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# Localization in *Yersinia pestis* of Peptides Associated with Virulence†

SUSAN C. STRALEY‡ AND ROBERT R. BRUBAKER*

*Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824*

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An avirulent guanine auxotroph of wild-type *Yersinia pestis* was used to select isogenic mutants lacking invasive determinants of virulence including V and W antigens ($Vwa^-$), genetically linked fibrinolysin, coagulase, and pesticin activities ($Pst^-$), and the capacity to absorb exogenous pesticin and pigments including hemin ($Pgm^-$). After growth in environments known to favor expression of these factors by the parent, cells were converted to spheroplasts and disrupted to obtain preparations of cytoplasm; particulate matter was separated into inner and outer membranes by sucrose gradient centrifugation. Peptides present in these fractions were then solubilized and compared by two-dimensional polyacrylamide gel electrophoresis. Components unique to $Vwa^+$ cells, including V antigen, were restricted to the cytoplasmic fraction. In contrast, peptides possibly corresponding to fibrinolysin and coagulase were located primarily within the outer membrane of the $Pst^+$ parent; pesticin was not identified. Similarly, a major outer membrane peptide, possibly representing the pesticin and pigment receptor, was peculiar to the $Pgm^+$ parent. Accordingly, two of the virulence factors examined ($Pst^+$ and $Pgm^+$) can interact directly with host cells or fluids by virtue of their location on the bacterial surface. The remaining cytoplasmic $Vwa^+$ determinant remains a candidate for a regulatory system whose role in pathogenicity is expression of functions required for intracellular survival.

*Yersinia pestis*, the causative agent of bubonic plague, has provided a classic system for the identification of phenotypic determinants necessary for expression of virulence by facultative intracellular parasites (10). Wild-type cells possess at least five such factors which are all required for a minimum intraperitoneal 50% lethal dose of <10 cells in experimental animals (5, 6). These determinants are known or assumed to mediate the biosynthesis of endogenous purines ($Pur^+$), virulence or V and W antigen-associated metabolic step-down at 37°C in $Ca^{2+}$-deficient medium ($Vwa^+$), invasiveness associated with the production of the bacteriocin pesticin and genetically linked fibrinolysin and coagulase activities ($Pst^+$), absorption of organic iron in the form of hemin via a nonspecific receptor of exogenous pigments ($Pgm^+$), and resistance to uptake by professional phagocytes promoted by a protein-lipid-polysaccharide (16) capsular antigen termed fraction 1 ($Fra^+$).

Avirulence resulting from mutation to $Pur^-$ also occurs in *Y. pseudotuberculosis* and *Y. enterocolitica* (unpublished data) and is especially pronounced in isolates of *Y. pestis* that lack the analogs of chromosomal gene products of *Escherichia coli* known to catalyze anabolic conversions past the level of inosine 5′-monophosphate (4). Attenuation in this case probably reflects an inability to grow within normally purine-deficient fluids of the host (1). All of the remaining virulence determinants probably promote interactions with the host which favor bacterial dissemination or ensure survival. This role is straightforward with the antiphagocytic $Fra^+$ factor. Similarly, the $Pgm^+$ determinants may assure a reservoir of iron in the form of hemin, which is nutritionally available to yersiniae (24), within an in vivo environment that is otherwise highly deficient in inorganic iron (29). Like $Pur^+$, the $Fra^+$ and $Pgm^+$ determinants are evidently controlled by chromosomal genes, since their expression was not correlated with a loss of plasmids normally present in *Y. pestis* (14).

In contrast, the $Pst^+$ determinant was detected only in isolates possessing a 6-megadalton plasmid (14) which probably contains structural genes for pesticin and its attendant invasive enzymes (7). Indirect evidence indicates that the receptor for pesticin is either identical to or expressed concomitantly with the pigment receptor of *Y. pestis* (3). Similarly, only yersiniae harboring an approximately 45-megadalton plas-

† Article no. 10062 from the Michigan Agricultural Experiment Station.

