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Divergence among Genes Encoding the Elongation Factor Tu of *Yersinia* Species▽

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Elongation factor Tu (EF-Tu), encoded by *tuf* genes, carries aminoacyl-tRNA to the ribosome during protein synthesis. Duplicated *tuf* genes (*tufA* and *tufB*), which are commonly found in enterobacterial species, usually coevolve via gene conversion and are very similar to one another. However, sequence analysis of *tuf* genes in our laboratory has revealed highly divergent copies in 72 strains spanning the genus *Yersinia* (representing 12 *Yersinia* species). The levels of intragenomic divergence between *tufA* and *tufB* sequences ranged from 8.3 to 16.2% for the genus *Yersinia*, which is significantly greater than the 0.0 to 3.6% divergence observed for other enterobacterial genera. We further explored *tuf* gene evolution in *Yersinia* and other *Enterobacteriaceae* by performing directed sequencing and phylogenetic analyses. Phylogenetic trees constructed using concatenated *tufA* and *tufB* sequences revealed a monophyletic genus *Yersinia* in the family *Enterobacteriaceae*. Moreover, *Yersinia* strains form clades within the genus that mostly correlate with their phenotypic and genetic classifications. These genetic analyses revealed an unusual divergence between *Yersinia tufA* and *tufB* sequences, a feature unique among sequenced *Enterobacteriaceae* and indicative of a genus-wide loss of gene conversion. Furthermore, they provided valuable phylogenetic information for possible reclassification and identification of *Yersinia* species.

The genus *Yersinia*, a member of the family *Enterobacteriaceae*, is composed of 14 known species: *Y. aldovae*, *Y. aleksiciae*, *Y. bercovieri*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. massiliensis*, *Y. mollaretii*, *Y. pestis*, *Y. pseudotuberculosis*, *Y. rohdei*, *Y. ruckeri*, and *Y. similis* (45, 49–51). Three of these species are important human pathogens. *Y. pestis* is the etiologic agent of plague, while *Y. enterocolitica* and *Y. pseudotuberculosis* usually cause self-limiting food-borne illnesses.

Interspecies and intraspecies genetic studies of relationships of yersiniae based on DNA-DNA hybridization and sequencing of housekeeping genes have provided additional information not encompassed by the current classification, which is based mainly on biochemical phenotypes (14, 16, 17, 36). Thus, *Y. enterocolitica*, *Y. frederiksenii*, and *Y. kristensenii* show more genetic diversity than other species in the genus (17, 36, 46). Indeed, more divergent strains that form their own clades could represent novel *Yersinia* species. Neubauer and colleagues (46) characterized two subspecies of *Y. enterocolitica*, *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica*, that have distinctive 16S rRNA sequences. Sprague and Neubauer (49) recently proposed a novel species, *Y. aleksiciae*, which was differentiated from *Y. kristensenii* based on a comparison of a region of the 16S rRNA gene and by the presence of lysine decarboxylase activity. On the other hand, based on DNA-DNA hybridization, *Y. pestis* and *Y. pseudotuberculosis* are a single genomospecies, even though they are classified as two distinct nomenspecies (6). Previous analyses revealed that *Y. pestis* emerged within the last 9,000 to 40,000 years from a *Y. pseudotuberculosis* predecessor (2, 48a). Although *Y. pestis* and *Y. pseudotuberculosis* are members of the same genomospecies, the medical implications for these organisms preclude establishment of a new nomenclature, which could endanger laboratory workers and general public health (30, 31, 58, 60). Finally, *Y. ruckeri* is the most distant *Yersinia* species, and its inclusion in the genus is still debated (28, 36, 51). Clearly, further genetic studies of the genus *Yersinia* are required in order to clarify the phylogeny and reinforce the taxonomy of this group.

Previous studies using 16S rRNA gene sequences exposed this method's limited ability to resolve the evolutionary history of *Yersinia* species (36). The high level of sequence conservation among 16S rRNA genes in some bacteria and the presence of multiple copies with sequence variations in many other bacteria limit the use of these genes for taxonomic resolution of closely related microorganisms (12, 13). Sequence analysis of the *tuf* gene has been proven to be valuable for accurate evaluation of genetic relationships among closely related microorganisms, such as members of the genus *Enterococcus* and the family *Enterobacteriaceae* (32, 47). Elongation factor Tu (EF-Tu), encoded by *tuf* genes, carries aminoacyl-tRNA to the

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TABLE 1. *Yersinia* strains used in this study[a]

Species or subspecies	Strain	Abbreviation	Isolation			Accession no.	
			Country	Source	Year	*tufA*	*tufB*
Y. aldovae	ATCC 35236^T	Yad1	Czechoslovakia	Drinking water	NA	EF113985	EF114034
	ATCC 35237	Yad2	Norway	Fish	NA	EF113986	EF114035
	CCUG 26532	Yad3	Germany	Feces	1983	EF113987	EF114036
	CCUG 26915	Yad4	Germany	Human feces	NA	EF113988	EF114037
Y. aleksiciae	LMG 22254^T	Yak1	Finland	Human feces	NA	EF113989	EF114038
Y. bercovieri	88–0762	Yb1	NA	NA	NA	EF113990	EF114039
	ATCC 43970^T	Yb2	France	Human feces	NA	EF113991	EF114040
	CCUG 26330	Yb3	France	Food	NA	EF113992	EF114041
	CCUG 26539	Yb4	Germany	Feces	1986	EF113993	EF114042
Y. enterocolitica subsp. *enterocolitica*	8081	Ye1	United States	Septicemia	NA	AM286415[b]	AM286415[b]
	ATCC 9610^T	Ye2	United States	Human tissue	NA	EF113994	EF114043
	ATCC 23715	Ye3	United States	Human blood	1968	EF113995	EF114044
	ATCC 27729	Ye4	Belgium	Human blood	1972	EF113996	EF114045
	CCUG 8238	Ye5	United States	Human	NA	EF113998	EF114047
Y. enterocolitica subsp. *palearctica*	CCRI-905	Ye6	Canada	Human blood	NA	EU566877	EU566908
	CCRI-952	Ye7	Canada	Human feces	NA	EU566878	EU566909
	CCRI-1044	Ye8	Canada	Human blood	NA	EU566875	EU566906
	CCRI-1139	Ye9	Canada	Human blood	NA	EU566876	EU566907
	CCRI-9984	Ye10	Canada	Human feces	NA	EU566879	EU566910
	CCRI-10035	Ye11	Canada	Human feces	NA	EU566872	EU566903
	CCRI-10046	Ye12	Canada	Human feces	NA	EU566873	EU566904
	CCRI-10098	Ye13	Canada	Human feces	NA	EU566874	EU566905
	CCRI-10461	Ye14	Canada	Human feces	1976	EU566881	EU566912
	CCRI-10462	Ye15	Canada	Human feces	1976	EU566882	EU566913
	CCRI-10463	Ye16	Canada	Human feces	1976	EU566883	EU566914
	CCRI-10464	Ye17	Canada	Human feces	1976	EU566884	EU566915
	CCRI-10465	Ye18	Canada	Human feces	NA	EU566885	EU566916
	CCRI-10603	Ye19	Finland	Human feces	1981	EU566886	EU566917
	CCUG 4586	Ye20	Sweden	Human MLN	1963	EF113997	EF114046
	CCUG 18381	Ye21	Sweden	Human feces	1986	EF113999	EF114048
	CCUG 21476	Ye22	Sweden	Human blood	1987	EF114000	EF114049
	CCUG 31436	Ye23	France	Bovine stools	NA	EF114001	EF114050
	CCUG 33553	Ye24	Denmark	Chinchilla	1960	EF114002	EF114051
	CCUG 46041	Ye25	Sweden	Human blood	2002	EU566880	EU566911
Y. frederiksenii	ATCC 29912	Yf1	Belgium	Human	NA	EF114003	EF114052
	ATCC 33641^T	Yf2	Denmark	Sewage	NA	EF114004	EF114053
	CCUG 8246	Yf3	NA	Human	NA	EF114005	EF114054
	CCUG 26594	Yf4	Norway	Forced pork	1983	EF114006	EF114055
	CCUG 26949	Yf5	Sweden	Human feces	1990	EF114007	EF114056
	CCUG 30114	Yf6	Sweden	Human feces	1992	EF114008	EF114057
	ER 5307	Yf7	NA	NA	NA	EF114009	EF114058
Y. intermedia	ATCC 29909^T	Yi1	NA	Human urine	NA	EF114010	EF114059
	ATCC 33647	Yi2	NA	Human feces	NA	EF114011	EF114060
	ATCC 33648	Yi3	NA	Human urine	NA	EF114012	EF114061
Y. kristensenii	ATCC 33638^T	Yk1	NA	Human urine	NA	EF114013	EF114062
	CCUG 26582	Yk2	Germany	Sewage	1980	EF114014	EF114063
	CCUG 26589	Yk3	Norway	Forced meat	1983	EF114015	EF114064
	CCUG 46842	Yk4	Sweden	NA	2002	EF114016	EF114065
Y. mollaretii	ATCC 43969^T	Ym1	United States	Soil	NA	EF114017	EF114066
	CCUG 26536	Ym2	Germany	Human feces	1984	EF114018	EF114067
	ER 4215	Ym3	NA	NA	NA	EF114019	EF114068
Y. pestis	91001	Ype1	NA	NA	NA	AE017042[b]	AE017042[b]
	Antiqua	Ype2	NA	NA	NA	CP000308[b]	CP000308[b]
	CO92	Ype3	United States	Human	NA	AL590842[b]	AL590842[b]
	KIM	Ype4	NA	Human	NA	AE009952[b]	AE009952[b]

