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Linkage of the Horizontally Acquired *ypm* and *pil* Genes in *Yersinia pseudotuberculosis*

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The superantigen-encoding *ypm* gene and the *pil* gene cluster governing type IV pilus biogenesis have been laterally acquired by *Yersinia pseudotuberculosis*. PCR assays on 270 unrelated strains from various environmental and animal sources revealed a significant association of *ypm* and *pil* in isolates.

To date, *Yersinia pseudotuberculosis* is the only known gram-negative bacterium that synthesizes a superantigenic toxin (YPM), a protein which strongly stimulates the proliferation of polyclonal T lymphocytes (1). YPM is a distinct member of the bacterial superantigen family (5) in that (i) its molecular mass (14 kDa) is much lower than that of superantigens produced by *Mycoplasma arthritidis* and the gram-positive species *Staphylococcus aureus* and *Streptococcus pyogenes* (22 to 29 kDa) and (ii) it does not show significant amino acid similarity to other proteins. The three-dimensional structure of YPM has been recently established and the closest structural neighbors found, besides members of the tumor necrosis factor superfamily, were viral capsid proteins (10). Genes coding for bacterial superantigens are frequently located within mobile genetic elements in general and within bacteriophages in particular (16). Two features strongly suggest that *Y. pseudotuberculosis* horizontally acquired the superantigen-encoding gene: *ypm* is not distributed in all strains of the species and its G+C content is significantly lower than that of the genomic core (35 versus 47%) (5, 19). We have reported that *ypm* is present on the *Y. pseudotuberculosis* chromosome, 245 bp downstream of a 26-bp sequence (called *yrs*) which is homologous to *dif* (4), a site-specific recombination target used by filamentous bacteriophages for host chromosome integration (14). However, we failed to detect any phage remnants in the vicinity of this nucleotide motif (4). The *yrs* site is also present on the chromosome of *Yersinia pestis*, a *ypm*-negative species derived from *Y. pseudotuberculosis* (4), and strikingly, it is surrounded by filamentous phage-like (CUS-2) genes (4, 12). Therefore, one can reasonably speculate that a bacteriophage was involved in the incorporation of *ypm* into *Y. pseudotuberculosis*.

Fimbriae (and especially type IV pili) may serve as bacteriophage receptors at the bacterial cell surface (2, 3, 13, 15, 17, 18). We recently reported the presence in *Y. pseudotuberculosis* of an 11-kb, 11-gene *pil* locus (*pilLMNOPQRSUVW*) that encodes a type IV pilus (7). It is located in the 5′ part of a large (98-kb) pathogenicity island (PAI) called YAPI (6). Like *ypm*, the *pil* gene cluster is not present in all *Y. pseudotuberculosis* strains (7). The aim of the present work was to analyze *ypm* and *pil* association in a large collection of isolates, in order to determine the potential role of type IV pili in the emergence of *ypm* in *Y. pseudotuberculosis*.

Thirty strains from each of the nine most frequent O serotypes (1a, 1b, 2b, 2c, 3, 4a, 4b, 5a, and 5b) were randomly chosen from a collection of 2,235 strains previously typed for presence of the *ypm* gene and the high-pathogenicity island (HPI) (11). The strains had been isolated from various environmental and animal sources (Fig. 1A). One hundred ninety-six of the 270 selected strains originated from Asia (mainly Japan with 188 strains, but also Korea and China). The remainder were collected from Europe (including Russia), America, and Oceania, with an unknown geographical origin for just two strains. Screening of *pil* segments was performed by PCR analysis as previously described, with primer sets 1 (forward, 5′ TATGTTGCTGGAGGCTCAG 3′; reverse, 5′ GCGAACTATCAGCTATACG 3′) and 2 (forward, 5′ GCA GGTTATTGTTGCTCCT 3′; reverse, 5′ GTCGTGGTATCA CTGAAGC 3′) amplifying fragments of *pilPQ* (569 bp) and *pilSUV* (1223 bp), respectively (6). Amplimers were analyzed by agarose gel electrophoresis. One hundred sixty-eight strains (62.2% of the total) were found to be *pil* positive and all generated an amplification product of the expected size with each primer set. As shown in Fig. 1, *pil* genes were detected in strains from a broad natural reservoir, with a notably high frequency (≈80%) for those recovered from water. PCR analyses were negative for all O:1a strains (which are mainly isolated in European countries [11]), whereas the non-O:1a strains yielded amplimers with a frequency that varied according to the O serotype and ranged from 46.7% (O:4b) to 96.7% (O:2c and O:5b). Like *ypm* (19), *pil* genes were predominantly distributed in isolates from Asian rather than non-Asian countries (74.5 versus 29.2%, $P < 10^{-7}$). However, in light of the geographical disparity of the O serotypes, this difference should be interpreted with caution. Indeed, O serotypes with the highest proportion of *pil*-positive strains (O:2c, O:5b, and to a lesser extent O:2b) were those with the highest percentages of Asian isolates (100, 100, and 82.8%, respectively). We

