

# Geographical Heterogeneity between Far Eastern and Western Countries in Prevalence of the Virulence Plasmid, the Superantigen *Yersinia pseudotuberculosis*-Derived Mitogen, and the High-Pathogenicity Island among *Yersinia pseudotuberculosis* Strains

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*Yersinia pseudotuberculosis* produces novel superantigenic toxins designated YPMa (*Y. pseudotuberculosis*-derived mitogen), YPMb, and YPMc and has a pathogenicity island termed HPI (high-pathogenicity island) and R-HPI (the right-hand part of the HPI with truncation in its left-hand part) on the chromosome. Analysis of the distribution of these virulence factors allowed for differentiation of species *Y. pseudotuberculosis* into six subgroups, thus reflecting the geographical spread of two main clones: the YPMa<sup>+</sup> HPI<sup>-</sup> Far Eastern systemic pathogenic type belonging to serotypes O1b, -2a, -2b, -2c, -3, -4a, -4b, -5a, -5b, -6, -10, and UT (un-typeable) and the YPMs<sup>-</sup> HPI<sup>+</sup> European gastroenteric pathogenic type belonging to serotypes O1a and -1b. The YPMa<sup>+</sup> HPI<sup>+</sup> pathogenic type belonging to serotypes O1b, -3, -5a, -5b, and UT and the YPMb<sup>+</sup> HPI<sup>-</sup> nonpathogenic type belonging to non-melibiose-fermenting serotypes O1b, -5a, -5b, -6, -7, -9, -10, -11, and -12 were prevalent in the Far East. The YPMc<sup>+</sup> R-HPI<sup>+</sup> European low-pathogenicity type belonging to non-melibiose-fermenting serotype O3 and the YPMs<sup>-</sup> HPI<sup>-</sup> pathogenic type belonging to 15 serotypes were found to be prevalent all over the world. This new information is useful for a better understanding of the evolution and spread of *Y. pseudotuberculosis*.

*Yersinia pseudotuberculosis* has a wide distribution in most countries with cold climates and is recognized as an important causal agent of sporadic and epidemic human enteric disease (45). *Yersinia* pathogenic to humans are known either as the causative agent of plague (*Y. pestis*) or as food-borne pathogens that cause intestinal diseases (*Y. enterocolitica*) (8). The pathogenicity of each of these species depends on the presence of 70-kb virulence plasmid pYV (for plasmid associated with *Yersinia* virulence) (4, 13, 25, 33). This plasmid is essential for virulence, and its presence differentiates pathogenic from non-pathogenic *Yersinia*. Additionally, *Y. pestis*, *Y. enterocolitica* biotype 1B, and almost all European strains of *Y. pseudotuberculosis* serotype O1 have a pathogenicity island termed the high-pathogenicity island (HPI) on the chromosome (7), and almost all Far Eastern strains of *Y. pseudotuberculosis* produce a novel superantigenic toxin designated *Y. pseudotuberculosis*-derived mitogen (YPM) encoded by a gene on the chromo-

some (1, 46). *Y. pseudotuberculosis* has been classified into serotypes O1 to O14 (43); serotypes O1 to O5 have been isolated in Europe and the Far East and almost all are pathogenic, while serotypes O6 to O14 have been isolated only from wild animals and environments in the Far East but never from clinical samples (1, 13, 17, 20, 21, 43). There are numerous reports of each virulence factor of *Y. pseudotuberculosis* (1, 5, 7, 14, 19, 34, 45, 46); however, comparisons of the prevalences of above virulence factors in the wild *Y. pseudotuberculosis* strains have not been documented.

Pathogenic *Yersinia* can be further subdivided into low-pathogenicity strains, i.e., strains that induce a mild intestinal infection in humans and at low doses are not lethal for mice, and high-pathogenicity strains, which cause severe systemic infection in humans and at low doses kill mice (7). This difference in the level of virulence correlates with the presence of a large chromosomal fragment with characteristics of an HPI, because its presence is essential for expression of a high-virulence phenotype (7). This chromosomal segment is involved in biosynthesis, regulation, and transport of the siderophore yersiniabactin (24, 35); thus the *Yersinia* HPI can be considered an iron capture island (7). The presence and size of the HPI

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TABLE 1. Sources and serotypes of *Y. pseudotuberculosis* strains<sup>a</sup> used