Continued on following page

TABLE 1—*Continued*

Species or subspecies	Strain	Abbreviation	Isolation			Accession no.	
			Country	Source	Year	*tufA*	*tufB*
Y. pseudotuberculosis	ATCC 13979	Yps1	Sweden	Hare	1952	EF114020	EF114069
	ATCC 27802	Yps2	Denmark	Mink	1943	EF114021	EF114070
	ATCC29833^T	Yps3	Turkey	NA	1952	EF114022	EF114071
	CCUG 7803	Yps4	Sweden	Humanblood	1978	EU566890	EU566921
	CCUG 17342	Yps5	Sweden	Hare	1951	EU566887	EU566919
	CCUG 17345	Yps6	Japan	Guinea pig	1951	EU566888	EU566920
	CCUG 37903	Yps7	NA	NA	1997	EU566888	EU566918
	ER 5271	Yps8	NA	NA	NA	EU566891	EU566922
	IP 32953	Yps9	NA	NA	NA	BX936398[b]	BX936398[b]
Y. rohdei	ATCC43380^T	Yro1	Germany	Dog feces	NA	EF114023	EF114072
	ATCC 43871	Yro2	Germany	Dog feces	NA	EF114024	EF114073
	ATCC 43873	Yro3	UnitedStates	Human feces	NA	EF114025	EF114074
	CCUG 26544	Yro4	Germany	Human feces	1978	EF114026	EF114075
	CCUG 26545	Yro5	Germany	Human feces	1989	EF114027	EF114076
Y. ruckeri	ATCC29473^T	Yru1	UnitedStates	Rainbow trout	NA	EF114028	EF114077
	CCRI-10643	Yru2	UnitedStates	Rainbow trout	NA	EF114029	EF114078
	CCUG 21537	Yru3	NA	NA	NA	EF114030	EF114079

[a] T = type strain. Abbreviations are those used in Fig. 2. NA, not available; MLN, mesenteric lymph node.
[b] Sequences retrieved from completed genome sequences.

programmed ribosome during protein synthesis. The synteny of gammaproteobacterial *tuf* gene regions is conserved. Most enterobacterial genomes possess two copies of the *tuf* gene, *tufA* and *tufB*, in distinct operons, designated *str* and tRNA-*tufB*, respectively (20, 39). The *fusA* gene, which encodes elongation factor G, is located upstream of *tufA* in the *str* operon of gammaproteobacteria (35, 39). Four tRNA structural genes are located upstream of *tufB* in the tRNA-*tufB* operon (20), and the *secE* and *nusG* genes are downstream of *tufB* in most *Enterobacteriaceae* (20, 35, 39). The *tufA* and *tufB* genes appear to evolve in concert through gene conversion events that maintain the sequence homology (1). A previous analysis of duplicated bacterial *tuf* genes revealed identical or very similar nucleic acid sequences that differ by less than 1.4% (39). In contrast, the data presented here show that the *tufA* and *tufB* gene copies in 12 species of the genus *Yersinia* have a remarkable level of variability (up to 16%). Chain and colleagues have observed a similar level of divergence in *Y. pestis* and *Y. pseudotuberculosis* (10). In the current work, we performed an in-depth study of *tuf* gene sequence variation in the genus *Yersinia*. The data obtained were juxtaposed with data for closely related members of the *Enterobacteriaceae*, and mechanisms for the remarkable diversity were examined. As described here, the divergence among the *Yersinia* intragenomic *tuf* gene sequences appears to be unique among sequenced *Enterobacteriaceae*, as demonstrated by phylogenetic analyses.

MATERIALS AND METHODS

Bacterial strains. Sixty-six *Yersinia* strains (Table 1) and 14 *Enterobacteriaceae* strains (*Enterobacter agglomerans* ATCC 27989, *Escherichia vulneris* ATCC 33821, *Hafnia alvei* ATCC 13337, ATCC 25927, ATCC 51873, CCRI-10616, CCRI-11829, and CCRI-16651, *Klebsiella pneumoniae* ATCC 13883, *Obesumbacterium proteus* ATCC 12841, *Plesiomonas shigelloides* ATCC 14029, *Serratia fonticola* DSM 4576, *Serratia marcescens* ATCC 13880, and *Yokenella regensburgei* ATCC 35313) were obtained from the American Type Culture Collection (Manassas, VA) (ATCC), the Belgian Coordinated Collections of Microorganisms (Ghent, Belgium) (LMG 22254), the Collection du Centre de Recherche en Infectiologie (Québec, Canada) (CCRI) (http://wdcm.nig.ac.jp/CCINFO/CCINFO.xml?861), the Culture Collection of the University of Göteborg (Göteborg, Sweden) (CCUG), the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) (DSM 4576), and the Public Health Agency of Canada (Winnipeg, Canada) (ER 5307, ER 4215, ER, 5271, and 88-0762). All strains were grown overnight at 30 or 37°C under aerobic conditions on Trypticase soy agar with 5% sheep blood.

DNA isolation. Bacterial DNA was isolated from mid-log-phase cultures of selected strains by using a BioSprint 15 DNA blood kit (Qiagen, Mississauga, Ontario, Canada) automated with a KingFisher mL instrument (Thermo Fisher Scientific, Waltham, MA). Alternatively, a GNOME DNA kit (Qbiogene Inc., Carlsbad, CA) was used (32).

Primer design. The organization of the genes surrounding the *tufA* and *tufB* genes was used to design primers for specific amplification of *tufA* and *tufB*. The bacterial *fusA* or gammaproteobacterial and *Deinococcus-Thermus nusG* gene sequences in public databases were assembled in a multiple alignment using the CLUSTAL W software (version 1.83) (11, 55). Conserved regions were identified at the 3′ end of the *fusA* gene and at the 5′ end of the *nusG* gene. The *fusA* primer (primer F1; 5′-GTICCIYTIKCIGARATGTTYGGITAYGC-3′; positions 1972 to 2000 in the *Escherichia coli* K-12 GenBank accession number U00096 sequence) was combined with a previously designed universal primer for the terminal region of *tuf* (primer T2; 5′-CCIACIGTICKICCRCCYTCRCG-3′; positions 1132 to 1154 in the *E. coli* K-12 sequence) (47) to amplify *tufA* sequences. The *nusG* primer (primer N1; 5′-AACGCCTGRACGACRTACCA-3′; positions 25 to 44 in the *E. coli* K-12 sequence) was used in combination with a second universal primer for the 5′ region of *tuf* (primer T1; 5′-AAYATGATIA CIGGIGCIGCICARATGGA-3′; positions 271 to 299 in the *E. coli* K-12 sequence) (47) to amplify *tufB* sequences.

