM5 pMT1 and the adjacent resolvase homologue were present in CO-92 (ORF 1.70) but not in pHCM2. Neither pFra/pMT1 nor pHCM2 had an obvious set of transfer or mobilization genes.

There is only one significant segment of low G+C compo-

TABLE 2. Overall sequence features of pFra, pMT1, and pHCM2

Parameters	Value for plasmid or specific host[a]		
	Y. pestis CO-92 pFra	*Y. pestis* KIM5 pMTI[b]	*S. enterica* serovar Typhi CT18 pHCM2
Chromosomal G+C (%)	47.6	47.6	52.05
Plasmid size (bp)	96,210	100984	106515
Plasmid G+C (%)	50.23	50.16	50.61
Proportion of plasmid with cross-species similarity (%)	58.6	59.6	52.6 (pFra)
			56.2 (pMT1)
G+C content of plasmid regions with cross-species similarity (%)	51.9	51.9	51.9
G+C content of nonhomologous regions (%)	47.8	47.8	49.1 non-pFra
			48.9 non-pMT1
Number of Chi and Chi complement sites on one plasmid sequence strand	19	20	32
Expected[c] Chi frequency (in same length sequence of random base order)	2.96	3.06	3.36
Probability[d] (rank) of random Chi occurrence in plasmid based on dinucleotide frequency	$<10^{-38}$ (1)	$<10^{-44}$ (1)	$<10^{-116}$ (1)

[a] Unless otherwise specified, nucleotide sequences as listed in Materials and Methods.
[b] GenBank accession no. AF053947.
[c] Calculated from the base composition of the sequence (41).
[d] Determined using R'MES software for a word length of 8, i.e., ranking 65,536 possible different octanucleotides (23).

sition in the regions common to pFra/pMT1 and pHCM2. This is adjacent to an IS*200*-like sequence, IS*1541*, in pFra (Fig. 3) and contains the pFra ORF 1.34 with no significant similarity on BLAST searching other than in pHCM2. The blocks of sequence similarity between pFra and pHCM2 occur in the same order in both plasmids. Most of the sequence present in *Y. pestis* KIM5 but not in *Y. pestis* CO-92 is present in pHCM2, and there are no inversions of any homologous sequence in pHCM2 compared with *Y. pestis* KIM5, while there is one such inversion in CO-92, reflecting the 37-kb inversion compared with KIM5 (Fig. 3).

Repeated elements. The three insertion elements encoded by pFra (Two IS*100* copies and a single IS*1541*) were outside the regions of sequence similarity with pHCM2 (Fig. 3). No insertion elements were apparent in the pHCM2 sequence. The *E. coli* Chi site GCTGGTGG and the complement CCA CCAGC occur 19 times in pFra (Table 2) and are restricted to the regions also found in pHCM2 (Fig. 3). This sequence is not found in the other two *Y. pestis* plasmids. The R'MES program (23) showed it to be the most overrepresented of the 65,536 possible octanucleotides in pFra, pMT1, and pHCM2 (Table 2), based on the dinucleotide composition of each plasmid. A novel 57-bp imperfect repeat is present upstream of three unidentified ORFs in both pHCM2 and pFra (data not shown). Another five copies of this repeat are present between unidentified ORFs in pHCM2. These repeat sequences were not found elsewhere in the *Y. pestis* or *S. enterica* serovar Typhi genome sequence and were not deposited in GenBank. They do not show the palindromic symmetry typical of integrons or super integrons (62).

DISCUSSION

***Y. pestis* biovar-associated plasmid differences.** The most obvious differences between the *Y. pestis* CO-92 and KIM5 plasmid sequences were those related to recombination between IS*100* elements or IS*100* transposition. No IS-related transposition differences were apparent in CO-92 pPst compared with KIM pCP1 despite the presence of an IS*100* se-

quence, possibly due to the limited number of potential insertion sites in this small plasmid. A 24-kb inversion between two IS*100* copies in pMT1 was previously noted on comparison of two sequences of the same strain of *Y. pestis* KIM5 (37, 42). From the loss of direct repeats flanking IS*100* in one of these sequences, this inversion can be attributed to IS*100* recombination, presumably during 30 years of laboratory storage (8) or during the *E. coli* subcloning of *Y. pestis* plasmids that was carried out to obtain the KIM plasmid sequences (37, 42). Laboratory strains of bacteria that are stored for many years are particularly subject to IS-related rearrangements (48). In contrast, *Y. pestis* CO-92 is a recent clinical isolate (1992) (21) with fewer laboratory passages, and the genomic shotgun method employed in this study did not require subcloning of the entire pFra plasmid, reducing the scope for in vitro rearrangement that could influence sequence results.