Area	Source	Total no. of strains	No. of strains of serotype (O-):																						
			1a	1b	1c	2a	2b	2c	3	4a	4b	5a	5b	6	7	9	10	11	12	13	14	UT			
West Europe	Humans	296	58	30		1	33		161	3		10													
	Pig	20	9	5			2		3		1														
	Horses	1							1																
	Rabbits	4		1						3															
	Guinea pig	3	2			1																			
	Cat	1	1																						
	Hares	1	1																						
	Birds	7	2	3				2																	
Australasia	Birds	75	34	18			13		1			9													
	Human	1							1																
	Pigs	66					7		59																
	Cows	5	2	1					2																
	Cattle	13	1						12																
	Goats	32	1						31																
S. America	Deer (domestic)	15	2				9		4																
	Buffalos	24	2						22																
N. America	Humans	3	1	2																					
	Monkeys	22							22																
	Pigs	3							3																
Far East Russia	Humans	1,939	5	213	2	12	115	65	253	104	565	208	209	50	38	10	18	12	2	4	1	53			
	Wild mice	54		20					11	5	18														
	Reindeer	37		23					6	7	1														
	Salmon	3	3																						
	Vegetables	2	2																						
	Environment	12		3					2	3	4														
	Environment	2									2														
China	Human	1							1																
	Pig	3							2		1														
	Rabbits	17		6			2		4				3								2				
	Wild mice	10			1	1	2		1		1			1							2	1			
Korea	Humans	65		1							23													41	
	Pigs	2							1		1														
	Chicken	1														1									
	Wild mouse	1									1														
	Water	19									7													12	
Japan	Humans	530		41		10	41	10	12	4	212	60	140												
	Pigs	312		40			17	20	146	8	78	2	1												
	Pork	2									2														
	Cow and goat	2		1			1																		
	Cats and dogs	69		18			3	3	8	1	29	4		2	1										
	Monkeys	41		17							19	1		3											
	Cape hyrax (in zoo)	5									5														
	Rabbits	17		4			1	1	1		2			8											
	Guinea pigs	8		2					2			2		2		2									
	Raccoon dogs	200		7			25	8	8	40	60	18	14	2	2		15	1							
	Wild mice	105		2			2	3	32	9	15	16	3	15	5	2		1							
	Moles	25									6	1	11	2	3			2							
	Others wild animals	30		1			6	1	6	2	8	3	1	2											
	Birds	10		1						6	3														
	Water	352		25	1	1	15	19		3	24	73	96	38	12	28	5	2	8	2					
	Soil	2		1						1															
	Total		2,235	63	243	2	13	148	65	414	107	565	218	209	50	38	10	18	12	2	4	1	53		

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within the *Y. pseudotuberculosis* species correlate with the serotype. At present, a complete island was found only in strains of serotype O1, HPI with a 9-kb truncation in its left-hand part is characteristic of serotype O3 strains, and HPI has not been detected in strains of other serotypes (5, 14, 34). Since data on only 43 strains, all of *Y. pseudotuberculosis* obtained from Eu-

rope (except for one strain of serotype O5a from Russia and two strains of serotype O4b from Japan [34]), have been reported (5, 14, 34), it is difficult to draw any conclusion concerning the definite prevalence of HPI, especially with regard to geographical distribution and the source of infection.

Although the main clinical manifestations of *Y. pseudotuber-*

TABLE 2. Primers of *virF*, YPMs, and HPI genes

Virulence factor	Target	Orientation <sup>a</sup>	Sequence (5'-3')	Size of product (bp)	Annealing temp. (°C)	Reference
pYV	<i>virF</i>	SP ASP	TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	590	55	48
YPMa	<i>ypmA</i>	SP ASP	CACTTTTCTCTGGAGTAGCG GATGTTTCAGAGCTATTGTT	350	55	27
YPMb	<i>ypmB</i>	SP ASP	TTTCTGTCTACTGACATTA CCTCTTCCATCCATCTCTTA	453	52	36
YPMc (for sequence)	<i>ypmA</i> and <i>ypmC</i>	SP ASP	ACACTTTTCTCTGGAGTAGCG ACAGGACATTTTCGTC	418	49	10
HPI	<i>IS100</i>	SP ASP	ATTGATCCACCGTTTTACTC CGAACGAAAAGCATGAAACAA	963	55	32
HPI	<i>psn</i>	SP ASP	CTTTCCACCAACACCATCC AAACCGCCACTTCGCTTC	1,062	55	6
HPI	<i>yptE</i>	SP ASP	CCCTTACCCATTGCCGAAC TCCCCACCTCATCCAGCC	1,198	55	16
HPI	<i>irp1</i>	SP ASP	AGAAACCGATGCTCACCC TCCTCTCCTGACGTAGCC	526	55	16
HPI	<i>irp2</i>	SP ASP	AAGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	280	55	39
HPI	<i>ybtP-ybtQ</i>	SP ASP	GCCGGGAACGTCAAAGAA AGGTGAGCTTTCATGTGCCT	1,816	55	3
HPI	<i>ybtX-ybtS</i>	SP ASP	TCAGTCGAATGTGAAACCGC GCAGCCGTGCCTGGCACCCCTT	1,453	55	3
HPI	<i>int</i>	SP ASP	TGCGCCATGCGGTCCATC GGTGCATAAGATTCTCGG	714	55	3
HPI	<i>asnT-int</i>	SP ASP	ATCGCTTTGCGGGCTTCTAGGT GAACGGCGGACTGTTAAT	1,396	55	3

<sup>a</sup> SP, sense primer; ASP, antisense primer.

*culosis* infection in Europe are fever, gastroenteric symptoms, and mesenteric lymphadenitis, those in Japan (21, 37), far-eastern Russia (42), and Korea (12) include not only gastrointestinal symptoms but also a variety of systemic manifestations such as fever, scarlatiniform rash, desquamation, erythema nodosum, and arthritis. The clinical pathophysiology of *Y. pseudotuberculosis* has many similarities to that of infections caused by *Staphylococcus aureus* and *Streptococcus pyogenes*, which are each known to produce a superantigen (38). *Y. pseudotuberculosis* is also known to produce a superantigenic toxin, known as YPM (1, 46) and then YPMa after the discovery of a variant (36). It was experimentally confirmed that YPMa is a virulence factor which exacerbates the toxicity of *Y. pseudotuberculosis* in systemic but not in gastroenteric infection in mice (11). Superantigen-producing strains could be separated into three clusters which contained YPMa, YPMb, or YPMc encoded by the *ypmA*, *ypmB* (36), and *ypmC* (10) genes, respectively. We and another research group (47, 49) reported that there was a distinct geographical heterogeneity between the Far East and Europe regarding the prevalence of the *ypmA* gene and that the *ypmA* gene was absent in strains belonging to serotypes O1a, O1b, and O2b from Europe but was present in almost all strains belonging to serotypes O1b, O2b, O2c, O4a, O4b, O5a, and O5b from the Far East. However, the relation-

ship between the clinical manifestations of *Y. pseudotuberculosis* infections and the prevalence of HPI and YPMa in *Y. pseudotuberculosis* has not been demonstrated.

