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Plague Virulence Antigens from *Yersinia enterocolitcia*

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The virulence of *Yersinia enterocolitica*, biotype 2, serotype O:8, in mice is related to its ability to produce plague V and W antigens. V and W antigens in *Y. enterocolitica* are shown to be immunologically identical to the previously described V and W antigens of *Yersinia pestis* and *Yersinia pseudotuberculosis*.

Release of exotoxins, production of capsules, and use of devices that confer a high degree of mobility are mechanisms used by extracellular microbial parasites to avoid uptake and killing by leukocytes. In contrast, obligate intracellular parasites are readily phagocytized but escape destruction by either inhibiting degranulation, with attendant release of lysozomal enzymes, or by penetrating directly into the cytoplasm of the host cell. Less is known about virulence mechanisms of facultative intracellular parasites which can grow in extracellular spaces or within macrophages. Classical examples of this latter group are *Yersinia pestis*, the causative agent of bubonic plague, and the closely related but less virulent *Yersinia pseudotuberculosis* (2). A major determinant of virulence in *Y. pestis* and *Y. pseudotuberculosis* is the ability to produce the plague V and W or virulence antigens (Vwa^+) (3, 5). The role of these proteins in causing disease is unknown; they may provide protection against phagocytosis by leukocytes possessing O_2-dependent killing mechanisms (4) or serve to permit intracellular growth within host cells lacking these mechanisms (1). The purpose of this report is to present information demonstrating that the third species of the genus, *Yersinia enterocolitica*, an enteric pathogen of humans, can share the ability to produce V and W antigens and that their production directly correlates with virulence in mice.

In vitro conditions for near optimal production of V and W antigens in yersiniae are aeration at 37°C in an enriched medium which lacks Ca^{2+} but contains at least 20 mM Mg^{2+} (1). These conditions mimic intracellular fluid with respect to the concentration of these cations and is bacteriostatic to Vwa^+ but not Vwa^- organisms. Growth of Vwa^+ yersiniae does, however, occur at 37°C if the amount of Ca^{2+} that is contained in mammalian plasma (2.5 mM) is added to the medium; this calcium requirement does not exist at 26°C where V and W are not produced. Accordingly, culture at 37°C in Ca^{2+}-deficient media is selective for growth of Vwa^- mutants which, in the case of *Y. pestis*, arise at the high rate of 10^{-4}, thereby contributing to a rapid shift to avirulence (1).

Y. enterocolitica is a rather recently defined organism that has received considerable attention as an important cause of gastroenteritis and acute terminal ileitis in humans (10). In contrast to other yersiniae, *Y. enterocolitica* is generally avirulent in laboratory animals (12), although a few exceptions have been reported. Among the latter is *Y. enterocolitica* strain WA (6) which exhibits an intravenous 50% lethal dose of <500 colony-forming units in mice (7). Since this organism was isolated and subsequently transferred at 25°C, we assumed that potential population shifts to avirulence would not have occurred if it was similar to other members of the genus. The organisms were grown at 25°C on slopes of blood agar base (BBL Microbiology Systems, Cockeysville, Md.), removed in 0.033 M potassium phosphate buffer (pH 7.0), and then plated at appropriate dilutions on blood agar base containing added $CaCl_2$ (4.0 mM) or $MgCl_2$ (20 mM) plus sodium oxalate (20 mM). After incubation for 24 h at 26°C, colonies (2 mm in diameter) were observed at equal frequency on both media. After similar incubation at 37°C, the same number of colonies (2 mm in diameter) occurred on the medium supplemented with Ca^{2+}, whereas only 1 to 0.1% of the plated bacteria formed colonies (4 mm in diameter) on the oxalated media. Upon subculture, cells from the latter grew at equal frequency on Ca^{2+}-deficient media. Independent clones of the large Ca^{2+}-independent variant were isolated and shown to possess biochemical and cultural properties identical to the prototroph. These isolates, however, were avirulent in 8-week-old Tru:ICR mice infected intraperitoneally (Table 1).

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The effect of Ca^{2+} on growth of the phototroph and an avirulent mutant selected on oxalated blood agar base was compared to that of Vwa^+ and Vwa^- *Y. pestis* EV76 and *Y. pseudotuberculosis* PB1 in the defined liquid medium of Higuchi et al. (9). The latter was modified by elimination of D-xylose and all D-amino acids except D-alanine, reduction of K_2HPO_4 to 2.5 mM, addition of 2.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) to maintain buffering capacity, and substitution of equimolar NH_4Cl for ammonium acetate; in all experiments the concentration of $MgCl_2$ was 20 mM. Organisms were agitated (50 ml/500-ml Erlenmeyer flask at 200 rpm on a model G76 Gyratory shaker, New Brunswick Scientific Co., New Brunswick, N.J.) at 26°C for about 10 generations and then transferred to fresh medium with or without addition of Ca^{2+} (4.0 mM). Increase in optical density was then recorded upon additional incubation at 26 or 37°C. As shown in Fig. 1, all yersiniae multiplied at 26°C in the absence of added Ca^{2+}. In contrast, however, growth of prototrophic *Y. enterocolitica*, Vwa^+ *Y. pseudotuberculosis*, and especially Vwa^+ *Y. pestis* was markedly inhibited at 37°C in Ca^{2+}-deficient medium compared to that of their respective avirulent mutants.