The deletions we have detected adjacent to one of the IS*100* elements in *Y. pestis* CO-92 pFra compared to KIM pMT1 (Fig. 1) are demonstrable in other *Y. pestis* bv. Orientalis strains but not in those of *Y. pestis* bv. Antiqua or Medievalis (Fig. 2). Consistent biovar-specific deletions are unlikely to be in vitro phenomena. These deletions are largely responsible for the 4.8-kb difference in size between *Y. pestis* CO-92 pFra and KIM5 pMT1. It is well recognized that pFra differs in size in strains of different origins (24). In particular, the pFra of two *Y. pestis* bv. Orientalis strains has previously been noted on restriction mapping to be 3 kb smaller than the pFra of two *Y. pestis* bv. Antiqua strains (57). However, not only is this sequence present in *Y. pestis* bv. Medievalis and Antiqua strains, but most of it forms part of the pFra plasmid-related sequence which is encoded by the *S. enterica* serovar Typhi plasmid. This suggests that the interbiovar difference is indeed due to a unique deletion in *Y. pestis* bv. Orientalis pFra strains rather than an insertion in *Y. pestis* bv. Medievalis and Antiqua strains. This is consistent with a reported Orientalis-specific chromosomal point mutation (11) and supports phylogenetic analysis based on restriction patterns on chromosomal hybridization with IS*100*, which suggested that this biovar had arisen after the other two (1). The definition of these deleted seg-
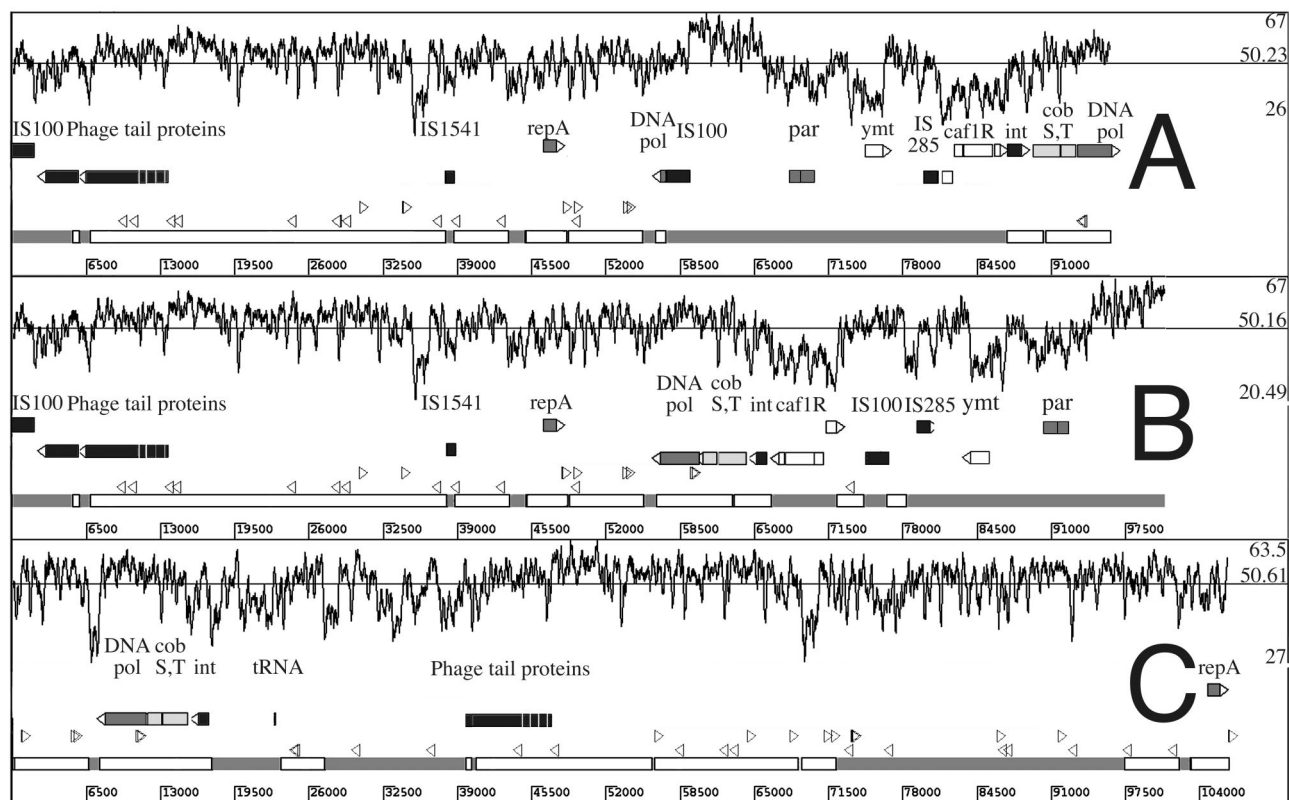
FIG. 3. Comparison of the *Y. pestis* CO-92 pFra plasmid (A) with *Y. pestis* KIM5 pMT1 (GenBank accession no. AF053947) (B) and *S. enterica* serovar Typhi CT18 pHCM2 (C). Numbers on the horizontal scales are base pairs. Numbers on the vertical scale correspond to the plasmid GC percentage determined on a 200-bp window shown graphically along the top of each panel. The three numbers shown in each panel correspond to the mean percent GC for the whole plasmid and regional maxima and minima. Each panel contains the same features in the following order below the GC percentage graph. (i) Selected ORFs are shown as labeled blocks. Those transcribed to the right are shown above those transcribed to the left. ORF block shades are coded as follows: black, insertion sequence or phage related; dark gray, plasmid or DNA replication; light gray, non-DNA metabolism; white, virulence related. ORF abbreviations: DNA pol, DNA polymerase III alpha subunit; int, phage integrase; caf1R, F1 capsular operon and regulatory genes; ymt, murine toxin. (ii) Arrowheads corresponding to the location of *E. coli* Chi sequences are shown. Those pointing to the right are 5′-GCTGGTGG-3′, and those to the left are 5′-CCACCAGC-3′. (iii) Clear blocks in panels A and B correspond to regions of the *Y. pestis* plasmid sequence with sequence identity to *S. enterica* serovar Typhi pHCM2. The grey shading between the clear blocks corresponds to regions of the *Y. pestis* plasmids with no sequence identity to pHCM2. In panel C, the clear blocks represent regions of *S. enterica* serovar Typhi pHCM2 with significant sequence identity to *Y. pestis* (pFra and pMT1). The dark gray blocks represent regions with no sequence identity with *Y. pestis* pFra/pMT1. This figure is based on Artemis (63) views of individual annotated plasmid sequences arranged in a composite image using Adobe Photoshop.

