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In Vivo Comparison of Avirulent Vwa$^-$ and Pgm$^-$ or Pst^r Phenotypes of Yersiniae†

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The abilities of *Yersinia pestis* to undergo restriction in Ca^{2+}-deficient medium with concomitant production of V and W antigens (Vwa$^+$) and to absorb exogenous pigments (Pgm$^+$) are established virulence factors. Mutation of *Y. pestis* to Pgm$^-$ is known to promote resistance to pesticin (Pst^r) and reduced lethality by peripheral routes of injection. Vwa$^+$ Pgm$^-$ isolates of *Y. pestis* were shown in this study to retain virulence in mice when injected intravenously. Although Pgm$^-$ in appearance, wild-type cells of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* may also be sensitive to pesticin. Pst^r mutants of Vwa$^+$ strains of these species were similarly of reduced virulence, especially by peripheral routes of injection. The consequences of mutation to Vwa$^-$ and Pgm$^-$ or Pst^r on growth and persistence in vivo were determined. After intravenous injection, Vwa$^+$ yersiniae of all species exhibited sustained growth in mouse spleen, liver, and lung and accumulated in blood. Septicemia was not observed after similar injection of Vwa$^-$ mutants which were unable to maintain comparable rates of net increase in tissues. Mutation to Pgm$^-$ or Pst^r did not influence proliferation but resulted in enhanced clearance from organs. It is known that reticuloendothelial cells serve as favored sites of replication for all wild-type yersiniae. Our results are consistent with the hypothesis that the Vwa$^+$ phenotype favors growth within macrophages and that the Pgm$^+$ and pesticin-sensitive phenotypes permit long-term, probably extracellular, retention within organs. Virulence in standard animal models (mice, rats, and guinea pigs) was not correlated with resistance to the bactericidal action of serum.

The central role of iron in influencing the outcome of bacterial infection (39, 40) was first observed with *Yersinia pestis* (22), the causative agent of bubonic plague. Wild-type organisms absorbed exogenous pigments, including hemin (21) and Congo red (35), on solid media and thus formed dark colonies (Pgm$^+$). Isogenic Pgm$^-$ mutants grew as light colonies and were avirulent by intraperitoneal injection in mice unless the animals received sufficient iron to saturate serum transferrin (22). Later work showed that both Pgm$^+$ and Pgm$^-$ organisms accumulated Fe^{3+} by an inducible siderophore-independent process and could utilize hemin as a sole source of iron (29). Similar transport processes were defined in wild-type but phenotypically Pgm$^-$ *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (29). Pgm$^+$ *Y. pestis* possess unique outer membrane peptides (34) and high-molecular-weight cytoplasmic iron storage componets (unpublished observations); these structures are probably encoded by chromosomal genes (16, 34). In addition to *Y. pestis*, wild-type shigellae and neisseriae can be Pgm$^+$ (28).

A 6-megadalton plasmid (16, 34) mediates the ability of *Y. pestis* to produce the bacteriocin pesticin (Pst$^+$) (2, 15, 20) and genetically linked coagulase and fibrinolytic activities (1, 10). As a consequence, pesticinogenic organisms are extremely lethal by peripheral routes of injection. This high level of invasiveness was markedly reduced upon loss of the pesticin plasmid, although full virulence was retained if such Pst$^-$ mutants were injected intravenously (9). Like Pgm$^-$ isolates (22), Pst$^-$ yersiniae could be phenotypically restored to virulence via intraperitoneal injection in mice receiving sufficient iron to saturate serum transferrin (9). Sensitivity of *Y. pestis* to the antibacterial activity of pesticin (Pst^s) required both mutation to Pst$^-$, resulting in loss of cellular immunity (14), and retention of the Pgm$^+$ phenotype, which evidently provides absorption sites for the bacteriocin (6). This relationship, which permitted determination of a mutation rate from Pgm$^+$ to Pgm$^-$ of 10^{-5} (6), indicates that pesticin-resistant (Pst^r) mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* share one or more deficiencies common to Pgm$^-$ isolates of *Y. pestis*.

