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The Plasmid-Encoded Outer-Membrane Proteins of *Yersinia pestis*

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The yersinial plasmid-encoded outer-membrane proteins (Yops) are encoded by the low-Ca^{++} response virulence plasmid present in the yersiniae pathogenic for humans. It has been shown that in *Yersinia pestis* KIM there are 11 of these proteins, which are expressed maximally during growth at 37°C in the absence of Ca^{++}. To recover these proteins in outer membranes, it was necessary to transfer the *Y. pestis* low-Ca^{++} response plasmid to *Yersinia pseudotuberculosis*. However, two of these proteins (Yops M and N) were present in the culture medium after growth of *Y. pestis*; no other Yops were released by the bacteria. This raises the possibility of multiple roles for Yops in the virulence of *Y. pestis*.

The Yops, yersinial plasmid-encoded outer-membrane proteins, are a set of major protein bands originally discovered in outer membranes of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* [1–3]. These proteins are encoded by a virulence plasmid ~75 kilobases (kb) in size [1, 2]. Yops are not seen in isolated outer membranes of *Yersinia pestis* grown in vitro [1]; however, if the 75-kb plasmid of *Y. pestis* is transferred to *Escherichia coli* or *Y. pseudotuberculosis*, several Yops are expressed that cross-react serologically with those of *Y. enterocolitica* [4, 5]. Further, a plague convalescent-phase serum recognized two of these cross-reactive Yops, a finding indicating that *Y. pestis* expresses Yops during human infection [6]. Likewise, human yersiniosis convalescent-phase sera recognized several serologically cross-reacting Yops of *Y. pseudotuberculosis* and *Y. enterocolitica* [6]. Thus, the Yops are potentially important surface antigens of the three species of *Yersinia* pathogenic for humans. My laboratory has identified and characterized some of the *yop* genetic loci in *Y. pestis* [7].

The 75-kb virulence plasmid mediates a virulence property called the low-Ca^{++} response (Lcr^+ phenotype), which confers on the bacteria a growth requirement for Ca^{++} at 37°C and the ability to express the plague virulence antigens V and W [8]. We used the transposon Mu dI(Ap *lac*) to make insertion mutations in pCD1, the *Y. pestis* KIM Lcr plasmid [7]. We obtained mutants with insertions in pCD1, which were normal with respect to the known properties of the low-Ca^{++} response. However, expression of the disrupted genes in response to temperature and Ca^{++} resembled that of V antigen [7]. These genes, like that for the V antigen, all showed maximal expression at 37°C in the absence of Ca^{++}.

We transferred five of these pCD1::Mu dI(Ap *lac*) plasmids to *Y. pseudotuberculosis* to see if the Mu dI(Ap *lac*) inserts had eliminated expression of Yops [7]. Figure 1 shows that each insert eliminated expression of at least one Yop (one of these inserts eliminated two Yops; *lane 8*). Therefore each insert lay within an operon containing at least one *yop* gene.

Comparisons of two-dimensional protein profiles for the bacteria studied in figure 1 confirmed that three of the Yops (A, G, and I) are present only in *Y. pseudotuberculosis* serotype I, whereas three others (M, K, L) apparently are unique to *Y. pestis* (figures 1 and 2) ([7] and data not shown). Thus, there is some species variability in the repertory of Yops. The two-dimensional separations also showed that the major Yop band at 45K (figure 1) actually contains two Yops, H and M, and that there is a minor Yop (N) obscured by the major band due to YopD in one-dimensional profiles (figure 2).

Measurements of the LD_{50} for our *Y. pestis* insertion mutants in mice showed that at least two Yops (E and K) are necessary for virulence [7]. YopJ evidently is not necessary for virulence in our mouse model [7], nor is YopL, which is disrupted by an insertion at 28.4 kb on the pCD1 map (figure 1) ([10] and unpublished data).