We investigated the distribution of virulence factors pYV, YPMs, and HPI among 2,235 wild *Y. pseudotuberculosis* strains of various serotypes isolated from patients, domestic and wild animals, and natural environments all over the world.

#### MATERIALS AND METHODS

**Bacterial strains.** The 2,235 strains of *Y. pseudotuberculosis* collected from all over the world are listed in Table 1. O serotype-untypeable (UT) strains, which are agglutinated against antiserum to both O1 and -5, are dominant in clinical strains in Korea. Melibiose fermentation of all strains was carried out at 28°C for 7 days. For this study, all strains were grown on Trypticase soy agar plates (BBL, Cockeysville, Md.) at 28°C for 48 h.

**Detection of *virF* gene, *ypmA*, and *ypmB* genes, and *Yersinia* HPI genes by PCR.** Bacteria grown on Trypticase soy agar plates were suspended in 50 µl of distilled water to achieve a concentration of 10<sup>8</sup> CFU/ml and boiled for 10 min. Aliquots of 4 µl were then examined using PCR. The different sets of primers used for PCR amplification were synthesized by Life Technologies (Tokyo, Japan). Eleven sets of primers for analyzing pYV, YPMa, YPMb, and HPI in this study are listed in Table 2. Strains PB1 (serotype O1a) (34), 334 (serotype O4b) (49), and R104 (serotype O6) (36) were used as positive controls for HPI and the YPMa and YPMb genes, respectively. PCR was carried out in a 20-µl reaction volume with the Gene Amp PCR System 2400 (Perkin-Elmer, Weiterstadt, Germany) with *TaqI* polymerase (Takara, Shiga, Japan). The initial denaturation step (94°C, 5 min) was followed by 25 cycles of denaturation (94°C, 1 min),

annealing (at each temperature in Table 2; 1 min), and extension (72°C, 1 min), with one final extension step (72°C, 7 min). PCR was used to detect the *irp2* gene in all strains to screen for the presence of HPI and then was used to identify other genes in the *irp2*-positive strains. All the primer pairs were used separately in PCR, except for multiplexing with the *irp1* and *ybtX-ybtS* genes and the *psn* and *ybtP-ybtQ* genes.

**Sequence analysis of *ypmA* and *ypmC* genes.** To sequence the *ypmA* and *ypmC* genes of serotype O3 strains (10 strains each of genetic groups 3 and 5 and 2 strains of group 1), 2 strains each from other serotypes of genetic group 3, and 1 strain each from non-melibiose-fermenting (NMF) serotypes O1c, -2c, and -6 of genetic group 3, PCR fragments were generated with *ypmA* primers described by Carnoy and Simonet (10) (Table 2). The PCR products were purified using the QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed using the Big Dye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer) and the ABI Prism 310 genetic analyzer (Perkin-Elmer).

## RESULTS

**Analysis of *virF* and *ypm* genes in wild *Y. pseudotuberculosis* strains.** The presence of *virF* and *ypm* genes in the strains examined is given in Table 3. The *ypmA*, *ypmB*, and *ypmC* genes were detected in 1,597 strains (71%), 94 strains (4%), and 235 strains (11%) of wild *Y. pseudotuberculosis*, respectively. The *ypmA* gene was detected in 84% of the strains of 14 serotypes, except for 6 serotypes (O1a, -9, -11, -12, -13, and -14) from the Far East, but not in any strain from Western countries, except for three strains of serotype O4a from horses in Denmark. In the Far East, the prevalence of the *ypmA* gene in strains belonging to serotypes O1b, -2a, -2b, -2c, -3, -4a, -4b, -5a, -5b, and UT, which are associated with human disease, was 86%, while that in the animal or environmental strains belonging to serotypes O1a, -1c, -6, -7, -8, -9, -10, -11, -12, -13, and -14 and not associated with human disease was 40%. The *ypmB* gene was detected in 12% of strains belonging to serotypes O1b, -5a, -5b, -6, -7, -9, -10, -11, and -12 isolated from moles, wild mice, a marten, and river water but never from clinical samples in Japan. Based on the sequences of the 418-bp internal regions of superantigen-encoding genes, *ypmC* and *ypmA* differed by one nucleotide, as described by Carnoy and Simonet (10). The *ypmC* gene was detected in serotype O3 strains from patients, monkeys, and livestock in Western countries, while it was found in 50% of the serotype O3 strains isolated from pigs and a patient but never in the isolates from wild animals in the Far East.