Whereas the Pgm$^+$ and Pst$^+$ phenotypes are limited to *Y. pestis*, expression of an in vitro nutritional requirement for Ca^{2+} and attendant ability to produce virulence or V and W antigens are shared by all wild-type yersiniae (Vwa$^+$). These antigens are mediated by approximately 45-megadalton plasmids, and they are distinct from the ancillary outer membrane peptides of Vwa$^+$ *Y. pseudotuberculosis* and *Y. enterocolitica*. The biological roles of the virulence antigens are unknown. Some properties attributed to expression of the Vwa$^+$ phenotype in various yersiniae are abilities to prevent phagocytosis, grow within fixed macrophages, cause host cell damage in vitro, promote adhesion to epithelial cells, and resist the bactericidal activity of serum (8). The purpose of this study was to define the consequences of mutation to Vwa$^-$ and to Pgm$^-$ or Pst^r on proliferation and retention of yersiniae in vivo.

MATERIALS AND METHODS

Bacteria. Unless stated otherwise, organisms used in experiments were *Y. pestis* KIM (5, 34), *Y. pseudotuberculosis* PB1 (30), and *Y. enterocolitica* WA (11, 30). Isogenic Vwa$^-$ mutants (16) of the organisms were selected on magnesium oxalate agar (19), and Pgm$^-$ mutants of *Y. pestis* were obtained on Congo red agar (35). Pst^r mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* were isolated on solid medium containing homogenous pesticin (20) by the procedure used previously for determining the mutation rate

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TABLE 1. Consequences of mutation of Vwa$^-$ and Pgm$^-$ or Pst^r on the 50% lethal dose of yersiniae in mice

Organism	Phenotype	50% lethal dose[a]		
		i.v.	i.p.	s.c.
Y. pestis KIM	Vwa$^+$ Pgm$^+$[b]	$<10^1$	$<10^1$	$<10^1$
	Vwa$^+$ Pgm$^-$	1.5×10^1	$>10^7$	$>10^7$
	Vwa$^-$ Pgm$^+$	$>10^7$	$>10^7$	$>10^7$
	Vwa$^-$ Pgm$^-$	$>10^7$	$>10^7$	$>10^7$
Y. pseudotuberculosis PB1	Vwa$^+$ Pst^s	2.0×10^1	2.4×10^4	1.6×10^5
	Vwa$^+$ Pst^r	10^3	1.2×10^6	$>10^7$
	Vwa$^-$ Pst^s	$>10^7$	$>10^7$	$>10^7$
	Vwa$^-$ Pst^r	$>10^7$	$>10^7$	$>10^7$
Y. enterocolitica WA	Vwa$^+$ Pst^s	10^2	2.1×10^2	2.3×10^3
	Vwa$^+$ Pst^r	2.3×10^3	2.3×10^4	1.1×10^5
	Vwa$^-$ Pst^s	$>10^7$	$>10^7$	$>10^7$
	Vwa$^-$ Pst^r	$>10^7$	$>10^7$	$>10^7$

[a] i.v., intravenous injection; i.p., intraperitoneal injection; and s.c., subcutaneous injection.
[b] The 50% lethal dose for wild-type *Y. pestis* KIM (Vwa$^+$ Pgm$^+$) was established in reference 5.

of *Y. pestis* to Pgm$^-$ (6) or by the method recently described for obtaining Pst^r clones of *Escherichia coli* (17). Auxotrophic mutants were induced by growth in heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 2-aminopurine (10 mg/ml) at 26°C in slanted tubes (20 by 180 mm) and identified by replica plating onto minimal synthetic medium either directly or after enrichment by selection with penicillin (5).

Cultivation. Slopes of tryptose blood agar base (Difco) were incubated for 1 day (*Y. pseudotuberculosis* or *Y. enterocolitica*) or 2 days (*Y. pestis*) at 26°C after inoculation from stock cultures of buffered glycerol maintained at −20°C as previously described (1). Organisms were suspended and appropriately diluted in 0.033 M potassium phosphate buffer (pH 7.0) (phosphate buffer) for use in injections.

Animals. Outbred female Swiss-Webster mice, 6 weeks of age, weighing 25 to 30 g (Harlan-Sprague Dawley, Inc., Indianapolis, Ind.), were used in all experiments. The animals received water and commercial food ad libitum in a room controlled at 18.5°C and 35% humidity.