‡ Present address: Department of Microbiology, University of Alabama in Birmingham, Birmingham, AL 35294.

mid are $Vwa^+$ (14, 15, 31); this determinant, like $Pur^+$, is common to *Y. pseudotuberculosis* (11) and *Y. enterocolitica* (12). Little is known about the physiological role of this important virulence factor. Its induction in $Ca^{2+}$-deficient medium enriched with $Mg^{2+}$, an environment similar to mammalian intracellular fluid, is consistent with a function permitting growth or at least survival within host cell cytoplasm (5, 6).

The purpose of this study was to characterize cytoplasmic, inner membrane, and outer membrane fractions of a $Pur^-$ auxotroph of *Y. pestis* which possesses the remaining determinants of virulence. Peptides unique to the $Pst^+$ and $Pgm^+$ phenotypes were located in the outer membrane as would be expected in extracellular parasites. In contrast, components peculiar to the $Vwa^+$ phenotype were limited to the cytoplasm, suggesting a regulatory function for a virulence factor that is possibly analogous to those constitutively present in obligate intracellular parasites.

## MATERIALS AND METHODS

**Bacteria.** A $Pur^-$ auxotroph (strain K25) of wild-type *Y. pestis* KIM (4) was used as the parent of $Vwa^-$, $Pst^-$, and $Pgm^-$ mutants. This isolate, blocked in the biosynthesis of GMP (*guaB*), is avirulent (intraperitoneal mouse 50% lethal dose of $>10^7$ cells) and thus is appropriate for routine experimental use when the otherwise potentially lethal $Vwa^+$, $Pst^+$, $Pgm^+$, and $Fra^+$ background is required. Strain K25 reverts to prototrophy at a frequency of $<10^{-9}$ (unpublished data). A $Vwa^-$ isolate was selected at 37°C on magnesium oxalate agar (17). After three monthly transfers at 4°C on slopes of blood agar base (Difco Laboratories, Detroit, Mich.), a $Pst^-$ mutant which represented about 40% of the total population was recovered. A $Pgm^-$ strain was obtained by plating and incubation at 26°C on Congo red agar (28) supplemented with 0.1 mM guanosine.

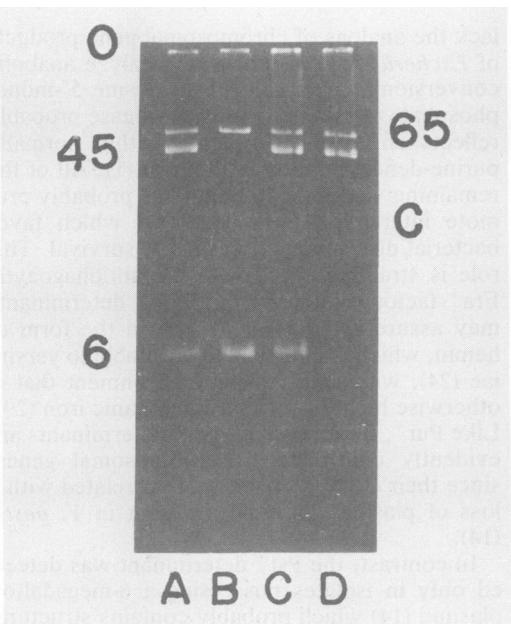



FIG. 1. Plasmids present in *Y. pestis* K25 (A) and in its isogenic $Vwa^-$ (B), $Pgm^-$ (C), and $Pst^-$ (D) mutants. Molecular weights were assigned by electrophoresis in adjoining lanes of plasmids of known molecular weight from *Y. pestis* EV76 (14). O, Origin; C, chromosomal fragments.