**Analysis of HPI in wild *Y. pseudotuberculosis* strains.** A complete HPI, which showed positive reactions for all of the *IS100*, *psn*, *yptE*, *irp1*, *irp2*, *ybtP-ybtQ*, *ybtX-ybtS*, *int*, and *asnT-Int* genes by PCR, was detected in all strains of serotype O1a, 12% of strains of serotype O1b, and 18 strains belonging to serotypes O3, -5a, -5b, -13, 14, and UT (Table 3). An HPI with a truncation of one or two regions of the *IS100*, *yptP-Q*, or *ans* tRNA gene was detected in eight strains belonging to serotypes O1a, -1b, -3, and -5b. One strain of serotype O3 was isolated from the sputum of a patient with a fever and pneumonia in China. In Western countries, HPI (including an incomplete HPI) was detected in all serotype O1a strains and 84% of serotype O1b strains. In the Far East, an HPI was detected in 5 strains of serotype O1a from reindeer and salmon in far-eastern Russia and in 20 strains belonging to serotypes O1b, -3, -5a, -5b, -13, -14, and UT. The latter strains were obtained from five patients, a pig, and wild animals in the Far East but never in Western countries. The right-hand part of the HPI (R-HPI) with a truncation of *IS100*, *yptE*, and *psn* genes in its

left-hand part was detected in 57% of serotype O3 strains but never in other serotypes. An R-HPI was found in all serotype O3 strains from patients, monkeys, and livestock from Western countries, except for one swine strain which did not harbor an R-HPI or an HPI. In the Far East, an R-HPI was found in 50% of the serotype O3 strains isolated from pigs and a patient but never from wild animals. HPI was not detected in serotype O1c, -2a, -2b, -2c, -4a, -4b, -6, -7, -9, -10, -11, and -12 strains from all over the world or in any serotype O1b, -5a, -5b, and UT strains, except for nine strains from the Far East. The presence of the products (pYU, YPMa, YPMb, and YPMc) of the *virF*, *ypmA*, *ypmB*, *ypmC*, and HPI genes in wild *Y. pseudotuberculosis* strains is shown in Table 3. The strains were separated into six genetic groups: group 1 (YPMa<sup>+</sup> HPI<sup>+</sup>; pathogenic type), group 2 (YPMs<sup>-</sup> HPI<sup>+</sup>; European gastroenteric pathogenicity type), group 3 (YPMa<sup>+</sup> HPI<sup>-</sup>; Far Eastern systemic pathogenicity type), group 4 (YPMb<sup>+</sup> HPI<sup>-</sup>; non-pathogenic type), group 5 (YPMc<sup>+</sup> R-HPI<sup>+</sup>; European low-pathogenicity type), and group 6 (YPMs<sup>-</sup> HPI<sup>-</sup>; pathogenic type).

**Relationship between melibiose fermentation and the presence of virulence-associated genes.** Genetic group 4, which consisted of 93 strains belonging to serotypes O1b, -5a, -5b, -6, -7, -9, -10, -11, and -12, except for one strain of serotype O9, and genetic group 5, which consisted of 235 strains of serotype O3, did not ferment melibiose (Table 3). All strains belonging to the other genetic groups fermented melibiose, except for four strains of serotypes O1c, -2b, -2c, and -14.

## DISCUSSION

The pathogenicity of *Y. pseudotuberculosis* depends on the presence of pYV (13), YPMa (11), and HPI (7). pYV is essential for virulence; its presence differentiates pathogenic from nonpathogenic *Yersinia* and is absolutely required for pathogenicity. However, pYV was absent from one-fourth of virulent serotypes (Table 3) and might be lacking in stock cultures. Analysis of the presence of pYV in this species is not sufficient to determine pathogenicity, and the restriction endonuclease analysis of virulence plasmid patterns is also insufficient for analyzing of the epidemiology of all strains of this species.

YPMa (11) and HPI (7) are closely correlated with clinical features of *Y. pseudotuberculosis* infections. The main clinical manifestations of *Y. pseudotuberculosis* infections in the Far East are usually more diverse and severe than they are in the West (12, 21, 37, 42); those seen in Europe include fever and gastroenteric symptoms with mesenteric lymphadenitis (8). The major difference in clinical symptoms between the Far East and Western countries is that rash and desquamation, which are not seen in patients in Western countries, are common in the Far East. We reported that YPM was detected in all clinical strains belonging to serotypes O1b, -2a, -2b, -2c, -3, -4a, -4b, -5a, and -5b, of which serotypes O1b, -2b, -4b, and -5b were dominant, in the Far East but never in European clinical isolates belonging to serotypes O1a and -1b, although it was found in serotype O3 (49). It was confirmed that YPM-producing strains could be separated into three clusters of strains which produce YPMa, YPMb, or YPMc encoded by the *ypmA*, *ypmB* (36), and *ypmC* (10) genes, respectively. In the present

TABLE 3. Geographical heterogeneity between the Far East and West regarding the prevalence of pYV, HPI, and YPMs among wild *Y. pseudotuberculosis* strains

Genetic group	Presence of:					pYV <sup>+</sup>	MF	Pathogenic type	Serotypes (O-) from:			% of strains from:			No. of isolates	Exceptions
	HPI	R-HPI	YPMa	YPMb	YPMc				West	Far East	Humans	Animals	Environ-ment			
1	+	-	+	-	-	56	+	Pathogenic		1b, 3, 5a, 5b, UT		44	56	9	IS100 negative (two strains each of serotypes: O1b and -5b) in HPI	
2	+	-	-	-	-	49	+	European gastroenteric <sup>a</sup>	1a, 1b	1a, 3, 5b, 13, 14		16	84	99	<i>yjyP-Q</i> and <i>ans</i> RNA gene negative (one strain of O1a); <i>yjyRQ</i> negative (one strain of O1b); IS100 gene negative (two strains of O5b) in HPI; O14 NMF	
3	-	-	+	-	-	77	+	Systemic (Far East; except for O1c and -7)	4a	1b, 1c, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 10, UT		39	42	1,589	<i>vif</i> negative (O1c and -7 strains); NMF (one strain each of O1c, -2c, and -6)	
4	-	-	-	+	-	0	-	Nonpathogenic		1b, 5a, 5b, 6, 7, 9, 10, 11, 12			54 <sup>b</sup>	46	93	MF (one strain of O9)
5	-	+	-	-	+	89	-	European low pathogenicity	3	3		2	98 <sup>c</sup>	235		
6	-	-	-	-	-	50	+	Pathogenic	1b, 2a, 2b, 3, 5a	1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 10, 11, 13, UT		8	74	210	NMF (one strain of O2b)	