Growth in vivo. At intervals after intravenous injection, mice were anesthetized and sacrificed by aseptic scission of the axillary artery, thereby permitting removal of blood samples with a micropipette. Lungs, liver, and spleen were then aseptically removed and homogenized separately at a concentration of 100 mg/ml of phosphate buffer. Appropriate dilutions of these preparations in phosphate buffer were cultured on tryptose blood agar base for 1 to 2 days at 26°C; the limits of detection by this procedure were 10 CFU/ml in blood and 100 CFU/g in organs. Groups of five mice were sacrificed at intervals, and proliferation was evaluated by comparison of group mean values. The method of Behrens-Kärber (25) was used to assay the 50% lethal dose. Surviving mice used in these determinations were maintained for at least 3 weeks after injection.

Sensitivity to serum. The bactericidal activity of fresh mouse, rat, guinea pig, rabbit, and human sera was assayed as previously described, except that 20% serum was utilized (30). Blood for preparation of mouse serum was collected by aseptic scission of the axillary artery.

RESULTS

The effect of mutation to Vwa$^-$ and Pgm$^-$ or Pst^r on the 50% lethal dose of yersiniae in mice was compared by intravenous and peripheral routes of infection (Table 1). Regardless of species or method of injection, the 50% lethal dose of Vwa$^-$ isolates exceeded 10^7 organisms. However, considerable variation in lethality occurred in Pgm$^-$ or Pst^r mutants of Vwa$^+$ isolates, and these differences were dependent on the route of injection.

For example, in accordance with earlier findings (9), the 50% lethal dose of wild-type *Y. pseudotuberculosis* was significantly less when injected intravenously than when given by the intraperitoneal or subcutaneous route. Mutation of Vwa$^+$ cells of this species to Pst^r resulted in an approximate 100-fold decrease in lethality by all routes of injection. Wild-type *Y. enterocolitica* was somewhat more lethal than *Y. pseudotuberculosis* via peripheral routes of injection, but Pst^r mutants of the former were also of reduced virulence. It has long been established that Pgm$^-$ mutants of Vwa$^+$ *Y. pestis* are avirulent when injected intraperitoneally (21, 22). An important finding of the present investigation was the observation that mutation of wild-type *Y. pestis* to Pgm$^-$ did not significantly increase the intravenous 50% lethal dose. All of 20 additional Vwa$^+$ Pst^r clones of *Y. pseudotuberculosis* and *Y. enterocolitica*, including genetically marked auxotrophs, exhibited similarly reduced lethality after intraperitoneal injection, and four distinct Vwa$^+$ Pgm$^-$ isolates of *Y. pestis* (strains KIM, EV76, G25, and M23) exhibited high virulence when injected by the intravenous route; these responses are therefore typical of the species.

To define the consequences of mutation to Vwa$^-$ and to Pgm$^-$ or Pst^r, mice were injected intravenously with 10^2 to 10^3 Vwa$^+$ and 10^6 Vwa$^-$ yersiniae. As shown in Fig. 1A, cells of wild-type *Y. pseudotuberculosis* were rapidly removed from the vascular system but continued to proliferate in liver, spleen, and lungs. As the infection progressed with time, the organisms reappeared within blood, evidencing pronounced septicemia at the time of death. A very similar pattern was detected with wild-type *Y. enterocolitica* (Fig. 1B), except that the infection was more prolonged and the organisms achieved higher populations within organs.

Although wild-type *Y. pestis* was not examined, intravenously injected Vwa$^+$ Pgm$^-$ mutants of this species also grew rapidly within organs and caused pronounced septicemia (Fig. 2A). Death occurred rapidly after intravenous injection even though terminal populations were less than those observed with wild-type *Y. pseudotuberculosis* and *Y. enterocolitica* (Fig. 1). This difference was assumed to

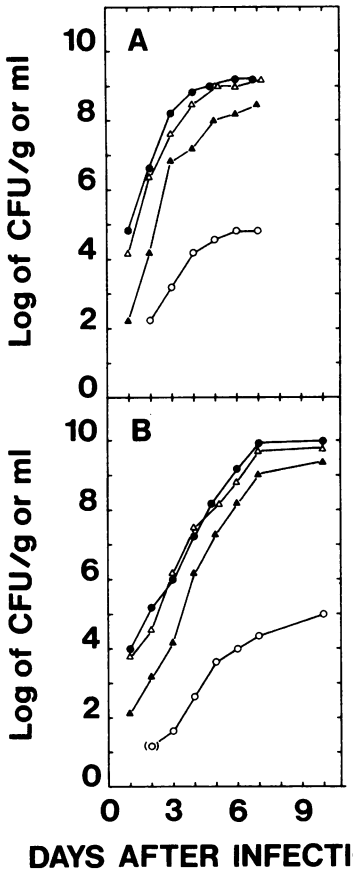

FIG. 1. Populations in blood (○), spleen (●), liver (△), and lungs (▲) of wild-type (Vwa^+ Pst^s) *Y. pseudotuberculosis* PB1 (A) and *Y. enterocolitica* WA (B) after respective intravenous injection of 10^2 and 10^3 viable cells. The value in parentheses was not statistically significant.