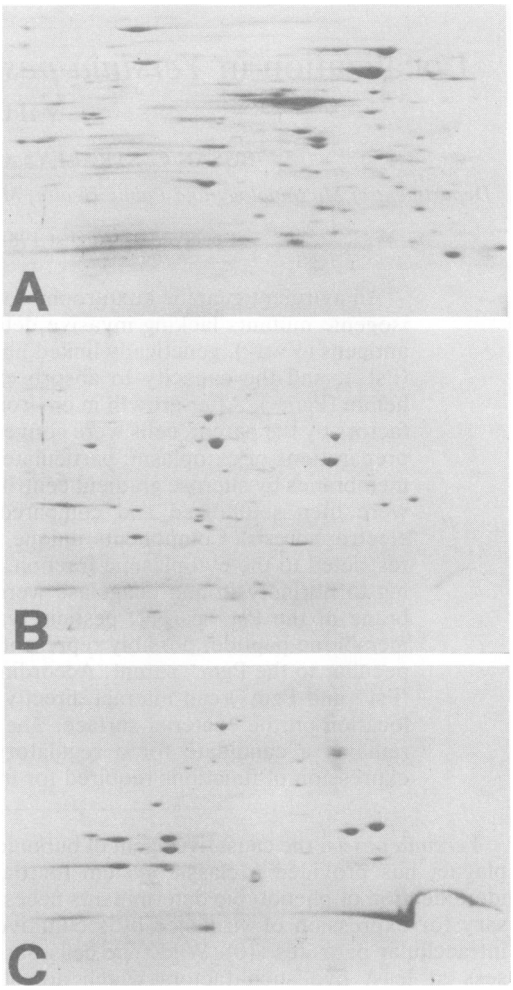



FIG. 2. Electropherograms of the cytoplasmic (A), inner membrane (B), and outer membrane (C) fractions of a $Vwa^-$ mutant of *Y. pestis* K25 grown under conditions that promote expression of this virulence determinant.

**Media and cultivation.** Parent and $Vwa^-$ cells were grown at 37°C as described previously (12, 27) except that the medium contained 0.5 mM guanosine and 0.5 mM hypoxanthine. The latter was added to prevent evident feedback inhibition caused by the presence of guanosine alone. The parent and the $Pst^-$ isolate were

TABLE 1. Specific activity of virulence determinants in Pur⁻ *Y. pestis* K25 grown in modified Higuchi medium (MHM), heart infusion broth (HIB), and minimal synthetic medium (MSM)

| Virulence determinant | Property assayed[a] | Sp act[b] | | |
|---|---|---|---|---|
| | | MHM | HIB | MSM |
| Vwa⁺ | V-antigen production | 4.9 | 0.4 | ND[c] |
| Pst⁺ | Coagulase production | 2.3 | 1.9 | 2.1 |
| | Fibrinolysin production | 1,700 | 940 | 140 |
| | Pesticin production | 160 | 58,000 | 320 |
| Pgm⁺ | Hemin-binding capacity | 0.25 | 0.63 | 3.4 |

[a] V antigen was assayed in cells grown at 37°C; other properties were determined in organisms cultivated at 26°C.
[b] Expressed as units (defined in the text) per milligram of protein.
[c] ND, Not determined.

cultivated at 26°C in heart infusion broth (Difco Laboratories), and the Pgm⁺ determinant was compared after growth in defined minimal medium (4) plus 0.5 mM guanosine and 0.5 mM hypoxanthine.

In all cases, cells were incubated at 26°C for 2 days on slopes of blood agar base, transferred to an appropriate liquid medium, and grown for at least six doublings at 26°C before dilution to an optical density of 0.1 to 0.2 at 620 nm (~$10^8$ cells per ml) into cultures of the same medium used in the experiments. For comparison of the Vwa⁺ factor, organisms were grown for about one generation at 26°C and then shifted to 37°C; after 5 h, restricted Vwa⁺ cells (optical density of 0.6) were collected by centrifugation (10,000 × *g* for 30 min). Vwa⁻ mutants and those grown for comparison of the Pst⁺ and Pgm⁺ determinants were harvested during logarithmic growth at an optical density of about 1.0; the latter were maintained at 26°C.

**Fractionation of bacteria.** The procedures used for the isolation of cytoplasmic and inner and outer membrane fractions were modified from those used for related enteric bacteria (23, 26) and have been described for yersiniae in detail (27). Briefly, the method involved the conversion of cells to spheroplasts with lysozyme and EDTA, followed by disruption by sonication. Particulate material was collected by centrifugation, and the supernatant fluid was retained as the cytoplasmic fraction. The sediment was then separated into inner and outer membranes by isopycnic sucrose gradient centrifugation.

**Two-dimensional gel electrophoresis.** The procedure of O'Farrell (22) was employed for the preparation of electropherograms; details of this method as used with concentrated samples of fractions from *Y. pestis* have been reported (27). The amounts of protein applied to gels were determined by the method of Lowry et al. (21).