<sup>a</sup> Except for Far East strains.  
<sup>b</sup> Males and wild mice.  
<sup>c</sup> Live stock in Western countries and only pigs in the Far East.

study, YPMa was detected in 98% of Far Eastern clinical strains belonging to serotypes O1b, -2a, -2b, -2c, -3, -4a, -4b, -5a, -5b, and UT and YPMc was detected in five strains of serotype O3 from Western countries and Japan (28), while YPMb was never detected in clinical strains. In contrast, it was reported that the HPI present in *Y. pestis* and *Y. enterocolitica* biotype 1B is found only in strains of serotypes O1a and -1b from Europe, never in those of other serotypes. HPI carries virulence genes, namely, those of the yersiniabactin system, involved in siderophore-mediated iron acquisition, which is essential for the expression of the high-virulence phenotype (7). Five genes, *psn*, *irp1*, *irp2*, *ybtP*, and *ybtQ*, in an HPI are involved in the yersiniabactin system (15, 24, 26). The *psn* and *irp2* genes are important for expression of the high-pathogenicity phenotype (7, 9). It was therefore considered that strains of serotypes O1a and -1b harboring a complete HPI are highly pathogenic but that strains of serotypes O2, -4, -5, and -6, which do not have a complete HPI, are low-pathogenicity strains (7). The present study demonstrated the presence of a complete HPI in some clinical strains of serotypes O3 and UT from the Far East. The distribution of YPMa and HPI in *Y. pseudotuberculosis* noted by Eastern and European investigators (1, 10, 14, 34, 47, 49), respectively, was confirmed in the present study, in which 10-fold more strains than in our previous study were tested. Therefore, the difference in clinical manifestations of *Y. pseudotuberculosis* infection between the Far East and Western countries is related to heterogeneity in the distribution of YPMa or HPI.

Almost all the clinical strains were classified into two main clones: the YPMs<sup>-</sup> HPI<sup>+</sup> European gastroenteric-pathogenicity type (group 2) belonging to serotypes O1a and -1b and the YPMa<sup>+</sup> HPI<sup>-</sup> Far Eastern systemic-pathogenicity type (group 3) belonging to serotypes O1b, -2a, -2b, -2c, -3, -4a, -4b, -5a, -5b, -6, -10, and UT (Table 3). The third clone was classified into the YPMc<sup>+</sup> R-HPI<sup>+</sup> European low-pathogenicity type (group 5) belonging to NMF serotype O3. This new information is useful for a better understanding of the evolution and spread of *Y. pseudotuberculosis*.

The clinical strains of group 2 are dominant in strains isolated from human *Y. pseudotuberculosis* gastroenteric infections with mesenteric lymphadenitis in Europe, Australasia, and North America but not in the Far East. These strains were mainly distributed among wild animals and livestock other than pigs in Western countries. This and previous molecular analyses (19, 41) of *Y. pseudotuberculosis* demonstrate that these *Y. pseudotuberculosis* strains may have been unknowingly introduced by human carriers or in shipments of livestock from Europe in the late 1700s and early 1800s. Thus, serotypes O1a and -1b in this group were recognized as the European gastroenteric-pathogenicity type. In contrast, the clinical strains of group 3 caused human *Y. pseudotuberculosis* systemic infections in the Far East but not in Western countries. This group was distributed in the Far East, except for three strains of serotype O4a from horses in Denmark, and thus the group, including serotypes O1b, -2a, -2b, -2c, -3, -4a, -4b, -5a, -5b, -6, -10, and UT, of which serotypes O1b, -2b, -4b, and -5b were dominant, was recognized as the Far Eastern systemic-pathogenicity type.

The other clinical strains were classified into three pathogenic groups, groups 1 (YPMa<sup>+</sup> HPI<sup>+</sup>), 5 (YPMc<sup>+</sup> R-HPI<sup>+</sup>),

and 6 (YPMs<sup>-</sup> HPI<sup>-</sup>), and nonpathogenic group 4 (YPMb<sup>+</sup> HPI<sup>-</sup>) (Table 3). Although an estimation of the pathogenicity of groups 1 and 6 awaits confirmation from investigations of more cases of human infections, the pathogenicity of groups 4 and 5 was discussed. The distribution of group 4, which includes NMF serotypes O1b, -5a, -5b, -6, -7, -9, -10, -11, and -12 and which does not have pYV, among wild animals and environments in Japan suggests that the origin of this group is the Far East and that this group is nonpathogenic, thereby supporting the proposal that serotypes O6, -7, -9, -10, -11, and -12 are not lethal for mice (30). Group 5 contained NMF serotype O3 strains originating from patients and pigs and other members of livestock in Western countries (29) and from a patient and pigs in the Far East (44) but never from wild animals anywhere. This group may also have come from Europe via the same route as group 2 (19, 41) and may have been introduced by the import of pigs and pork from Western pig-producing countries to Japan (19, 20), just as is the case with the transport of pathogenic *Y. enterocolitica* after the 1970s (18). These theories were confirmed in the present study, which demonstrates a European origin for this group.