reflect the presence of plague exotoxin (4). Patterns observed for Vwa^+ Pst^r *Y. pseudotuberculosis* (Fig. 2B) and *Y. enterocolitica* (Fig. 2C) were analogous, except that septicemia and retention within organs were less pronounced than observed with the wild type (Fig. 1). As a consequence, the host eventually contained the infection and then eliminated the invading organisms.

Maximum populations of Vwa^- yersiniae detected within organs scarcely exceeded the number of organisms provided by injection. However, if these Vwa^- isolates remained Pgm^+ or Pst^s, they were retained for an extended period within the host (Fig. 3). In contrast, those Vwa^- mutants which were also Pgm^- or Pst^r were rapidly cleared from organs (Fig. 4).

To test the hypothesis that proliferation in vivo is associated with ability to resist the antibacterial activity of serum, the ability of Vwa^- yersinia to survive within fresh 20% mouse, rat, guinea pig, rabbit, and human sera was determined. As expected, all such mutants were fully resistant to sera from mice, rats, and guinea pigs (Table 2); patterns of sensitivity to rabbit and human sera were similar to those reported earlier (26, 27, 30). These findings demonstrate that sensitivity to serum is not a significant parameter in the standard mouse, rat, and guinea pig models traditionally used to define virulence in yersiniae.

DISCUSSION

As already noted, the physiological advantages conferred in vivo by expression of the Vwa^+, Pgm^+, and Pst^s phenotypes are not fully resolved. Satisfactory explanations of their significance must be consistent with the following sequence of events, which is common to *Y. pestis*, *Y. pseudotuberculosis*, and invasive isolates of *Y. enterocolitica*. After penetration of the dermal barrier by flea bite (*Y. pestis*) or the gastrointestinal tract (*Y. pseudotuberculosis* and *Y. enterocolitica*), the organisms enter the highly iron-deficient environments constituting lymph, blood, and inter-

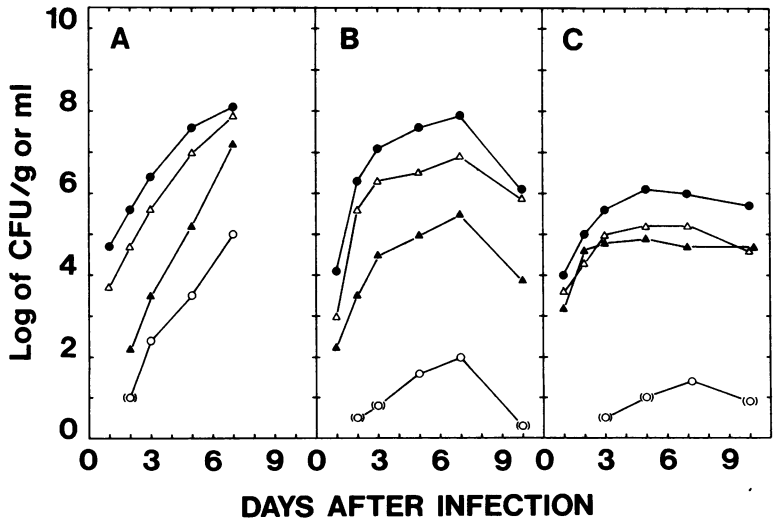

FIG. 2. Populations in blood (○), spleen (●), liver (△), and lungs (▲) of Vwa^+ and Pgm^- or Pst^r *Y. pestis* KIM (A), *Y. pseudotuberculosis* PB1 (B), and *Y. enterocolitica* WA (C) after respective intravenous injection of 10^2, 10^2, and 10^3 viable cells. The values in parentheses were not statistically significant.