**Assay of virulence determinants.** The production of V antigen was determined by gel diffusion (20) with cell-free extracts prepared by sonication for 1 min in 0.05 M Tris-hydrochloride buffer, pH 7.8. Pesticin was assayed by the ability of such extracts to inhibit growth of *E. coli* ϕ (8), and attendant fibrinolytic and

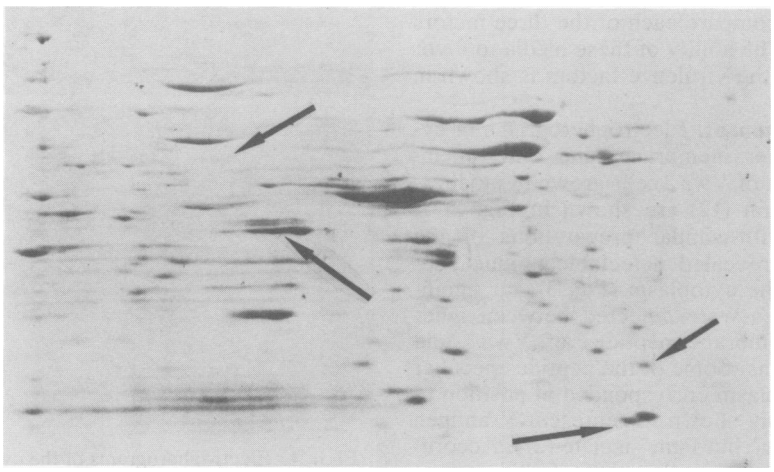


FIG. 3. Electropherograms of the cytoplasmic fraction of *Y. pestis* K25 grown under conditions promoting expression of the Vwa⁺ virulence determinant. Arrows designate peptides unique to this Vwa⁺ parent; those at lower right were not removed by antiserum to V antigen (27).

coagulase activities were estimated by the methods of Beesley et al. (2). One unit of activity was defined as the least amount of sample capable of yielding detectable activity. Expression of the $Pgm^+$ determinant was compared by defining the ability of cells to remove hemin from solution (19); in this case, 1 U of activity was defined as that capable of binding 1 μg of the compound.

**Characterization of plasmids.** Plasmids in the parent and those retained in its $Vwa^-$, $Pst^-$, and $Pgm^-$ mutants were isolated and determined by electrophoresis in agarose gels as previously described (14).

## RESULTS

**Content of plasmids.** To confirm that plasmids within the $Pur^-$ parent (strain K25) were typical of those reported for other isolates (14), the plasmid content of the former was compared with that of its $Vwa^-$, $Pst^-$, and $Pgm^-$ mutants. Mutation to $Vwa^-$ and $Pst^-$ was correlated with the loss of a 42-megadalton plasmid and a 6-megadalton plasmid, respectively (Fig. 1). No detectable alteration of plasmid content occurred upon mutation to $Pgm^-$, and the cryptic 65-megadalton plasmid of *Y. pestis* (14), now known to be associated with expression of the plague murine toxin (R. R. Brubaker, unpublished data), was retained in all isolates. These results indicate that an organism possessing all known invasive determinants of virulence contains the previously predicted content of plasmids.

**Expression of virulence factors.** In preliminary experiments, results obtained with a variety of media were compared for the ability to promote expression of the $Vwa^+$, $Pst^+$, and $Pgm^+$ phenotypes. No single medium was capable of supporting maximal induction of more than one such determinant. Accordingly, different media were used to compare each of the three factors under study. The ability of these media to favor production of the virulence factors is shown in Table 1.

**$Vwa^+$ determinant.** Electropherograms of cytoplasmic, inner membrane, and outer membrane fractions of $Vwa^-$ cells grown in modified Higuchi medium (12) are shown in Fig. 2. A comparison with similar preparations of the $Vwa^+$ parent revealed detectable unique peptides only in the cytoplasm (Fig. 3); no significant differences were detected between inner and outer membrane peptides of $Vwa^+$ and $Vwa^-$ organisms. Some of the peptides peculiar to $Vwa^+$ cytoplasm corresponded in position to those previously shown to represent V antigen in a related $Pur^+$ but $Pgm^-$ isolate (27). Accordingly, the cytoplasmic location of this antigen was not influenced by its production in a $Pgm^+$ background.