All strains harboring YPMb or YPMc (groups 4 or 5) did not ferment melibiose. All strains isolated from pigs and other sources in Europe (29) and about one-half of all strains isolated from healthy pigs in Japan were NMF serotype O3 (44). The NMF serotype O3 strains from pigs were avirulent and atoxic, compared with strains of melibiose-fermenting (MF) serotype O3 from other sources in Japan (31, 44) or serotype O1 from healthy pigs (29). In the present study, serotype O3 strains from pigs were divided into NMF group 5 and MF groups 1, 2, 3, and 6. In contrast, all serotype O3 strains from wild animals in Japan were classified into MF groups 1, 2, 3, and 6. These MF strains were virulent for mice but NMF strains were not (data not shown), as previously described (29, 31, 44). However infections with NMF serotype O3 strains were found in some humans in Europe, Australia, and Japan (31) and in ruminant flocks in Australasia (41). In a case of human infection with NMF serotype O3 in Japan, a patient with terminal ileitis did not have systemic symptoms (28). This phenomenon contrasts with human infections with MF serotype O3, distributed widely among pigs and wild animals in the Far East. Thus the pathogenicity of YPMc, whose gene differs by one nucleotide from that of YPMa (10), and that of R-HPI, which lacked the *psn* gene encoding the outer membrane receptor for the yersiniabactin, the bacteriocin, and the pesticin (15) of this group, is lower than those of YPMa and HPI. Thus, NMF serotype O3 does not have a serious pathogenicity. Although those findings suggest a close relationship between the presence of YPMb or YPMc and the NMF strains in groups 4 and 5, the relationship between the presence of YPMb or YPMc, pathogenicity, and melibiose fermentation in *Y. pseudotuberculosis* remains unclear.

The geographical association between the prevalence of YPMs and HPI genes does not always depend on geographical differences among serotypes but rather on the geographical disparity in the YPMs and HPI genes. *Y. pestis*, which evolved from *Y. pseudotuberculosis* 1,500 to 20,000 years ago, is a highly uniform clone of *Y. pseudotuberculosis* that arose shortly before the first known pandemics of plague and that has three biovars that are phylogenetically distinct (2). In China after the third

pandemic, foci of plague endemicity persisted in 10 areas for which the epidemiology was known and the distinct biovars of *Y. pestis* were characterized by geographic locations (22, 23). Skurnik et al. (40) suggested that the cryptic O-antigen gene cluster of *Y. pestis* bv. Orientalis showed that *Y. pestis* is most closely related to and has evolved from *Y. pseudotuberculosis* serotype O1b, isolated from a patient in Japan. Moreover, there is the possibility that the individual *Y. pseudotuberculosis* serotype O1b strain that is a direct ancestor to *Y. pseudotuberculosis* represents a substrain of *Y. pseudotuberculosis* serotype O1b (40). This speculation may be supported by differences in the YPMs and HPI genes of *Y. pseudotuberculosis* serotype O1b strains originating from different parts of the world, in addition to differences of restriction endonuclease analysis of virulence plasmid patterns of this serotype (19, 20). These findings suggest that the distribution of biochemical varieties of *Y. pestis* in China depends on the evolution of various types of *Y. pestis* from *Y. pseudotuberculosis* strains with different genotypes and serotypes in wide areas of the Far East over 20,000 years. When *Y. pseudotuberculosis* strains from China, Africa, the Middle East, and central Asia become available for analysis, the ancestor to *Y. pestis* may be identified.

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#### REFERENCES

1. Abe, J., T. Takeda, Y. Watanabe, H. Nakao, N. Kobayashi, D. Y. M. Leung, and T. Kohsaka. 1993. Evidence for superantigen production by *Yersinia pseudotuberculosis*. *J. Immunol.* **151**:4183–4188.
2. Achtman, M., K. Zurth, G. Morelli, G. Torrea, A. Guiyoule, and E. Carniel. 1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. USA* **96**:14043–14048.
3. Bach, S., A. De Almeida, and E. Carniel. 2000. The *Yersinia* high-pathogenicity island is present in different members of the family *Enterobacteriaceae*. *FEMS Microbiol. Lett.* **183**:289–294.
4. Brubaker, R. R. 1991. Factors promoting acute and chronic diseases caused by yersiniae. *Clin. Microbiol. Rev.* **4**:309–324.
5. Buchrieser, C., R. Brosch, S. Bach, A. Guiyoule, and E. Carniel. 1998. The high-pathogenicity island of *Yersinia pseudotuberculosis* can be inserted into any of the three chromosomal *asn tRNA* genes. *Mol. Microbiol.* **30**:965–978.
6. Buchrieser, C., M. Prentice, and E. Carniel. 1998. The 102-kilobase unstable region of *Yersinia pestis* comprises a high-pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement. *J. Bacteriol.* **180**:2321–2329.
7. Carniel, E. 1999. The *Yersinia* high-pathogenicity island. *Int. Microbiol.* **2**: 161–167.
8. Carniel, E., and H. H. Mollaret. 1990. Yersiniosis. *Comp. Immunol. Microbiol. Infect. Dis.* **13**:51–58.
9. Carniel, E., I. Guilvout, and M. Prentice. 1996. Characterization of a large chromosomal “high-pathogenicity island” in biotype 1B *Yersinia enterocolitica*. *J. Bacteriol.* **178**:6743–6751.
10. Carnoy, C., and M. Simonet. 1999. *Yersinia pseudotuberculosis* superantigenic toxins, p.611–622. In J. E. Alouf and J. H. Freer (ed.), *Bacterial protein toxins: a comprehensive sourcebook*, 2nd ed. Academic Press, London, United Kingdom.
11. Carnoy, C., C. Mullet, H. Muller-Alouf, E. Leteurtre, and M. Simonet. 2000. Superantigen YPMa exacerbates the virulence of *Yersinia pseudotuberculosis* in mice. *Infect. Immun.* **68**:2553–2559.
12. Cheng, H. L., E. H. Choi, I. S. Ha, H. J. Lee, and Y. Choi. 1995. Acute renal failure associated with *Yersinia pseudotuberculosis*. *Nephron* **70**:319–323.
13. Cornelis, G. R., T. Biot, C. L. de Rouvroit, T. Michiels, B. Mulder, C. Sluiter, M.-P. Sory, M. Van Bouchaute, and J.-C. Vanootehem. 1989. The *Yersinia yop* regulon. *Mol. Microbiol.* **3**:1455–1459.
14. De Almedia, A. M. P., A. Guiyoule, I. Guilvout, I. Iteman, G. Baranton, and E. Carniel. 1993. Chromosomal *irp2* gene in *Yersinia*: distribution, expression, deletion and impact on virulence. *Microb. Pathog.* **14**:9–21.
15. Fetherston, J. D., J. W. Lillard, Jr., and R. D. Perry. 1996. YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. *Mol. Microbiol.* **22**:315–325.
16. Filippov, A. A., P. N. Oleinikov, V. L. Motin, O. A. Protsenko, and G. B. Smirnov. 1995. Sequencing of two *Yersinia pestis* IS elements, IS285, and