Sequencing of *tufA* and *tufB* genes. A 1,369-bp fragment of the *str* operon (*tufA*) was amplified using the F1 and T2 primers. The N1 and T1 primers were used for specific amplification of a 1,594-bp fragment of the *tufB* region. The PCR mixtures contained primers F1 and T2 or primers T1 and N1 (each at a concentration of 1.0 μM), 200 μM deoxyribonucleoside triphosphates (Amersham Biosciences, Piscataway, NJ), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 3.3 μg/μl bovine serum albumin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), 0.06 μg/μl methoxsalen (Sigma-Aldrich Canada Ltd.), and 0.025 U/μl *Taq* DNA polymerase (Promega, Madison, WI) combined with the TaqStart antibody (Clontech Takara Bio, Mountain View, CA) (33). Decontamination of the PCR mixtures prior to PCR was performed by using a Spectrolinker model XL-1000 UV cross-linker (Spectronics Corporation, Westbury, NY). Purified genomic DNA (3 ng) was added to each PCR mixture, which was subjected to thermal cycling with a PTC-200 DNA Engine thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) as follows: 5 min at 94°C, followed by 40 cycles of 1 s at 95°C, 1 min at 60°C (for *tufA*) or 58°C (for *tufB*), and 1 min at 72°C and then a final extension for 7 min at 72°C. Amplicons were

TABLE 2. *tuf* sequence variations in *Yersinia*[a]

Species	*n*	Intragenomic variation		Intraspecies variation			
				Nucleic acid identity (%)		Amino acid similarity (%)	
		Nucleic acid identity (%) of *tufA* and *tufB*	Amino acid similarity (%) of EF-TuA and EF-TuB	*tufA*	*tufB*	EF-TuA	EF-TuB
Y. aldovae	4	87.4–87.7	96.1	99.9–100.0	98.8–100.0	100.0	100.0
Y. aleksiciae	1	83.8	96.1	NA	NA	NA	NA
Y. bercovieri	4	83.9	95.7–96.1	99.6–100.0	99.9–100.0	100.0	99.6–100.0
Y. enterocolitica	25	90.5–91.7	96.1–98.1	93.9–100.0	97.7–100.0	97.7–100.0	100.0
Y. frederiksenii	7	86.0–87.8	95.3–95.7	92.5–100.0	92.0–100.0	96.9–100.0	99.6–100.0
Y. intermedia	3	84.7–85.2	94.9–95.3	99.7–99.9	98.7–99.6	100.0	99.6–100.0
Y. kristensenii	4	88.8–89.9	96.1	100.0	96.9–100.0	100.0	100.0
Y. mollaretii	3	84.7–85.1	94.9–95.7	96.9–99.7	99.9–100.0	98.8–100.0	100.0
Y. pestis	4	85.7–85.9	96.5	100.0	99.9–100.0	100.0	98.8–100.0
Y. pseudotuberculosis	9	85.9–86.0	96.5	99.7–100.0	97.5–100.0	100.0	99.6–100.0
Y. rohdei	5	84.7–85.1	96.1	99.7–100.0	99.2–100.0	100.0	100.0
Y. ruckeri	3	85.2	94.9	100.0	100.0	100.0	100.0
Yersinia	72	83.8–91.7	94.9–98.1	92.5–100.0	92.0–100.0	96.9–100.0	98.8–100.0
H. alvei	6	96.4–97.0	97.7–98.8	98.4–100.0	95.3–100.0	100.0	98.4–100.0
O. proteus	1	97.1	97.3	NA	NA	NA	NA
Other *Enterobacteriaceae*	12	98.8–100.0	99.6–100.0	NA	NA	NA	NA
V. cholerae (outgroup)	1	99.1	99.6	NA	NA	NA	NA

[a] *n*, number of strains; NA, not applicable.

detected using 1.2% agarose gel electrophoresis with 0.25 μg/ml of ethidium bromide in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Amplicon sizes were verified with a 1-kb DNA ladder used as a molecular weight marker (Invitrogen, Carlsbad, CA). During all of these steps, precautions were taken to avoid carryover of amplified DNA, as previously described (42). Amplification products were purified using gel electrophoresis (1.2% agarose at 120 V for 1 h), followed by staining with methylene blue (Mallinckrodt Baker, Inc., Phillipsburg, NJ) and DNA purification with a QIAquick gel extraction kit (Qiagen), as previously described (32). Both strands of each amplicon were sequenced using an automated ABI sequencer (Applied Biosystems, Foster City, CA) with 5 μM sequencing primer (primer F1, T1, or T2 for *tufA* and primer T1 or T2 for *tufB*). Chromatogram assembly and analysis were performed using the Sequencher 3.1 software (Gene Codes Corp., Ann Arbor, MI).

Phylogenetic analyses. In order to investigate *tuf* genetic variability within the genus *Yersinia*, 72 strains were chosen to represent 12 species; greater proportions of strains of known genetically diverse species were used (Table 1). Representative *Enterobacteriaceae* other than *Yersinia* were selected based on their close relationships at the phylogenetic level as determined by 16S rRNA gene sequence analysis (28, 47). *Vibrio cholerae* N16961 was used as a nonenterobacterial outgroup. The *tuf* gene sequences of *Erwinia carotovora* subsp. *atroseptica* SCRI1043 (accession number BX950851), *E. coli* K-12 (accession number U00096), *Photorhabdus luminescens* subsp. *laumondii* TTO1 (accession number BX470251), *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain LT2 (accession number AE006468), *Shigella flexneri* 2a 2457T (accession number AE014073), *V. cholerae* O1 biovar El Tor strain N16961 (accession number AE003852), *Y. enterocolitica* subsp. *enterocolitica* 8081, *Y. pestis* strains 91001, Antiqua, CO92, and KIM, and *Y. pseudotuberculosis* strain IP 32953 (Table 1) were retrieved from complete genome sequences available from the GenBank database (2, 4, 9, 10, 18, 23, 40, 44, 55a, 59, 61). A multiple-sequence alignment of the newly generated *tuf* sequences and sequences from the GenBank database was constructed using the CLUSTAL W software. The alignment was verified by visual inspection using the SeqLab editor (GCG Wisconsin Package, version 10.3; Accelrys Software Inc., San Diego, CA). A 771-bp region of the *tufA* and *tufB* genes (positions 316 to 1086 in *Y. pestis* CO92) was analyzed. The nucleic acid sequences were translated into amino acid sequences (residues 105 to 361) using the Translate function of the GCG Wisconsin Package and then inspected with the SeqLab editor to identify variations in residues between *Yersinia* EF-TuA (encoded by *tufA*) and EF-TuB (encoded by *tufB*). Levels of identity between *tufA* and *tufB* nucleic acid sequences and levels of similarity between the gene products were calculated with the GAP function of the GCG Wisconsin Package (BLOSUM62 amino acid substitution matrix) (Tables 2 and 3) (24). Phylogenetic analyses of comparative (see Fig. 1A) and concatenated (see Fig. 1B) *tufA* and *tufB* nucleic acid sequences were performed with the MEGA4 software (52) in order to compute evolutionary distances using the maximum composite likelihood method (52, 54). The differences in composition bias among sequences were considered in evolutionary comparisons (53). One thousand bootstrap analyses were performed to estimate the robustness of the phylogenetic inference (19). A split network was computed using SplitsTree 4.8 for Unix, and genetic distances were calculated using the uncorrected P method (27). A split network was also constructed using the Neighbor-Net method (7) and EqualAngle split transformation settings.