ments offers the first plasmid-based test for *Y. pestis* biovar differentiation by molecular means.

Horizontal DNA transfer between *Yersinia* and other *Enterobacteriaceae*. *Salmonella* species are clearly distinct from *Yersinia* species in 16S rRNA (15, 72) and other sequenced loci (46). Therefore, the presence of such a high degree of sequence identity on a potentially mobile element implies recent horizontal genetic exchange. There is some evidence of chromosomal DNA exchange between *S. enterica* serovar Typhimurium and *Yersinia* species other than *Y. pestis*. Intervening sequences of 100 bp in the *rrl* genes (23S rRNA genes) of highly pathogenic *Yersinia enterocolitica* strains are more closely related to sequences in *S. enterica* serovar Typhimurium than those of other *Yersinia* strains (68). This intervening sequence is not present in *Y. pestis* (68) or *S. enterica* serovar Typhi (44).

Short regions of similarity in nonstructural genes have been reported between the virulence-associated plasmids of *S. en-*

terica serovar Dublin and *Y. pseudotuberculosis* (40). Numerous short stretches of pMT1 (maximum length, 1,150 bp) over a 30-kb region have been previously noted to show high levels of DNA similarity with other plasmid and DNA sequences (42). The IS*100* and IS*285* insertion sequences found on *Y. pestis* plasmids and in particular pFra are present in the enteropathogenic *E. coli* adherence factor plasmid (74). IS*285* incorporates a 117-bp region with more than 76% identity to the gene encoding the 38-amino-acid enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) which is found in a variety of diarrhea-associated *E. coli* strains (76). The 225-amino-acid ORF pMT1.64c is 89% identical to a 227-amino-acid ORF from the virulence plasmid of *E. coli* O157:H7 (12).