- IS100. *Contrib. Microbiol. Immunol.* **13**:306–309.
17. **Fukushima, H., M. Gomyoda, and S. Kaneko.** 1990. Mice and moles inhabiting mountainous areas of Shimane Peninsula as sources of infection with *Yersinia pseudotuberculosis*. *J. Clin. Microbiol.* **28**:2448–2455.
  18. **Fukushima, H., K. Hoshina, H. Itogawa, and M. Gomyoda.** 1997. Introduction into Japan of pathogenic *Yersinia* through imported pork, beef and fowl. *Int. J. Food Microbiol.* **35**:205–212.
  19. **Fukushima, H., M. Gomyoda, S. Kaneko, M. Tsubokura, N. Takeda, T. Hongo, and F. N. Shubin.** 1994. Restriction endonuclease analysis of virulence plasmid for molecular epidemiology of *Yersinia pseudotuberculosis* infections. *J. Clin. Microbiol.* **32**:1410–1413.
  20. **Fukushima, H., M. Gomyoda, N. Hashimoto, I. Takashima, F. N. Shubin, L. M. Isachikova, I. K. Paik, and X. B. Zheng.** 1998. Putative origin of *Yersinia pseudotuberculosis* in western and eastern countries. A comparison of restriction endonuclease analysis of virulence plasmids. *Zentbl. Bakteriol.* **288**:93–102.
  21. **Fukushima, H., M. Tsubokura, K. Otsuki, Y. Kawaoka, R. Nishio, S. Moriki, Y. Nishino, H. Mototsune, and K. Karino.** 1985. Epidemiological study of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* infections in Shimane Prefecture, Japan. *Zentbl. Bakteriol. Hyg. Abt. 1 Orig.* **B180**:515–527.
  22. **Fukushima, H., Q. Hao, K. Wu, X. Hu, J. Chen, Z. Guo, H. Dai, C. Qin, S. Lu, and M. Gomyoda.** 2001. *Yersinia enterocolitica* O9 as a possible barrier against *Y. pestis* in natural plague foci in Ningxia, China. *Curr. Microbiol.* **42**:1–7.
  23. **Gao, Z., and Y. Zong.** 1995. *Yersinia pestis* and prevention against plague epidemics in mainland China. *Media-Circle* **40**:2–8. (In Japanese.)
  24. **Gehring, A. M., E. Demoll, J. D. Fetherston, I. Mori, G. F. Mayhew, F. R. Blattner, C. T. Walsh, and R. D. Perry.** 1998. Iron acquisition in plague—modular logic in enzymatic biogenesis of yersiniabactin by *Yersinia pestis*. *Chem. Biol.* **5**:573–586.
  25. **Gemski, P., J. R. Lazere, T. Casey, and J. H. Wohlhieter.** 1980. Presence of a virulence-associated plasmid in *Yersinia pseudotuberculosis*. *Infect. Immun.* **28**:1044–1047.
  26. **Heesemann, J., K. Hantke, T. Vocke, E. Saken, A. Rakin, I. Stojiljkovic, and R. Berner.** 1993. Virulence of *Yersinia enterocolitica* is closely associated with siderophore production, expression of an iron-repressible outer membrane polypeptide of 65,000 Da and pesticin sensitivity. *Mol. Microbiol.* **8**:397–408.
  27. **Ito, Y., J. Abe, K. Yoshino, T. Takeda, and T. Kohsaka.** 1995. Sequence analysis of the gene for a novel superantigen produced by *Yersinia pseudotuberculosis* and expression of the recombinant protein. *J. Immunol.* **154**:5896–5906.
  28. **Kanazawa, Y., K. Ikemura, I. Sasagawa, and N. Shigeno.** 1974. A case of terminal ileitis due to *Yersinia pseudotuberculosis*. *J. Jpn. Assoc. Infect. Dis.* **48**:220–228.
  29. **Mair, N. S., E. Fox, and E. Thal.** 1979. Biochemical, pathogenicity and toxicity studies of type III strains of *Yersinia pseudotuberculosis* isolated from the fecal contents of pigs. *Contrib. Microbiol. Immunol.* **5**:359–365.
  30. **Nagano, T., T. Kiyohara, K. Suzuki, M. Tsubokura, and K. Otsuki.** 1997. Identification of pathogenic strains within serogroups of *Yersinia pseudotuberculosis* and the presence of non-pathogenic strains isolated from animals and the environment. *J. Vet. Med. Sci.* **59**:153–158.
  31. **Nagano, T., K. Ichimura, N. Haji, K. Nagao, K. Someya, T. Kiyohara, K. Suzuki, M. Tsubokura, and K. Otsuki.** 1997. Characteristics and pathogenicity of non-melibiose-fermenting strains of *Yersinia pseudotuberculosis* O3. *Microbiol. Immunol.* **41**:175–183.
  32. **Podladchikova, O. N., G. G. Dikhanov, A. V. Rakin, and J. Heesemann.** 1994. Nucleotide sequence and structural organization of *Yersinia pestis* insertion sequence IS100. *FEMS Microbiol. Lett.* **121**:269–274.