Nucleotide sequence accession numbers. Partial *tufA* and *tufB* gene sequences have been deposited in the GenBank database under the following accession numbers: *Yersinia* strains, EF113985 to EF114030, EU566872 to EU566891, EF114034 to EF114079, and EU566903 to EU566922 (Table 1); *E. agglomerans* ATCC 27989, EU566892 and EU566923; *E. vulneris* ATCC 33821, EU566893 and EU566924; *H. alvei* ATCC 13337, ATCC 25927, ATCC 51873, CCRI-10616, CCRI-11829, and CCRI-16651, EF114031, EU566894 to EU566898, EF114080, and EU566925 to EU566929; *K. pneumoniae* ATCC 13883, EF114032 and EF114081; *O. proteus* ATCC 12841, EU566899 and EU566930; *P. shigelloides* ATCC 14029, EU566900 and EU566931; *S. fonticola* DSM 4576, EU566901 and EU566932; *S. marcescens* ATCC 13880, EF114033 and EF114082; and *Y. regensburgei* ATCC 35313, EU566902 and EU566933.

RESULTS

***tufA* and *tufB* gene similarities.** All 72 *Yersinia* strains analyzed in this study possessed two divergent copies of the *tuf* gene, *tufA* and *tufB*. The levels of intragenomic identity for *tufA* and *tufB* nucleic acid sequences varied from 83.8 to 91.7% for the 771-bp region studied (Table 2). In contrast, 12 *Enterobacteriaceae* strains (*E. agglomerans* ATCC 27989, *E. carotovora* SCRI1043, *E. coli* K-12, *E. vulneris* ATCC 33821, *K. pneumoniae* ATCC 13883, *P. luminescens* TTO1, *P. shigelloides* ATCC 14029, *S. enterica* subsp. *enterica* serovar Typhimurium LT2, *S. fonticola* DSM 4576, *S. marcescens* ATCC 13880, *S. flexneri* 2457T, and *Y. regensburgei* ATCC 35313), as well as a *Vibrionaceae* strain (*V. cholerae* N16961), had levels of intragenomic *tufA* and *tufB* identity ranging from 98.8 to 100.0% (Table 2). Interestingly, all six strains of *H. alvei* (ATCC 13337, ATCC 25927, ATCC 51873, CCRI-10616, CCRI-11829, and

TABLE 3. Interspecies variations of *tuf* sequences in *Yersinia*

Species (no. of strains)	% *tuf* identity (% EF-Tu similarity)[a]					
	Y. aldovae	*Y. aleksiciae*	*Y. bercovieri*	*Y. enterocolitica*	*Y. frederiksenii*	*Y. intermedia*
Y. aldovae (4)		95.7–95.9 (100.0)	95.3–95.6 (100.0)	91.8–93.9 (97.3–97.7)	92.1–95.7 (96.9–100.0)	96.1–96.4 (99.6)
Y. aleksiciae (1)	84.7–85.2 (97.3)		99.4–99.5 (100.0)	92.6–94.6 (97.3–97.7)	93.8–97.8 (96.9–100.0)	95.9–96.0 (99.6)
Y. bercovieri (4)	85.2–85.6 (96.9–97.3)	94.8–94.9 (99.6–100.0)		92.1–94.2 (97.3–97.7)	93.3–97.8 (96.9–100.0)	95.5–95.7 (99.6)
Y. enterocolitica (25)	92.3–93.4 (100.0)	84.7–85.1 (97.3)	85.5–85.9 (96.9–97.3)		91.6–95.7 (97.3–99.2)	91.6–93.7 (96.9–98.1)
Y. frederiksenii (7)	86.3–87.7 (97.3–97.7)	86.1–87.8 (99.2–99.6)	86.5–87.7 (98.8–99.6)	87.9–88.8 (97.3–97.7)		92.1–95.7 (96.5–99.6)
Y. intermedia (3)	85.2–86.3 (97.3–97.7)	85.0–85.2 (99.2–99.6)	83.9–84.4 (98.8–99.6)	85.9–87.0 (97.3–97.7)	89.1–90.8 (99.2–100.0)	
Y. kristensenii (4)	90.8–92.5 (100.0)	83.9–84.7 (97.3)	83.7–84.6 (96.9–97.3)	92.1–94.7 (100.0)	85.9–87.5 (97.3–97.7)	84.6–86.0 (97.3–97.7)
Y. mollaretii (3)	87.4–87.9 (96.9)	87.8 (98.4)	87.7–87.8 (98.1–98.4)	88.8–89.4 (98.8)	85.0–86.6 (98.4–98.8)	84.8–85.0 (98.4–98.8)
Y. pestis (5)	83.7–84.2 (96.9)	86.0–86.1 (98.8)	85.5–85.7 (98.4–98.8)	83.4–84.6 (96.9)	86.6–86.9 (99.2–99.6)	85.6–86.0 (98.8–99.2)
Y. pseudotuberculosis (4)	83.9–84.8 (96.9)	86.0–86.3 (98.4–98.8)	84.8–85.9 (98.1–98.8)	83.5–84.8 (96.9)	86.5–87.7 (98.8–99.6)	85.5–86.3 (98.4–99.2)
Y. rohdei (5)	86.6–87.3 (97.3)	86.6–87.2 (99.2)	87.2–87.8 (98.8–99.2)	86.5–87.2 (97.3)	90.7–91.8 (99.6–100.0)	88.6–89.1 (99.2–99.6)
Y. ruckeri (3)	84.8–85.1 (97.3)	83.5 (98.4)	82.4–82.5 (98.4)	83.9–84.2 (97.3)	85.2–85.6 (98.4–98.8)	83.9–84.8 (98.4–98.8)

[a] The data above the diagonal are for *tufA* and EF-TuA sequences, and the data below the diagonal are for *tufB* and EF-TuB sequences.

CCRI-16651) and *O. proteus* ATCC 12841 exhibited intermediate levels of identity for *tufA* and *tufB*, which ranged from 96.4 to 97.1%; this distinguished these strains from both *Yersinia* and other genera of the *Enterobacteriaceae*. The levels of intragenomic amino acid similarity for EF-TuA and EF-TuB exhibited a similar pattern and ranged from 94.9 to 98.1% for *Yersinia* strains, from 97.3 to 98.3% for *H. alvei* and *O. proteus* strains, and from 99.6 to 100% for other *Enterobacteriaceae* strains, as well as *V. cholerae* N16961 (Table 2). Amino acid sequence similarities for all EF-TuA and EF-TuB sequences from strains of *Yersinia*, strains of other *Enterobacteriaceae*, and *Vibrionaceae* strains were also examined (residues 105 to 361). Amino acid residues in functionally important sites are conserved in *Yersinia* (38). However, two amino acid positions that can distinguish all *Yersinia tufA* sequences from all *tufB* sequences were found (at position 167, Ile or Leu in EF-TuA and Thr in EF-TuB; at position 361, Thr in EF-TuA and Asn in EF-TuB). Therefore, these residues can be used as signature residues to discriminate between the *tufA* and *tufB* genes in *Yersinia* strains. Residue 167 is different (Ile, Leu, or Thr) in the other genera of the *Enterobacteriaceae* studied, and therefore the difference between EF-TuA and EF-TuB reported here is not specific to the genus *Yersinia*. In contrast, EF-Tu residue 361 is conserved among most of the *Enterobacteriaceae* strains (Thr) investigated in this study; the exceptions are the residues in EF-TuB in the genera *Yersinia* (Asn), *Hafnia* (Asn or Ser), and *Obesumbacterium* (Asn).

The levels of interspecies similarity for *tufA* and *tufB* genes and their products are shown in Table 3. Most notably, when the EF-TuA amino acid sequences of *Y. rohdei* were compared to those of *Y. pestis* and *Y. pseudotuberculosis*, the levels of interspecies similarity were 100%, even though the levels of *tufA* nucleotide sequence identity were 95.9 to 96.4% (Table 3). Also, the level of EF-TuB amino acid sequence similarity for *Y. aldovae*, *Y. enterocolitica*, and *Y. kristensenii* was 100.0%, while the levels of *tufB* nucleic acid sequence identity ranged from 90.8 to 94.7%. These examples suggest that there is selective pressure for both the EF-TuA and EF-TuB proteins to maintain amino acid sequence stability.