The region of pFra/pMT1 forming a mosaic of DNA identities and insertion sequences is also the region that encodes the known *Y. pestis* virulence-associated factors (capsule and the murine toxin). Interestingly, these genes have already been noted to be composed of a lower GC percentage than the

remainder of the plasmid (42) and the *Y. pestis* host. Strikingly, similarity to the pHCM2 plasmid is almost absent from this region, apart from the IS*100*-flanking sequence not present in *Y. pestis* bv. Orientalis strains.

The overrepresentation of the canonical *E. coli* Chi sequence seen on pHCM2 and the corresponding regions of pFra has not previously been reported for naturally occurring plasmids. R'MES analysis of the 23 complete plasmid sequences from *Enterobacteriaceae* represented on the Entrez Genomes database (http://www.ncbi.nlm.nih.gov:80/PMGifs/Genomes/eub_p.html) revealed only one further plasmid with a similar degree of Chi overrepresentation: *S. enterica* serovar Typhi plasmid pR27 (66) contains Chi as the 4th most overrepresented octanucleotide. This plasmid shows extensive sequence similarity to pHCM1, the other plasmid present in *S. enterica* serovar Typhi CT18 (http://www.sanger.ac.uk/Projects/S_typhi) in which Chi is the 5th most overrepresented octanucleotide. The contrasting complete absence of Chi sequences in the other two *Y. pestis* plasmids and the virulence-associated regions of pFra is intriguing. In *E. coli*, Chi sequences regulate the different functions of the RecBCD complex (exonuclease-recombinase) in repairing double-stranded breaks in host DNA and destroying foreign DNA cleaved by host endonucleases (71, 73). Different sequences may fulfill the function of Chi sequences in different bacterial hosts (71), and the presence of a specific Chi sequence in plasmid genes may be a signature of origin from a specific bacterial genome. Phylogenetic analysis suggests that multiple horizontal transfers of chromosomal bacterial genes to plasmids commonly occur (47), and these genes would necessarily include the respective specific bacterial Chi sequence, because Chi sequences tend to be in coding DNA (41). RecBCD-induced recombination due to Chi sequences may promote the survival of horizontally transferred DNA between closely related bacteria (32). It has been suggested that Chi sequences overlapping the start codons of plasmid replication proteins may promote recombination in replicon evolution (49). None of the Chi sequences identified in pFra or pHCM2 overlap start codons in this way. Related *Enterobacteriaceae* may use the same Chi sequence; e.g., *S. enterica* serovar Typhimurium has RecBCD-like enzymes active at *E. coli* Chi sites (45). Specific Chi sequence activity in *Yersinia* species and *S. enterica* serovar Typhi remains to be determined.

pFra is known to integrate into the *Y. pestis* chromosome (58), and the plasmid regions shared with pHCM2 contain Chi sequences and apparent metabolic genes (*cobS* and *cobT*) not found on plasmids in other organisms. Another recently described *Y. pestis* plasmid containing ORFs highly homologous to *E. coli* chromosomal genes (22) does not contain Chi sequences. The *pla* gene on pPst encodes a serine protease whose amino acid sequence is 70% identical (69) to those of products of chromosomal genes from *E. coli* and *S. enterica* serovar Typhimurium.

Homologous recombination between identical sequence on the plasmid and the chromosome (e.g., copies of IS*100*) is the suggested mechanism for the observed phenomenon of chromosomal integration of pFra (58). Other site-specific integration processes may have been functional in the acquisition of chromosomal genes by pFra and pHCM2. A predicted asparagine tRNA gene is present in the pHCM2-specific region at one boundary of the sequence common to both plasmids (Fig. 3) upstream of an integrase gene. tRNA genes are known to be integration sites of pathogenicity islands in the bacterial chromosome (30), and the *Yersinia* high-pathogenicity island is apparently inserted into an asparagine tRNA (10). There are no direct repeats of portions of the tRNA gene elsewhere in pHCM2 to suggest a currently excisable pathogenicity island. The integrase, *cobS*, and *cobT* genes are also present in pFra with no obvious interrupted genes at the boundaries of shared sequence in either plasmid.