**$Pst^+$ determinant.** Electropherograms of fractions of $Pst^-$ organisms grown in heart infusion broth are shown in Fig. 4. A comparison with corresponding preparations of the $Pst^+$ parent revealed two major outer membrane peptides unique to the latter (Fig. 5). Traces of these peptides were also observed in the inner membrane sample but not in the cytoplasmic sample of the parent (not shown). Attempts to locate pesticin, a known cytoplasmic protein (17), were not successful, although a unique smaller and more acidic $Pst^+$-dependent cytoplasmic peptide was observed (not shown).

**$Pgm^+$ determinant.** Electropherograms of fractionated $Pgm^-$ cells grown in an austere environment favoring absorption of hemin (4) are shown in Fig. 6. No significant differences

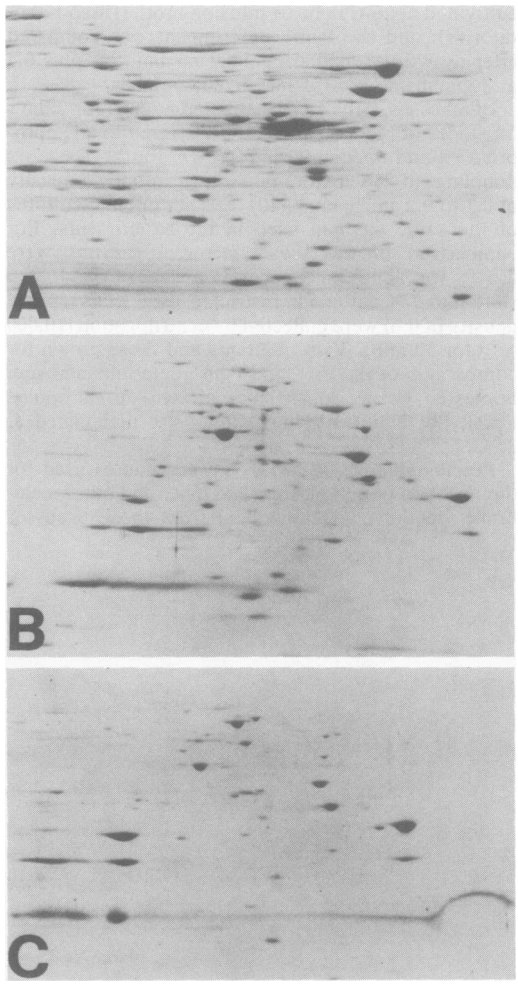



FIG. 4. Electropherograms of the cytoplasmic (A), inner membrane (B), and outer membrane (C) fractions of a $Pst^-$ mutant of *Y. pestis* K25 grown under conditions that promote expression of this virulence determinant.

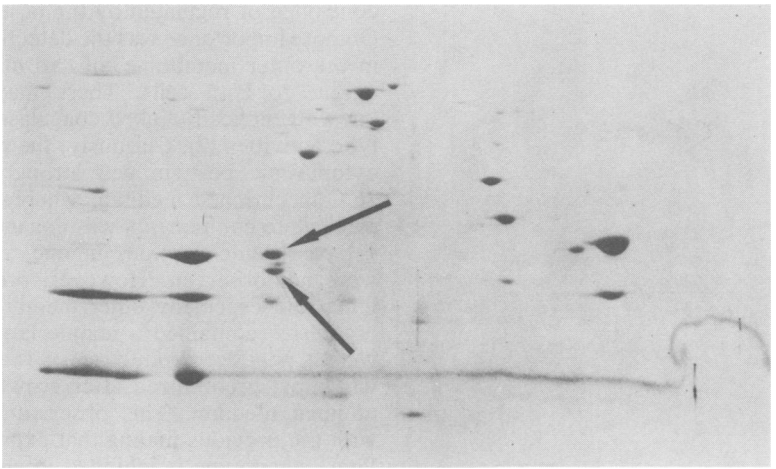


FIG. 5. Electropherograms of the outer membrane fraction of *Y. pestis* K25 grown under conditions promoting expression of the $Pst^+$ virulence determinant. Arrows designate peptides unique to this $Pst^+$ parent.

were noted upon comparison of the cytoplasmic fraction with that of the $Pgm^+$ parent (not shown). However, outer membrane preparations of the latter contained a single major and a few minor unique peptides (Fig. 7); again, traces of the major component occurred in samples of inner membrane (not shown).