Phylogenetic tree. A phylogenetic tree based on *tufA* and *tufB* nucleic acid sequences was constructed to study the evolution of orthologous and paralogous *tuf* genes in *Yersinia* compared to other genera in the family. Non-*Yersinia* enterobacterial reference strains were selected based on their relatively close relationships to the genus *Yersinia* at the phylogenetic level, based on 16S rRNA gene sequences. The paralogous genes of most of the non-*Yersinia* species are very similar and group together, forming an organismal *tufA-tufB* clade. On the other hand, the paralogous *tuf* genes of *Yersinia* strains showed the distinctive evolution of these strains (Fig. 1A). Based on the data obtained, it is clear that intragenomic *tufA* and *tufB* genes in *Yersinia* have diverged significantly. *H. alvei* and *O. proteus* are minor exceptions within the *Enterobacteriaceae*, because they form two other clades with separated *tufA* and *tufB* genes. The *Yersinia*, *H. alvei*, and *O. proteus tufB* interspecies distances (branch lengths) are approximately twice the interspecies distances for the *tufA* genes. The topology of the phylogenetic tree shows that *Yersinia tufA* gene sequences are monophyletic, while the *tufB* sequences are diphyletic. The *Yersinia tufA* clade branches with the *Y. aldovae-Y. enterocolitica-Y. kristensenii tufB* clade, which is separated from the *tufB* sequence cluster of other *Yersinia* species by other enterobacterial genera. However, this topology is not supported by the results of the bootstrap analysis.

Phylogenetic network. While phylogenetic trees describe evolutionary relationships based on mutational events, phylogenetic networks allow incorporation of more complex models of evolution, such as recombination and gene duplication (27). Because the evolution of *tuf* genes in *Enterobacteriaceae* was driven not only by mutation but also by gene conversion events, the Neighbor-Net method was used to visualize and analyze incompatible phylogenetic signals that are represented by edges (7, 34). *Yersinia tufA* and *tufB* sequences were analyzed by the Neighbor-Net method implemented in the SplitsTree software (Fig. 2). The Neighbor-Net analysis results support the clustering of *Yersinia tufA* and *tufB* sequences previously observed in the phylogenetic tree (Fig. 1A) and display the paralogous *tufA* and *tufB* genes in *Yersinia* as separate clusters linked by edges near the origin. In contrast, other paralogous enterobacterial *tufA* and *tufB* sequences are grouped and linked by edges at the extremities. This indicates that there was ancestral divergence of *Yersinia tufA* from *tufB*, while in other *Enterobacteriaceae* the *tufA* and *tufB* genes are still coevolving. The *H. alvei-O. proteus* group is an exception in which there are two separate *tufA* and *tufB* clusters, which have intermediate distances of the edges to the origin compared to *Yersinia* strains and the other *Enterobacteriaceae* strains. This suggests that there was more recent divergence compared to the genus

TABLE 3. —*Continued*

% *tuf* identity (% EF-Tu similarity)[a]					
Y. kristensenii	*Y. mollaretii*	*Y. pestis*	*Y. pseudotuberculosis*	*Y. rohdei*	*Y. ruckeri*
93.3–93.4 (97.7)	93.5–96.0 (98.8–100.0)	92.6–92.7 (97.7)	92.6–92.7 (97.7)	93.3–93.6 (97.7)	93.3–93.4 (97.7)
94.0 (97.7)	96.1–97.9 (98.8–100.0)	92.6 (97.7)	92.6 (97.7)	92.6–92.9 (97.7)	93.4 (97.7)
93.5–93.6 (97.7)	96.0–97.9 (98.8–100.0)	92.1–92.2 (97.7)	92.1–92.2 (97.7)	92.1–92.5 (97.7)	92.9–93.0 (97.7)
93.9–98.1 (97.7–100.0)	92.1–95.1 (97.3–98.4)	94.4–95.3 (98.1–99.6)	94.2–95.3 (98.1–99.6)	94.0–95.6 (98.1–99.6)	94.0–94.9 (98.1–98.8)
92.5–94.2 (97.3–97.7)	93.1–96.1 (96.9–100.0)	91.1–96.4 (97.7–99.6)	91.1–96.4 (97.7–99.6)	91.6–97.4 (97.7–99.6)	92.3–95.2 (97.7–98.8)
93.8–94.0 (98.1)	94.0–96.2 (98.4–99.6)	92.3–92.5 (97.3)	92.3–92.5 (97.3)	93.0–93.4 (97.3)	93.9–94.0 (97.3)
	93.6–94.4 (97.7–98.4)	94.0 (98.1)	93.8–94.0 (98.1)	94.0–94.3 (98.1)	94.3 (98.1)
86.8–87.9 (98.8)		92.9–94.0 (97.7–98.4)	92.9–94.0 (97.7–98.4)	92.7–93.6 (97.7–98.4)	93.8–94.7 (97.7–98.4)
83.7–85.0 (96.9)	83.4–83.5 (98.1)		99.7–100.0 (100.0)	96.1–96.4 (100.0)	95.7 (99.2)
83.7–85.2 (96.9)	83.7–84.0 (98.1)	97.5–99.9 (99.6–100.0)		95.9–96.4 (100.0)	95.5–95.7 (99.2)
84.3–85.5 (97.3)	85.1–85.5 (98.4)	85.1–85.6 (99.6)	85.3–85.9 (99.2–99.6)		96.6–96.9 (99.2)
83.8–84.4 (97.3)	81.3 (97.7)	84.0–84.2 (98.4)	84.2–84.6 (98.1–98.4)	85.2–85.3 (98.4)	

Yersinia. There is no evidence that *H. alvei* and *O. proteus tuf* paralogs are still coevolving.

***Yersinia* species.** The remarkable evolution of *tuf* genes in *Yersinia* has resulted in genetic variations that can be used to infer species clustering. Therefore, phylogenetic analyses of *tuf* genes in *Yersinia* provide information for reclassification and identification of *Yersinia* species. In order to enhance the number of sites analyzed simultaneously, we constructed a phylogenetic tree using concatenated *tufA* and *tufB* sequences (Fig. 1B). This tree showed the monophyletic nature of the genus *Yersinia*, its separation from other genera of the *Enterobacteriaceae*, and *Yersinia* species clustering, all of which were strongly supported by bootstrap analysis.

Based on the analysis of concatenated *tufA* and *tufB* sequences, *Y. aldovae*, *Y. aleksiciae*, *Y. bercovieri*, *Y. intermedia*, *Y. kristensenii*, *Y. mollaretii*, *Y. rohdei*, and *Y. ruckeri* strains form distinct clusters that correlate with the current species classification (Fig. 1B). *Y. aleksiciae* type strain LMG 22254 is clearly distinct from the *Y. kristensenii* strain cluster.

Nucleic acid sequences and tree topology showed the genotypic diversity of *Y. enterocolitica* (Fig. 1B). *Y. enterocolitica* concatenated *tufA* and *tufB* sequences form two distant clades supported by a high bootstrap value (99%). One clade includes 17 strains of *Y. enterocolitica* subsp. *palearctica*, which were designated group I (CCRI-905, CCRI-952, CCRI-1044, CCRI-1139, CCRI-9984, CCRI-10035, CCRI-10046, CCRI-10098, CCRI-10461, CCRI-10462, CCRI-10463, CCRI-10464, CCRI-10465, CCRI-10603, CCUG 4586, CCUG 21476, and CCUG 33553) and were isolated in Canada, Finland, Sweden, and Denmark (Table 1). The other clade contains two subgroups. One subgroup includes five *Y. enterocolitica* subsp. *enterocolitica* strains (8081, ATCC 9610, ATCC 23715, ATCC 27729, and CCUG 8238) isolated in the United States and Belgium, while the other subgroup contains two strains of *Y. enterocolitica* subsp. *palearctica* designated group II (CCUG 18381 and CCUG 31436) isolated in France and Sweden (46) (Table 1).