Mechanism of transfer. A transferable plasmid, pIPI202, conferring multiple drug resistances has recently been found in a clinical *Y. pestis* isolate (27). This 150-kb plasmid has an Inc6-C origin of replication (unlike pFra and pHCM2). Although there are no obvious mobilization genes on the pFra/pMT1 plasmid, cointegration (19) with another conjugative plasmid (27) or an *ori-T*-independent method of transfer, such as that mediated by the Constin element SXT from *Vibrio cholerae* (36), remain possibilities. The presence of endogenous transducing phages in *Y. pestis* has not been demonstrated (55), but phage genes are represented on the common regions of pFra and pHCM2. Both *Y. pestis* and *S. enterica* serovar Typhi are among the seven species not known to be naturally competent which contain homologues of the HP0333 gene involved in natural transformation in *Helicobacter pylori* (3). They may have been naturally competent in the past or require specific conditions to show this property. Recently it has been shown that genes on another nonconjugative plasmid (*E. coli* pO157) may be transferred from *E. coli* to other enteric bacteria via membrane vesicles (77). This has not been shown for *Salmonella* or *Yersinia* species.

Whatever the mode or direction of transfer, extensive recombination with chromosomal or other plasmid DNA has occurred in at least one of the new hosts after the transfer event to achieve the sharp delineations observed between plasmid regions common to both replicons and those unique to each plasmid (Fig. 3). Of the two plasmids, pFra shows more mosaicism, evidenced by variability in G+C composition and Chi sequence distribution, suggesting that it has recombined more than pHCM2, probably due to the presence of multiple insertion sequences. This implies that pFra originally resembled pHCM2 even more closely than it does now. The G+C composition of the regions common to both plasmids is much closer to the *S. enterica* serovar Typhi chromosomal background than that of *Y. pestis*, and insertion sequences present in pFra are found elsewhere in *Y. pestis* and not in pHCM2 or the *S. enterica* serovar Typhi chromosome, suggesting that any direct transfer was from *S. enterica* serovar Typhi to *Y. pestis* or *Y. pseudotuberculosis* and not in the other direction. Experiments are planned to search for conjugative plasmids related to pFra and pHCM2 in other *S. typhi* strains. The source for the virulence-related and lifestyle-specific genes on the unique portions of *Y. pestis* pFra remains obscure, but the G+C content and other sequence features (lack of Chi sequences) suggest an origin outside *Enterobacteriaceae*.

Date of plasmid transfer. The opportunities for direct contact between *S. enterica* serovar Typhi and *Y. pestis* are limited. Host adaptation limits typhoid fever to humans and a few higher primates (39). Although *Y. pestis* is invasive for epithelial cells in vitro (17) and by the oral route in animal models

(14), it does not colonize the gut (14) and has a restricted capacity for survival in the environment (55). This would confine contact between the two organisms to a dually infected human host or the gut of a flea vector which had fed on multiple hosts. *Y. pseudotuberculosis* however, is, a gastrointestinal pathogen with a wide host range likely to bring it into contact with *Salmonella* species in the environment or vertebrate gut. Like *Y. pestis*, *S. enterica* serovar Typhi is homogenous in population genetic analysis (65), forming only two clones. However, unlike the *Y. pestis* relationship with *Y. pseudotuberculosis*, phylogenetic analysis does not place it as a typical subclone of another *S. enterica* serovar (7, 65)—there are distinct serovar Typhi-specific polymorphisms not falling within the allelic variation seen within other currently identifiable *Salmonella* serovars. The very recent origin proposed for the *Y. pestis* clone (1,500 to 20,000 years ago) (1) is not therefore applicable to *S. enterica* serovar Typhi, and it is likely to have been in existence before *Y. pestis*.

The presence of most of the pFra biovar-specific deletion in the cryptic *S. enterica* serovar Typhi plasmid pHCM2 suggests that horizontal transfer of the plasmid occurred before emergence of the Orientalis biovar or involved a non-Orientalis biovar, which would be unusual in Vietnam. Plague arrived in Vietnam during the third pandemic in 1898, and most current isolates reported are *Y. pestis* bv. Orientalis, although some *Y. pestis* bv. Medievalis strains have been reported from the High-Plateaux (T. Mai, A. Guiyoule, D. Vinh, D. Tuyet, D. Thung, and E. Carniel, Ned. Tidjschr. Med. Microbiol. 6, Suppl. II, Abstr. P-70, 1998). The simplest route for direct plasmid transfer would be between an ancestral *Y. pseudotuberculosis* strain that gave rise to *Y. pestis* and an *S. enterica* serovar Typhi strain. The degree of identity retained by the plasmids is compatible with the hypothesis (1) that pFra plasmid acquisition and *Y. pestis* emergence could have been as recent as 2,000 years ago.

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