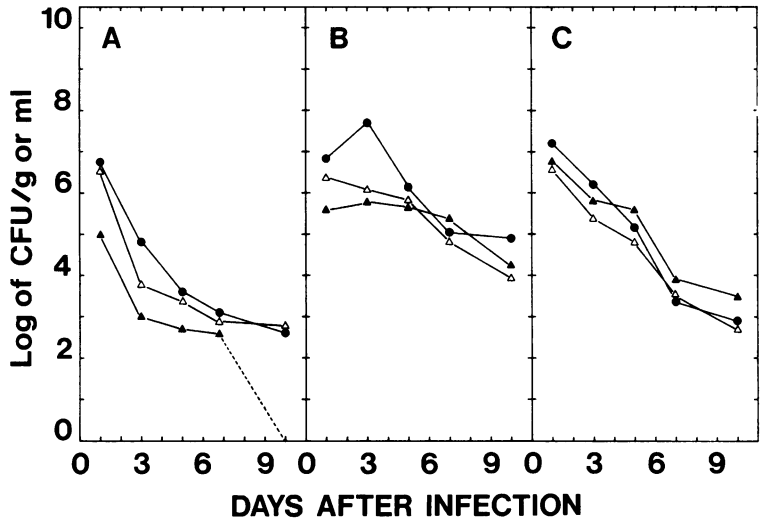

FIG. 3. Populations in spleen (●), liver (△), and lungs (▲) of Vwa$^-$ and Pgm$^+$ or Pst^s *Y. pestis* KIM (A), *Y. pseudotuberculosis* PB1 (B), and *Y. enterocolitica* WA (C) after intravenous injection of 10^6 viable cells. The dashed line indicates decrease to nondetectable level; septicemia was never observed.

stitial fluid. Proliferation in these extracellular niches is limited, and, unless the organisms obtain residence within appropriate nonprofessional phagocytes (3, 13), they become vulnerable to phagocytosis and killing by neutrophils. However, all yersiniae are capable of growth within macrophages (12, 24, 32, 36–38), which serve as favored sites of multiplication (24, 36–38). An important consequence of this dependence on macrophages is a correlation between high virulence in experimental infections and ability to gain access to the reticuloendothelial system. As shown in this study and in earlier work (9), all wild-type yersiniae are highly virulent by intravenous injection, a route which permits immediate interaction with fixed macrophages.

An important new observation was the finding that Vwa$^+$ Pgm$^-$ *Y. pestis* was as virulent as the wild type when injected intravenously into mice. This discovery will permit comparative studies of all yersiniae in laboratories not equipped for containment of wild-type *Y. pestis*. It is now evident that Pgm$^-$ and Pst$^-$ mutants of this species are similar in that both are virulent by intravenous injection and that invasiveness of both by intraperitoneal injection is phenotypically restored in mice receiving exogenous Fe^{3+} (9, 22). These responses may reflect distinct abilities of injected iron to overcome some lesion in iron metabolism present in Pgm$^-$ mutants and to overcome nonspecific mechanisms of host defense, thereby enabling Pst$^-$ mutants to eventually encounter target macrophages (7). Tacit to this argument is the understanding that extracellular environments are highly deficient in available iron, whereas the cation may be easily obtained by bacteria during intracellular residence. This partition of iron in vivo was also invoked to account for siderophore-independent iron transport in legionellae (31), another facultative intracellular parasite.