Y. frederiksenii, which also is known to be a genotypically heterogeneous species, consists of three concordant clades that correlate with three of the four previously characterized genomic groups (genomic groups 1a, 1b, and 3; no genomic group 2 reference strain was used in this study) (56). The intraspecies distances (branch length) of *Y. frederiksenii* ATCC 33641 (genomic group 1a type strain) from *Y. frederiksenii* CCUG 26594 (genomic group 1b) and *Y. frederiksenii* ER 5307 (unknown genomic group) are greater than those between strains of other *Yersinia* species. *Y. frederiksenii* strains ATCC 29912, CCUG 26949, and CCUG 30114 (all genomic group 3 strains) cluster with strain CCUG 8246 (unknown genomic group). The concatenated *tuf* gene tree clearly separates the genomic group 3 clade from genomic groups 1a and 1b. *Y. pestis* and *Y. pseudotuberculosis* strains cluster together based on the concatenated *tuf* gene tree and thus are presented as a unique genomospecies, as previously revealed by DNA-DNA hybridization analysis (6). Finally, although the taxonomy of *Y. ruckeri* is controversial, its *tuf* gene sequences cluster with those of other *Yersinia* species and support the conclusion that this species should be included in the genus (17, 28, 36, 51). In addition, the *tufA* and *tufB* genes of each *Y. ruckeri* strain exhibit a level of identity (85.2%) which is in the range observed for other *Yersinia* species (Table 2).

DISCUSSION

In this study, sequence analyses revealed that intragenomic *tufA* and *tufB* genes are divergent in 12 *Yersinia* species. In comparison, 12 non-*Yersinia* enterobacterial species investigated contained two intragenomic *tuf* genes which were very similar to one another. However, the intragenomic *tuf* sequences of the members of the *H. alvei-O. proteus* clade exhibited an intermediate level of divergence. The *tufA* and *tufB* genes have been described previously as genes evolving in concert through gene conversion events which maintain their remarkable level of nucleotide sequence identity (1, 3, 26, 39). Gene conversion driven by homologous recombination mechanisms explains the high levels of similarity usually observed for duplicated *tuf* genes of members of the *Enterobacteriaceae*, as shown in *S. enterica* serovar Typhimurium (1). Conversely, the remarkable divergence between the *tufA* and *tufB* genes in *Yersinia* strains may result from (i) the acquisition of an exogenous *tuf* gene by horizontal transfer, (ii) the gradual or spontaneous loss of effective conversion mechanisms (due either to defects in the mechanism or the level of dissimilarity of sequences), or (iii) either loss of function or neofunctionalization of one EF-Tu copy.

High levels of divergence (21 to 32%) of intragenomic *tuf* gene sequences have been observed previously for 11 enterococcal species, while six other enterococcal species contained only one *tuf* gene (32). Acquisition of an exogenous copy of the *tuf* gene by the ancestor of the 11 *Enterococcus* species having two *tuf* gene copies was the mechanism proposed to explain the

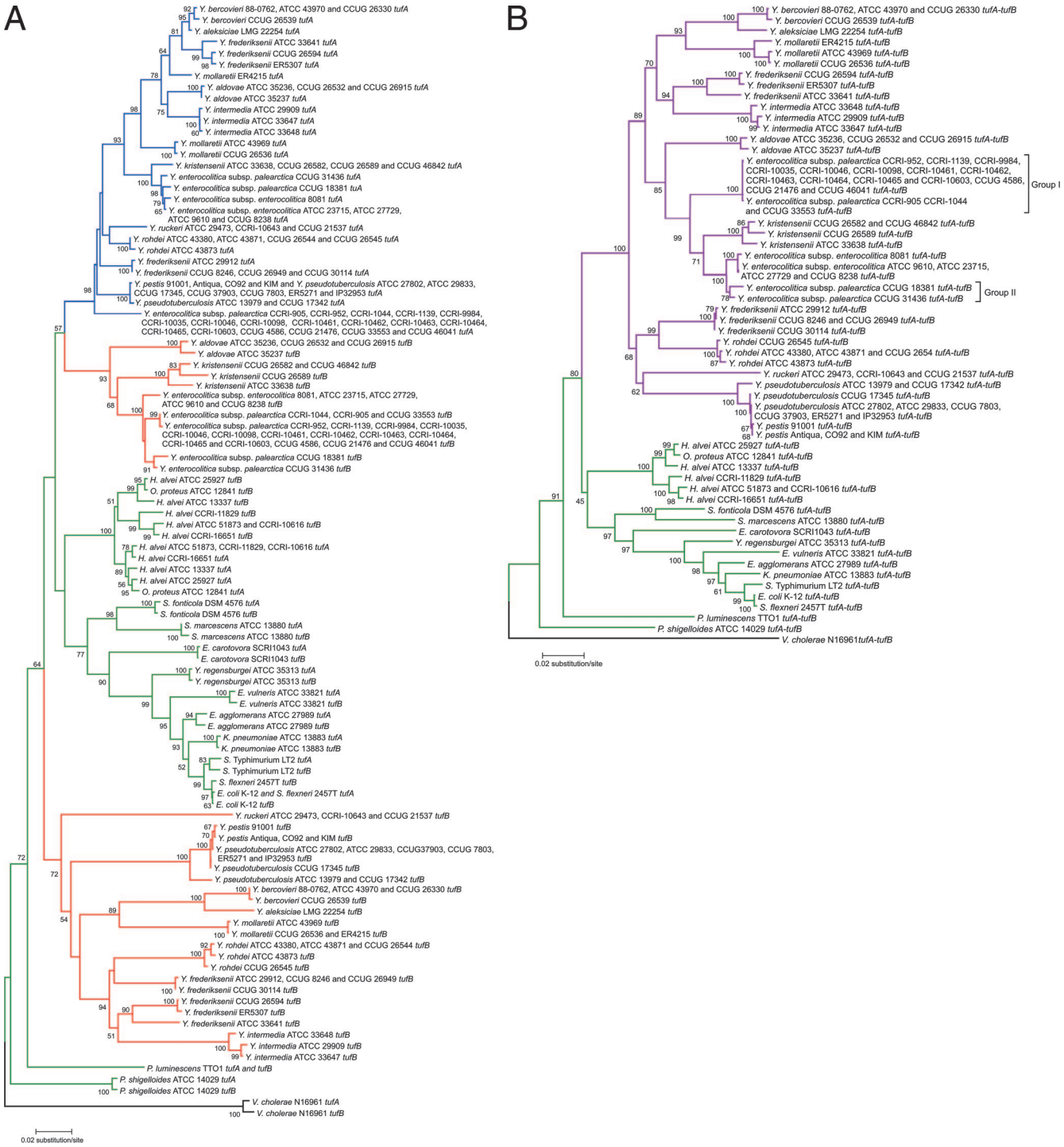

FIG. 1. Phylogenetic trees for *tufA* and *tufB* nucleic acid sequences from *Yersinia* and non-*Yersinia* enterobacterial strains. (A) Phylogenetic tree based on a comparison of *tufA* and *tufB* nucleic acid sequences. *Yersinia tufA* and *tufB* tree branches are blue and red, respectively. (B) Phylogenetic tree based on concatenated *tufA* and *tufB* nucleic acid sequences. *Yersinia* concatenated *tufA* and *tufB* tree branches are purple. The *tuf* gene branches for other enterobacterial species and *V. cholerae* (outgroup) are green and black, respectively. Evolutionary distances were computed using the maximum composite likelihood method of the MEGA4 software. The topological accuracy of the tree was evaluated using 1,000 bootstrap replicates.