We located the genes for the five inserts that we

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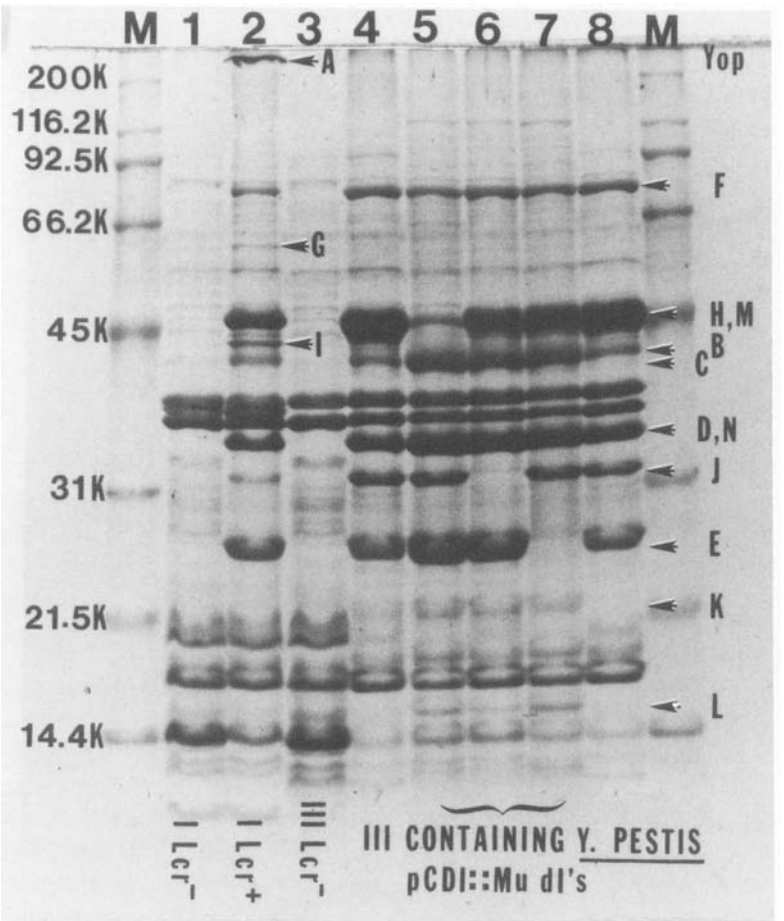

Figure 1. Electrophoretic protein profiles for outer membranes of *Yersinia pseudotuberculosis*. *Y. pseudotuberculosis* strains were grown at 37°C in defined medium without added Ca^{++}, and outer membranes were prepared and boiled in electrophoresis sample buffer [7]. Lanes 1–8 of the 12% polyacrylamide gel contained 50 μg of protein. *Lane M*, molecular weight standards; *lane 1*, *Y. pseudotuberculosis* PB/o (serotype I; Lcr^-); *lane 2*, *Y. pseudotuberculosis* PB1/+ (serotype I; Lcr^+); *lane 3*, *Y. pseudotuberculosis* 43 (pGW600) (serotype III; Lcr^-); *lanes 4–8*, *Y. pseudotuberculosis* 43 (pGW600) containing pCD1::Mu dI(Ap *lac*) with the Mu dI(Ap *lac*) inserted in pCD1, respectively, at 28.4 kb on the pCD1 map [7] ($YopL^-$), 69.5 kb ($YopH^-$), 73 kb ($YopJ^-$), 22 kb ($YopE^-$), and 31 kb ($YopK^-$ $YopL^-$). Other details about the bacteria and their handling are in [7].

had found to disrupt *yop* genes [7]. They represent four separate *yop* operons widely spaced on pCD1; the gene encoding the V antigen is in yet another operon [9]. Therefore these coordinately regulated virulence genes probably represent a virulence regulon.

Genes that mediate the coordinate regulation of V and Yops lie within a 17-kb region often called the Ca^{++}-dependence region, because transposon insertions in this region result in loss of the Ca^{++} requirement for growth [10]. Yersiniae with a transposon insertion in this Ca^{++}-dependence region have greatly decreased expression of V [10] and Yops [5, 11]. Apparently, regulatory Ca^{++}-dependence genes on pCD1 function to induce the coordinate expression of V antigen and Yops under appropriate conditions; V and Yops are thought to be end products of this system, with direct roles in pathogenesis.

The maximal expression of Yops in the absence of Ca^{++} and weak expression in its presence suggested that these proteins might be made when yersiniae are intracellularly located. Accordingly, we tested for expression of *yop* operons by *Y. pestis* grown in human monocyte-derived macrophages [12].