  33. **Portnoy, D. A., and S. Falkow.** 1981. Virulence-associated plasmids for *Yersinia enterocolitica* and *Yersinia pestis*. *J. Bacteriol.* **148**:877–883.
  34. **Rakin, A., P. Urbitsch, and J. Heesemann.** 1995. Evidence for two evolutionary lineages of highly pathogenic *Yersinia* species. *J. Bacteriol.* **177**:2292–2298.
  35. **Rakin, A., C. Noelting, S. Schubert, and J. Heesemann.** 1999. Common and specific characteristics of the high-pathogenicity island of *Yersinia enterocolitica*. *Infect. Immun.* **67**:5265–5274.
  36. **Ramamuthy, T., K. Yoshino, J. Abe, N. Ikeda, and T. Takeda.** 1997. Purification, characterization and cloning of a novel variant of the superantigen *Yersinia pseudotuberculosis*-derived mitogen. *FEBS Letters* **413**:174–176.
  37. **Sato, K., K. Ouchi, and M. Takai.** 1983. *Yersinia pseudotuberculosis* infection in children, resembling Izumi fever and Kawasaki syndrome. *Pediatr. Infect. Dis.* **2**:123–126.
  38. **Scherer, M. T., L. Ignatowicz, G. M. Winslow, J. W. Kappler, and P. Marack.** 1993. Superantigens: bacterial and viral proteins that manipulate the immune system. *Annu. Rev. Cell Biol.* **9**:101–128.
  39. **Schubert, S., A. Rakin, H. Karch, E. Carniel, and J. Heesemann.** 1998. Prevalence of the “high-pathogenicity island” of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. *Infect. Immun.* **66**:480–485.
  40. **Skurnik, M., A. Peippo, and E. Erelva.** 2000. Characterization of the O-antigen gene clusters of *Yersinia pseudotuberculosis* and the cryptic O-antigen gene cluster of *Yersinia pestis* shows that the plague bacillus is most closely related to and has evolved from *Y. pseudotuberculosis* serotype O:1b. *Mol. Microbiol.* **37**:316–330.
  41. **Slee, K., and N. W. Skilbeck.** 1992. Epidemiology of *Yersinia pseudotuberculosis* and *Y. enterocolitica* infections in sheep in Australia. *J. Clin. Microbiol.* **30**:712–715.
  42. **Somov, G. P., and I. L. Martinevsky.** 1973. New facts about pseudotuberculosis in the USSR. *Contrib. Microbiol. Immunol.* **2**:214–216.
  43. **Tsubokura, M., and S. Aleksic.** 1995. A simplified antigenic scheme for serotyping of *Yersinia pseudotuberculosis*: phenotypic characterization of reference strains and preparation of O and H factor sera. *Contrib. Microbiol. Immunol.* **13**:99–105.
  44. **Tsubokura, M., K. Otsuki, Y. Kawaoka, and T. Maruyama.** 1984. Characterization and pathogenicity of *Yersinia pseudotuberculosis* isolated from swine and other animals. *J. Clin. Microbiol.* **19**:754–756.
  45. **Tsubokura, M., K. Otsuki, K. Sato, M. Tanaka, T. Hongo, H. Fukushima, M. Maruyama, and M. Inoue.** 1989. Special features of distribution of *Yersinia pseudotuberculosis* in Japan. *J. Clin. Microbiol.* **27**:790–791.
  46. **Uchiyama, T., T. Niyoshi-Akiyama, H. Kato, W. Fujimaki, K. Imanishi, and X.-J. Yan.** 1993. Superantigenic properties of a novel mitogenic substance produced by *Yersinia pseudotuberculosis* isolated from patients manifesting acute and systemic symptoms. *J. Immunol.* **151**:4407–4413.
  47. **Ueshiba, H., H. Kato, T. Miyoshi-Akiyama, M. Tsubokura, T. Nagano, S. Kaneko, and T. Uchiyama.** 1998. Analysis of the superantigen-producing ability of *Yersinia pseudotuberculosis* strains of various serotypes isolated from patients with systemic or gastroenteric infections, wildlife animals and natural environments. *Zentbl. Bakteriol.* **288**:277–291.
  48. **Wren, B. W., and S. Tabaqchali.** 1990. Detection of pathogenic *Yersinia enterocolitica* by the polymerase chain reaction. *Lancet* **336**:693.
  49. **Yoshino, K., T. Ramamuthy, G. B. Nair, H. Fukushima, Y. Otomo, N. Takeda, S. Kaneko, and T. Takeda.** 1995. Geographical heterogeneity between Far East and Europe in prevalence of *ypm* gene encoding the novel superantigen among *Yersinia pseudotuberculosis* strains. *J. Clin. Microbiol.* **33**:3356–3358.