Comparison of the fate of Pgm$^-$ and Pst^r isolates in vivo

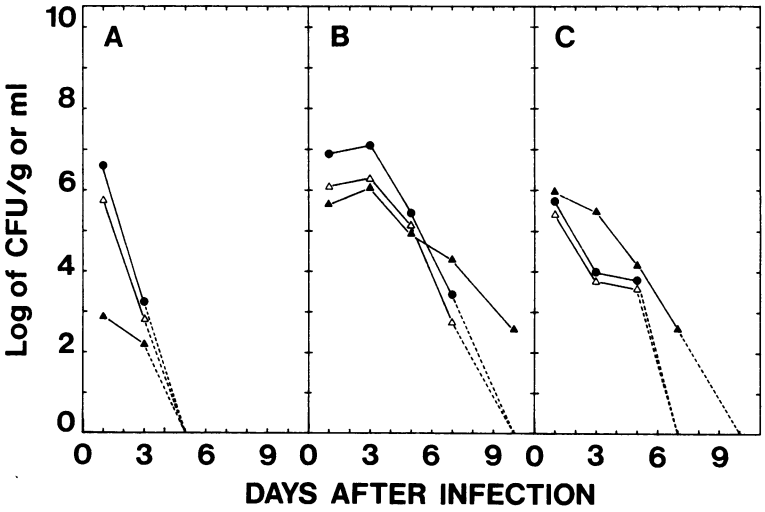

FIG. 4. Populations in spleen (●), liver (△), and lungs (▲) of Vwa$^-$ and Pgm$^-$ or Pst^r *Y. pestis* KIM (A), *Y. pseudotuberculosis* PB1 (B), and *Y. enterocolitica* WA (C) after intravenous injection of 10^6 viable cells. The dashed lines indicate decreases to nondetectable levels; septicemia was never observed.

TABLE 2. Resistance of yersiniae to various sera (20%) after growth in vitro at 26 and 37°C[a]

Organism	Vwa	Growth temp (°C)	% Survival after incubation for 1 h at 37°C in:					
			Phosphate buffer	Human serum	Rabbit serum	Rat serum	Mouse serum	Guinea pig serum
Y. pestis KIM	+	26	103	175	97	139	124	122
	0		117	137	138	124	126	117
	+	37	137	141	124	125	130	133
	0		126	147	123	127	109	128
Y. pseudotuberculosis PB1	+	26	69	0.26	40	57	193	129
	0		163	2.5	47	209	407	327
	+	37	41	160	97	87	91	117
	0		94	155	99	106	131	127
Y. enterocolitica WA	+	26	106	0.35	65	67	134	137
	0		104	0.15	0.36	133	129	161
	+	37	76	187	121	140	108	109
	0		117	0.05	61	133	124	141

[a] Conditions of growth and assay are defined in reference 29, except that 20% sera were used.

with the wild type revealed that the mutants were rapidly cleared from organs. This failure to persist in tissues could reflect an inability to acquire extracellular iron. It could also reflect mutational loss in Pgm^- and Pst^r organisms of an adhesin or other determinant capable of promoting retention in tissues. This question may be resolved by further study of the Pst^r phenotype which, in *E. coli*, can involve both resistance and tolerance. Pesticin-resistant mutants of this species evidenced no detectable lesion in accumulation of siderophores or storage of iron. In contrast, pesticin-tolerant *E. coli* was $TonB^-$, $ExbB^-$, or $ExbC^-$ and thus blocked in all high-affinity processes of iron transport (17).

The Vwa^+ phenotype, in contrast, was required for proliferation in vivo. These findings are essentially in accord with those reported earlier in similar experiments with single species, in which the significance of the Pgm^+ or Pst^s phenotype was not considered (11, 18). It is now amply clear that the Vwa^+ factor is necessary for sustained multiplication in organs and for attendant septicemia. The latter is assumed to result from spillover of bacteria into the vascular system from intracellular and interstitial reservoirs rather than from significant multiplication in blood which, at least in mice, is bacteriostatic (due to iron deficiency) but not bactericidal (23). To emphasize that the results obtained in this study were not attributable to serum resistance, we showed that mouse, rat, and guinea pig sera were not inhibitory to yersiniae under conditions in which rabbit and human sera were lethal. Accordingly, serum resistance in standard animal systems is not a parameter involved in expression of virulence. These findings are not, of course, in conflict with the hypothesis that the ancillary outer membrane peptides of *Y. enterocolitica* account for resistance to bactericidal sera (26). This study further showed that once peripheral barriers were bypassed by intravenous injection, wild-type cells of *Y. pseudotuberculosis* and *Y. enterocolitica* were capable of sustained organ-associated growth typical of highly virulent *Y. pestis*.

Not yet resolved is identification of the gene product(s) encoded on the Vwa plasmid which accounts for expression of virulence. Since the ancillary outer membrane peptides are not produced by *Y. pestis* (33), these structures are unlikely candidates. The V and W antigens are now known to be synthesized by all wild-type yersiniae, including the less-virulent serotypes of *Y. enterocolitica* (30). Accordingly, one or both of these components probably accounts for the differences in net in vivo proliferation described in this report. Development of an in vitro or cell culture system capable of reflecting the in vivo differences described above would facilitate definition of the Vwa^+, Pgm^+, and Pst^s phenotypes.

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