presence of two different intragenomic *tuf* genes (32). In contrast, the organization of *tuf* genetic regions shows that in *Enterobacteriaceae* containing two *tuf* gene copies an ancestral duplication of *tuf* gene was conserved (35, 39). The organization of the *tuf* genetic regions (*fusA-tufA* and *tufB-secE-nusG*) in *E. coli* K-12 is conserved in *Yersinia* and all of the other *Enterobacteriaceae* genomes studied here except the *P. luminescens* subsp. *laumondii* strain TTO1 genome (27). The latter strain has an unusual chimeric gene order (*fusA-tuf-secE-nusG*), whereas the other copy of the *tuf* gene is located down-

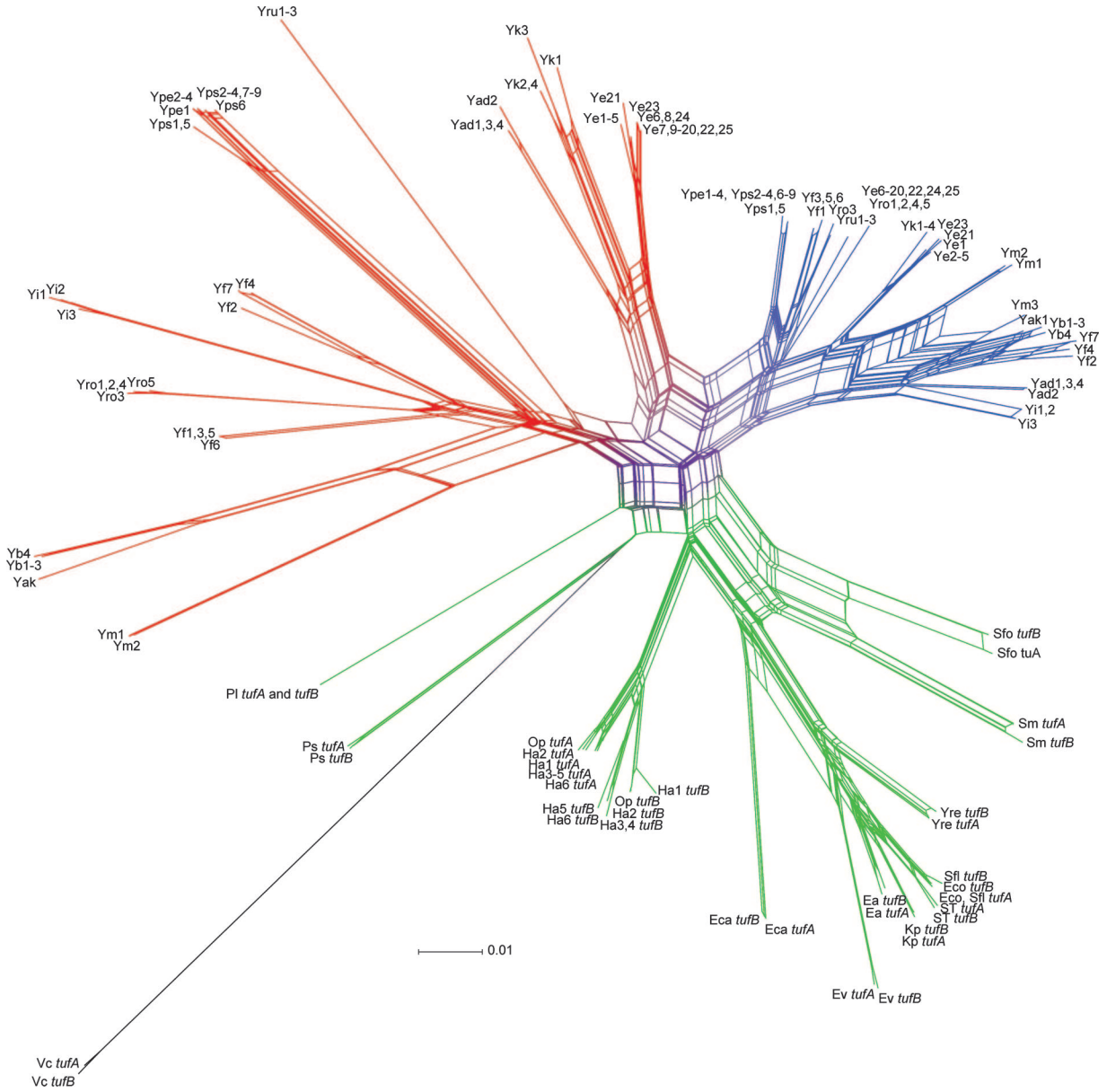

FIG. 2. Phylogenetic network for *tufA* and *tufB* nucleic acid sequences from *Yersinia* and non-*Yersinia* enterobacterial strains. The Neighbor-Net graph was computed by using the SplitsTree 4.8 software. *Yersinia tufA* and *tufB* branches are blue and red, respectively. The *tuf* gene branches of other enterobacterial species and *V. cholerae* (outgroup) are green and black, respectively. Abbreviations for *Yersinia* strains are shown in Table 1. Abbreviations for non-*Yersinia* strains are as follows: Ea, *E. agglomerans* ATCC 27989; Eca, *E. carotovora* subsp. *atroseptica* SCRI1043; Eco, *E. coli* K-12; Ev, *E. vulneris* ATCC 33821; Ha1, *H. alvei* ATCC 13337; Ha2, *H. alvei* ATCC 25927; Ha3, *H. alvei* ATCC 51873; Ha4, *H. alvei* CCRI-10616; Ha5, *H. alvei* CCRI-11829; Ha6, *H. alvei* CCRI-16651; Kp, *K. pneumoniae* ATCC 13883; Op, *O. proteus* ATCC 12841; Pl, *P. luminescens* subsp. *laumondii* TTO1; Ps, *P. shigelloides* ATCC 14029; ST, *S. enterica* subsp. *enterica* serovar Typhimurium strain LT2; Sfo, *S. fonticola* DSM 4576; Sm, *S. marcescens* ATCC 13880; Sfl, *S. flexneri* 2a strain 2457T; Yre, *Y. regensburgei* ATCC 35313; and Vc, *V. cholerae* O1 biovar El Tor strain N16961.

stream from tRNA genes. This abnormal arrangement of the *tuf* regions in strain *P. luminescens* subsp. *laumondii* TTO1 can be explained by recent homologous recombination between the *tufA* and *tufB* genes, resulting in the observed chimeric configurations. The conserved synteny of the *tuf* gene neighborhood in the genus *Yersinia*, as well as in the other *Enterobacteriaceae* studied here, also shows that there was conservation of two ancestral duplicated copies.

The higher levels of divergence between *tufA* and *tufB* sequences in the 12 *Yersinia* species examined in this study suggest that gene conversion became inefficient or ceased to function in the ancestor that gave rise to the modern *Yersinia* species. This could have been due to loss of specific or general gene conversion mechanisms or simply to sequence divergence beyond the divergence that these mechanisms allowed. Gene conversion mechanisms require recombination between very similar sequences. It has been proposed that recombination events played a large role in the evolution and emergence of *Y.*

pestis from *Y. pseudotuberculosis* and that active genome rearrangements in the form of inversions or translocations are responsible for a highly plastic genome with noticeable strain-to-strain variation (9, 15, 48). Moreover, multiple copies of the 16S and 23S rRNA genes are also influenced by gene conversion mechanisms (22, 43). The seven copies of the 16S and 23S rRNA genes in *Y. enterocolitica* subsp. *enterocolitica* 8081, *Y. pestis* strains 91001, Antiqua, CO92, and KIM, and *Y. pseudotuberculosis* IP 32953 have very similar nucleic acid sequences. Therefore, some gene conversion mechanisms are likely to still be operational in the genus *Yersinia*. More general models, in which the conversion frequency gradually declines as genes diverge via the accumulation of point mutations, have been studied previously (57). The *tufA* and *tufB* genes in the ancestor of the genus *Yersinia* could have mutated gradually, and thereby affected conversion mechanisms. The genes would then have evolved independently in parallel. This mutational evolution model could explain the high and relatively wide intragenomic *tuf* nucleic acid sequence divergence in *Yersinia* species, which ranges from 8.3 to 16.2%. To our knowledge, this is the first example of possible *tuf* gene conversion inefficiency. The intermediate level of sequence variation between intragenomic *tuf* genes in the *H. alvei-O. proteus* clade could be an attenuated result of the same phenomenon. The 16S rRNA gene sequence analysis performed by Ibrahim and colleagues (28) showed that *H. alvei* is the member of the *Enterobacteriaceae* most closely related to the genus *Yersinia*, but it was not included in this genus. However, the number of non-*Yersinia Enterobacteriaceae* included in this study was relatively small.