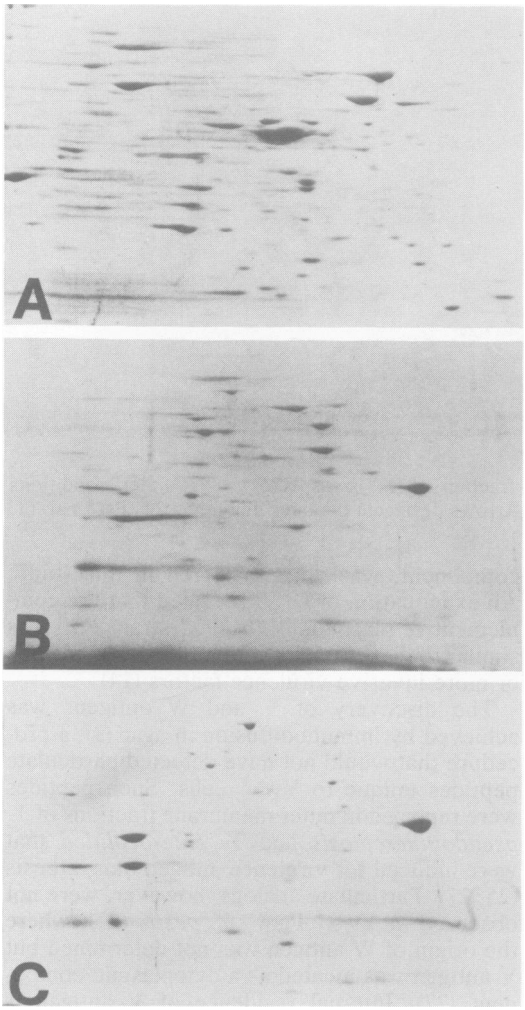
## DISCUSSION

Loss of virulence associated with purine auxotrophy reflects mutation of chromosomal genes which were necessarily conserved during evolution of the parasitic habit. Distinct from this form of avirulence, which results in nutritional privation in vivo, is that shared by variants lacking exotoxins or surface structures such as adhesins, capsules, and invasive enzymes. These virulence factors are often coded by extrachromosomal genes unique to extracellular parasites (13); their loss usually results in an inability of the organisms to resist nonspecific mechanisms of host defense.

It is generally assumed that the established virulence factors of *Y. pestis* are expressed independently and that gene products of separate determinants do not interact to cause disease (5, 10). This notion is probably correct with regard to the noninvasive $Pur^+$ factor. However, since the physiological functions of the $Vwa^+$ and $Pgm^+$ determinants are uncertain and since a casual relationship between products of the $Pst^+$ and $Pgm^+$ factors is known (3), the possibility of interaction among the invasive factors remains real. To favor the detection of such interactions should they occur, a $Pur^-$ but otherwise wild-type isolate was used as the parent of isogenic $Vwa^-$, $Pst^-$, and $Pgm^-$ mutants. The $Fra^+$ determinant, a known surface component, was not considered in this study. An examination of DNA revealed that the complement of plasmids in the $Pur^-$ strain K25 was similar to that obtained with mutants lacking one or more invasive virulence factors (14).

The discovery of V and W antigens was achieved by immunodiffusion in agar (9), a procedure that would not have detected particulate peptides unique to $Vwa^+$ cells. Such peptides were reported in outer membrane fractions of *Y. pseudotuberculosis* and *Y. enterocolitica* that were induced for virulence antigen biosynthesis (25, 27). Particulate analogs, however, were not observed in $Vwa^+$ $Pgm^-$ *Y. pestis* (27), where the origin of W antigen was not determined but V antigen was located as a cytoplasmic component (27). Internal residence of V antigen is consistent with a role in mediating temperature-dependent metabolic step-down in $Ca^{2+}$-deficient medium (30). However, it is difficult at present to reconcile this cytoplasmic location with the reported observation that antibody to V antigen can passively protect mice against plague (20). In the present study, we also detected cytoplasmic peptides corresponding to V antigen in $Pgm^+$ cells, plus additional unique cytoplasmic components. Further study will be necessary to determine whether any of the latter correspond to W antigen.