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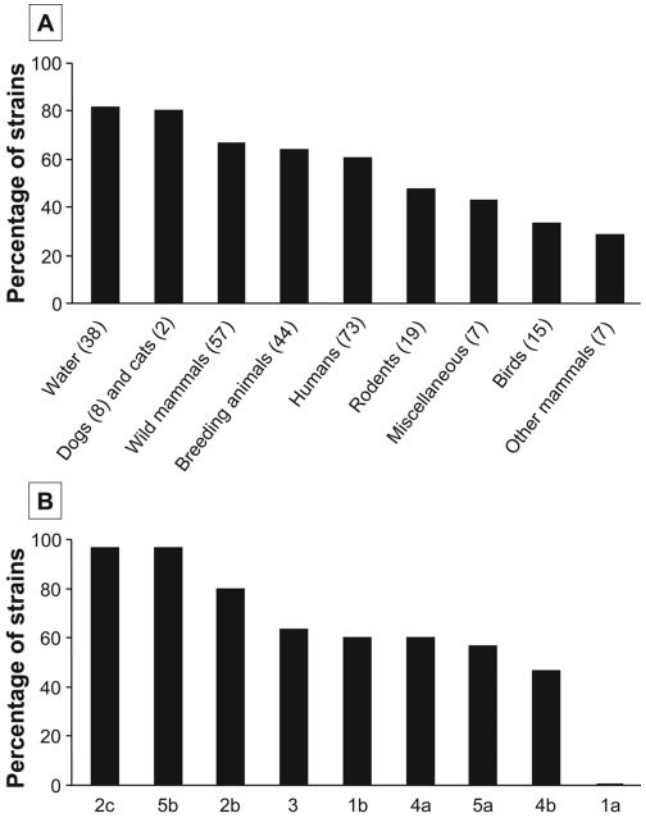

FIG. 1. *pil* distribution in *Y. pseudotuberculosis* strains in relation to their ecological niche (A) and O-antigen type (B). The number of strains in each class is given in parentheses. Water sources: mountain water (20), well water (5), river water (2), unknown origin (11). Wild mammal sources: raccoon dog (32), marten (7), hare (6), deer (5), reindeer (2), fox (2), mole (2), wild boar (1). Breeding animal sources: pig (29), rabbit (8), ruminant (7). Rodent sources: wild mouse (11), guinea pig (2), house rat (4), wild rat (1), unspecified (1). Miscellaneous sources: fish (2), plant (2), soil (1), unknown (2). Bird sources: pet birds (8), duck (2), pigeon (2), unspecified (3). Other mammal sources: monkey (3), goat (2), horse (2).

next examined the distribution of *pil* and *ypm* genes in the *Y. pseudotuberculosis* collection (Table 1). Of the 168 *pil*-positive strains, 135 (80.4%) contained *ypm*, whereas the gene was detected in only 46 out of 102 (45.1%) *pil*-negative strains (odds ratio [OR], 4.98; 95% confidence interval [95% CI], 2.79 to 8.93; $P < 10^{-7}$). Statistical tests also showed that *pil* genes were more often than not associated with *ypm* in both Asian and non-Asian isolates (OR, 2.52; 95% CI, 1.12 to 5.63; $P = 0.013$; and OR, 4.33; 95% CI, 1.30 to 14.80; $P = 0.005$; respectively). In contrast, the HPI was present in 19 out of 168 (11.3%) *pil*-positive strains and in 46 out of 112 (41.1%) *pil*-negative strains (OR, 0.25; 95% CI, 0.16 to 0.40; $P < 10^{-7}$). The lower frequency of the HPI in Pil$^+$ *Y. pseudotuberculosis* strongly argues that these strains have no tendency to accumulate virulence genes nor to laterally acquire mobile genetic elements more readily.

TABLE 1. Distribution of *pil* and *ypm* genes in 270 *Y. pseudotuberculosis* strains

Genotype	No. of strains (%)			
	Total	Asian origin	Non-Asian origin	Unknown area origin
pil-positive *ypm*-positive	135 (80.4)	123 (84.2)	12 (57.1)	
pil-positive *ypm*-negative	33 (19.6)	23 (15.8)	9 (42.9)	1
pil-negative *ypm*-positive	46 (45.1)	34 (68.0)	12 (23.5)	
pil-negative *ypm*-negative	56 (54.9)	16 (32.0)	39 (76.5)	1

The present work thus establishes that *pil* and *ypm* are specifically associated in *Y. pseudotuberculosis*. *pil* genes are present in both *Y. pseudotuberculosis* and *Yersinia enterocolitica* (8) and were probably acquired by the common *Yersinia* ancestor through PAI (YAPI) transfer (F. Collyn, C. A. Roten, L. Guy, M. Simonet, and M. Marceau, submitted for publication). In contrast, *ypm* is harbored by *Y. pseudotuberculosis* but not by *Y. enterocolitica* (5) and the gene may have arisen (most probably by transduction) after divergence from the *Yersinia* progenitor. It is therefore tempting to propose an evolutionary scenario for the origin of *Y. pseudotuberculosis* superantigen producers reminiscent of that suggested for the emergence of enterotoxinogenic *Vibrio cholerae* (9). Firstly, the *Yersinia* would have acquired the *pil* operon (the counterpart in *V. cholerae* is the PAI [VPI]-borne *tcp* operon) via YAPI transfer before it speciated, and secondly, some Pil$^+$ strains would have been infected and lysogenized by an *ypm*-encoding prophage (the counterpart in *V. cholerae* is a filamentous temperate phage CTXϕ encoding cholera toxin) using type IV pili as receptors. A previous in vitro study demonstrated that the PAI can be lost from *Y. pseudotuberculosis* (6), and nonproducers and superantigen producers lacking the *pil* operon would most probably result from spontaneous excision of YAPI. Characterization of the phage family that may have transferred *ypm* is a prerequisite step for validating the proposed evolutionary model. However, since there are no prophage remnants close to *ypm* (4), this identification cannot be driven using a helper bacteriophage. Comparative genometric analysis of the *ypm* locus with streptococcal and staphylococcal superantigen-encoding genes (especially those of phage origin) represents an alternative way of shedding light on this latter point.

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