In the *tuf* mutational evolution model, it is possible that one of the two *Yersinia* EF-Tu copies ceased to function or evolved to perform new functions. The levels of similarity between EF-TuA and EF-TuB amino acid sequences are significantly lower in *Yersinia* (94.9 to 98.1%) than in other *Enterobacteriaceae* (99.6 to 100%). The *H. alvei-O. proteus* group exhibits intragenomic *tuf* nucleic acid sequence divergence lower than that observed for *Yersinia*. However, amino acid sequence divergence is similar for the two groups (97.3 to 98.3% similarity). Thus, the nucleic acid sequence divergence resulted in significant amino acid sequence changes. Although evolution of new functions for duplicate genes may be rare (41), EF-Tu proteins have been linked to functions other than elongation, including chaperone properties (8). EF-Tu residue 361 (Thr) is conserved among all of the *Enterobacteriaceae* strains investigated here except for EF-TuB in the genera *Yersinia* (Asn), *Hafnia* (Asn or Ser), and *Obesumbacterium* (Asn). However, this amino acid residue could not be linked to known functional activities of the protein (5, 38). Although *tuf* DNA sequences are divergent in *Yersinia* species, it appears that some EF-Tu proteins have identical or very similar sequences. The *tufA* nucleic acid sequences of *Y. rohdei* and the *Y. pseudotuberculosis*/*Y. pestis* genomospecies were clearly divergent, but the EF-TuA amino acid sequences of these organisms were identical. Also, the levels of *tufB* nucleic acid sequence identity for *Y. aldovae*, *Y. enterocolitica*, and *Y. kristensenii* ranged from 90.8 to 94.7%, while the levels of EF-TuB amino acid sequence similarity were 100.0%. These observations suggest that functional convergent evolution occurred in these species for EF-TuA and EF-TuB. The recently described genome sequence of *Y. pestis* Nepal516 revealed a 58-amino-acid C-terminal deletion in EF-TuB that might have led to a nonfunctional copy of this elongation factor (10). However, such a truncated *tufB* gene has been found only in this isolate and may be a strain-specific mutation that occurred since the original isolation. There is no evidence suggesting that a loss of EF-Tu function occurred in other *Yersinia* strains. Differential expression studies of *tufA* and *tufB*, as well as gene inactivation mutagenesis analysis, may help elucidate specific functions of EF-TuA and EF-TuB under different physiological conditions. Han and colleagues (21) recently compiled microarray data from numerous studies investigating the expression of *Y. pestis* strain 201 genes under 25 different stress conditions in vitro. Cold shock stimulation appears to downregulate *tufB*, while the presence of an antibacterial peptide (polymyxin B) apparently upregulates *tufA* expression.

This study of the genus *Yersinia* was performed with a wide diversity of strains representing 12 *Yersinia* species. The evolution of *tuf* genes in *Yersinia* has resulted in genetic variations that provide a high level of resolution within the genus and represent a powerful tool for the classification of the *Yersinia* genomospecies. Moreover, the greater divergence between the *tufA* and *tufB* genes in *Yersinia* species than in other *Enterobacteriaceae* helps distinguish the genus *Yersinia* from other genera. The concatenated *tuf* gene tree was used to infer species clustering (Fig. 1B). *tuf*-based genetic analyses of *Y. aldovae*, *Y. aleksiciae*, *Y. bercovieri*, *Y. intermedia*, *Y. kristensenii*, *Y. rohdei*, and *Y. ruckeri* showed that their distinctive clades correlate with the phenotypic classification.

Y. enterocolitica strains show great diversity genetically as well as phenotypically. Studies of *Y. enterocolitica* based on DNA-DNA hybridization, 16S rRNA gene sequences, and G+C content have subdivided *Y. enterocolitica* into two subspecies (*Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica*) (29, 46). A more recent study, based on DNA-DNA microarray hybridization with the genome of strain *Y. enterocolitica* subsp. *enterocolitica* 8081, separated *Y. enterocolitica* strains into three groups (high-pathogenicity, low-pathogenicity, and nonpathogenic clades) (25). Here, the *tuf* results also support the biodiversity of *Y. enterocolitica* strains. Great evolutionary distances separate *Y. enterocolitica* subsp. *palearctica* (group I) strains from *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica* (group II) strains. Currently, it is not clear what caused the deviation in tree topology for these two clades. The divergence could not be completely explained by geographical biodiversity as both clusters contain strains isolated in North America and Europe. Based on *tuf* data, *Y. enterocolitica* subsp. *palearctica* strains were clearly separated into two groups (groups I and II). All 13 *Y. enterocolitica* strains isolated in Canada were members of *Y. enterocolitica* subsp. *palearctica* and did not cluster with strains of *Y. enterocolitica* subsp. *enterocolitica* isolated in the United States. Instead, the Canadian isolates clustered with other *Y. enterocolitica* subsp. *palearctica* strains isolated in Europe. Our analyses, based on *tuf* genes, showed that there are three groups of *Y. enterocolitica*, thus supporting previous findings (25).

Y. frederiksenii includes different genomic groups (genomic groups 1a, 1b, 2, 3, and 4) that are sufficiently different as determined by DNA-DNA hybridization, multilocus enzyme electrophoresis, and 16S rRNA gene and *gyrB* sequence anal-

yses to belong to at least three different *Yersinia* genomospecies (14, 16, 17, 56). Our *tufA* and *tufB* phylogenetic analyses also showed that there are three distinct clades that could represent three distinct genomospecies and thus further support the need to reevaluate the classification of these organisms.

Finally, inclusion of *Y. ruckeri* in the genus *Yersinia* has been controversial ever since this organism was classified. The *Y. ruckeri* G+C content is more similar to those of *Yersinia* species even though DNA relatedness showed that *Y. ruckeri* strains were only 30% related to *Yersinia* and *Serratia* species (51). Analysis based on multilocus sequence typing identified *Y. ruckeri* as the most distant species of the genus (36). However, a previous study based on 16S rRNA gene sequence analysis supported inclusion of *Y. ruckeri* in the genus *Yersinia* (28). Phylogenetic trees (Fig. 1) and a network (Fig. 2) of *tufA* and *tufB* gene sequences, as well as the level of divergence, showed that *Y. ruckeri* should be included in the genus *Yersinia* and thus supported its current classification.

In summary, to our knowledge, this is the first report of significant divergence between *tufA* and *tufB* genes throughout a genus. The high level of divergence between *tufA* and *tufB* genes in *Yersinia* strains is a characteristic hallmark of this genus. Here, the evolution of *tuf* genes in *Yersinia* was used to investigate species clustering, and the results provided information useful for reclassification and identification. Our analyses suggest that further investigation using a wider diversity of strains, as well as other genetic analyses, is needed to clarify the taxonomic classification of *Y. enterocolitica*, *Y. frederiksenii*, and *Y. ruckeri*. *tufA* and *tufB* genes could also be genetic targets that are useful for identification of *Yersinia* species for diagnostic purposes. In this study, we provide the first evidence, to our knowledge, supporting the hypothesis that there may be gene conversion inefficiency for the *tuf* gene encoding EF-Tu. It is not known whether the genetic drift seen in duplicated *tuf* genes in *Yersinia* resulted in evolution of a new function or resulted in the nonfunctionalization of the product of one copy of the gene. Determining the reason for the natural selection of the high level of divergence between *tufA* and *tufB* sequences in *Yersinia* requires further investigation and could lead to a better understanding of multigene family evolution in bacteria.

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