In a separate experiment, the bacteria were harvested by centrifugation (17,000 × *g* for 15 min) after 12 h of incubation at 37°C without added Ca^{2+}, suspended in phosphate buffer, and disrupted by treatment with a 100-W ultrasonic disintegrator (MSE Ltd., London, England) for 60 s. Insoluble debris was removed by centrifugation and the resulting extracts were adjusted to about 5 mg of protein per ml in phosphate buffer and assayed for virulence antigens by gel diffusion, using standards purified essentially as described by Lawton et al. (11). Both V (Fig. 2) and W (not illustrated) were detected in virulent but not avirulent *Y. enterocolitica* WA, using an exhaustively absorbed rabbit antiserum against Vwa^+ *Y. pestis*. (This serum gave a reaction of identity with anti-VW serum obtained from T. W. Burrows.) Additional studies failed to reveal other established or assumed plague virulence determinants in *Y. enterocolitica* WA (e.g., ability to produce capsular or fraction 1 antigen, murein toxin, coagulase, and fibrinolysin, or to absorb exogenous hemin [2]). The only other unusual property noted for strain WA was its sensitivity to pesticin, a bacteriocin produced by

TABLE 1. *Virulence of Ca^{2+}-independent variants of Y. enterocolitica WA for mice*[a]

Variant	Mortality
LOX-1	0/10[b]
LOX-2	1/10
LOX-3	0/10
LOX-4	0/10
LOX-5	0/10
LOX-6	0/10
LOX-7	0/10
LOX-8	0/10
WA parent	10/10
WA parent[c]	7/10

[a] An amount of 2.5 × 10^7 colony-forming units, injected intraperitoneally. Study terminated at day 30.
[b] Number dead/total.
[c] An amount of 5 × 10^2 colony-forming units, injected intraperitoneally.

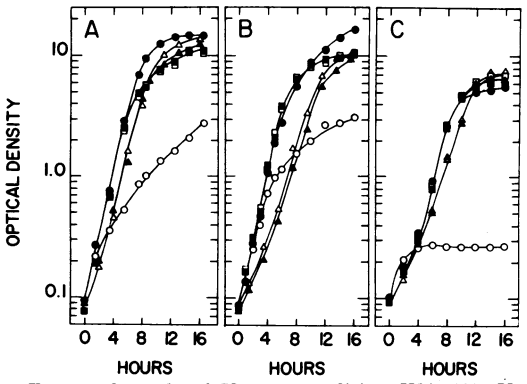

FIG. 1. *Growth of Y. enterocolitica WA (A), Y. pseudotuberculosis PB1 (B), and Y. pestis EV76 (C) in synthetic medium containing 20 mM Mg^{2+} at 37°C without added Ca^{2+} (○, wild type; ●, Ca^{2+}-independent), at 26°C without added Ca^{2+} (▲, wild type; △, Ca^{2+}-independent), and at 37°C with added 4.0 mM Ca^{2+} (■, wild type; □, Ca^{2+}-independent).*

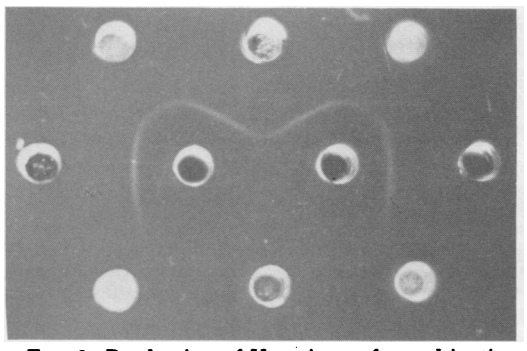

FIG. 2. *Production of V antigen after cultivation at 37°C in Ca^{2+}-deficient medium containing 20 mM Mg^{2+} by Vwa^+ Y. pestis EV76 (upper left), Y. pseudotuberculosis PB1 (upper center), and prototrophic Y. enterocolitica WA (upper right) but not by Ca^{2+}-independent Y. pestis EV76 (lower left), Y. pseudotuberculosis PB1 (lower center), or Y. enterocolitica WA (lower right); center wells contained monospecific anti-V serum, and wells at the far left and right contained homogenous V antigen purified from Vwa^+ Y. pestis EV76.*

Y. pestis which infrequently attacks isolates of *Y. enterocolitica* (2).

The discovery that *Y. enterocolitica* WA is Vwa^+ does not, of course, indicate that possession of this determinant is typical of the species. Nevertheless, clinical procedures used for recovery of this organism frequently involve primary isolation at 37°C. If the medium used for this purpose lacks sufficient Ca^{2+} to ensure growth of Vwa^+ organisms, which may often be the case, then only avirulent cells would be recovered. Attention to the possibility of inadvertent selection of Vwa^- *Y. enterocolitica* during primary isolation may help resolve the question of whether or not this species is normally Vwa^+ as it exists in nature.

The observed high frequency of conversion of Vwa^+ *Y. enterocolitica* WA to Vwa^- (0.1 to 1%) was greater than that of most chromosomal mutations. The probability that Vwa^+ *Y. enterocolitica* strains carry a plasmid which codes for virulence antigens gains support from recent work by Gemski et al. (8). These workers have demonstrated a 42-megadalton plasmid in *Y. enterocolitica* WA and other *Y. enterocolitica* strains, the presence of which correlates directly with virulence of the strain for mice.

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