Although pesticin is known to be a neutral 63,000-dalton monomeric protein of cytoplasmic origin (18), its location in noninduced cells could not be determined with certainty. The minor peptide unique to the cytoplasm of the parent was found in a region of the electropherogram distinct from that of purified pesticin (unpublished data); further work will be necessary to determine whether this component represents immunity protein or some additional activity




controlled or regulated by the pesticin plasmid. Of more importance was the detection, primarily in the outer membrane, of two major peptides unique to $Pst^+$ cells. These may reflect the known genetically linked coagulase and fibrinolytic activities (2). Curiously, the production of cytoplasmic pesticin was strongly favored at 26°C in enriched medium, whereas that of the particulate components was enhanced at 37°C.

Cytoplasmic peptides unique to $Pgm^+$ cells were not observed. However, preparations of inner and especially outer membranes of such organisms contained a unique large-molecular-weight peptide. Production of this component was most pronounced after growth at 26°C in minimal medium. This observation correlates with the previous finding that expression of the $Pgm^+$ phenotype is inhibited in enriched medium, especially at 37°C (2, 19). Since the minimal medium which favored the production of this peptide contained 100 μM $Fe^{2+}$, it is clearly not an iron-stress protein. The $Pgm^+$-dependent peptide may, however, serve as the pesticin and pigment receptor of *Y. pestis*.

All species of yersiniae are established facultative intracellular parasites (6), and all share the plasmid-mediated (14, 15, 31) $Vwa^+$ determinant of virulence and the associated nutritional requirement for $Ca^{2+}$ (12). Expression of this factor in *Y. pestis* was correlated with the appearance of V antigen and possibly W antigen in

FIG. 6. Electropherograms of the cytoplasmic (A), inner membrane (B), and outer membrane (C) fractions of a $Pgm^-$ mutant of *Y. pestis* K25 grown under conditions that promote expression of this virulence determinant.

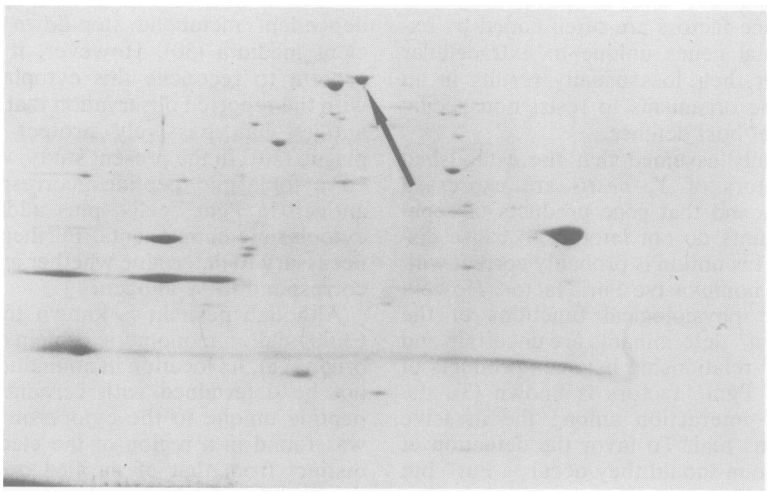


FIG. 7. Electropherograms of the outer membrane fraction of *Y. pestis* K25 grown under conditions promoting expression of the $Pgm^+$ virulence determinant. Arrow designates peptides unique to this $Pgm^+$ parent.

the cytoplasm; no significant changes in the peptides of the inner or outer membrane were detected (27; see above). Similarly, a peptide assumed to represent V antigen was observed in the cytoplasm of $Ca^{2+}$-starved $Vwa^+$ cells of *Y. pseudotuberculosis* and *Y. enterocolitica* (26). Unlike *Y. pestis*, however, cells of the latter two species also exhibited ancillary $Vwa^+$-dependent outer membrane peptides (25, 27). The significance of these components is unknown; they may not be required for expression of virulence since they are absent in $Vwa^+$ *Y. pestis*. Alternatively, they may duplicate functions performed by the remaining determinants of virulence unique to *Y. pestis*, all of which are known ($Fra^+$) or were shown in this study ($Pst^+$ and $Pgm^+$) to also be expressed, at least in part, at the cell surface.

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