Bacteria with a Mu dI(Ap *lac*)::Tn*9* insert in the *yopKL* operon were grown within the macrophages. Then the monolayers were permeabilized and assayed for β-galactosidase at pH 8, a condition permitting high activity of the bacterial enzyme but no detectable activity of the macrophage enzyme. These intracellular yersiniae showed strong expression of β-galactosidase, reflecting strong transcriptional activity of the disrupted *yopKL* operon, whereas expression by extracellular, nonphagocytosed yersiniae was weak [12]. We have obtained similar results for three other *yop* operons (unpublished data). These

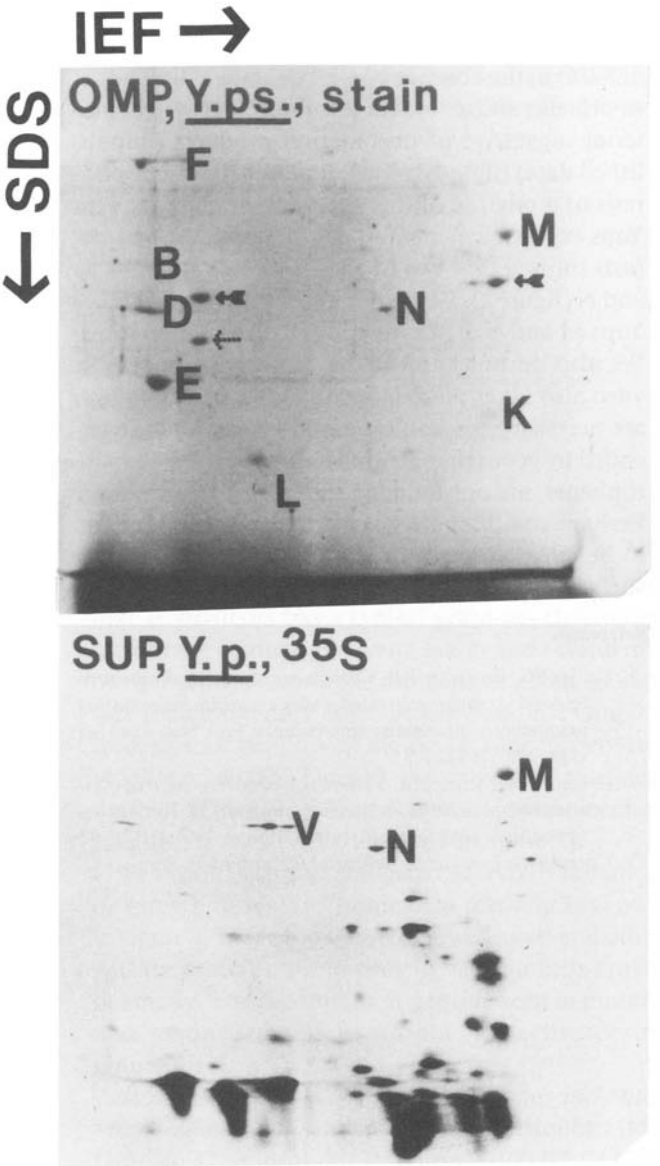

Figure 2. Comigration of outer-membrane proteins (Yops) M and N with proteins in the supernatant of a *Yersinia pestis* culture. ^{35}S-labeled proteins were harvested from cell-free spent medium after growth of *Y. pestis* KIM5 (Lcr^+) 6 hours at 37°C in the absence of Ca^{++} in defined medium [7] and labeling for 30 minutes with [^{35}S]methionine [9]. The cells were removed by centrifugation followed by filtration through 0.22-μm pores, and the resulting solution was concentrated 20-fold on a Bio-Molecular Dynamics MicroProDiCon concentrator (Beaverton, Ore.) with a 10K cut-off. A sample containing 1.6×10^4 cpm was mixed with 37 μg of protein from the same preparation of $YopJ^-$ *Yersinia pseudotuberculosis* membranes electrophoresed in *lane 6* of figure 1. The mixture was subjected to equilibrium two-dimensional electrophoresis [7] and stained with Coomassie Brilliant Blue G-250 to reveal the (nonlabeled) Yops (*top panel*). The letters designate Yops; the arrows indicate major outer-membrane proteins (present in Lcr^- bacteria). Fluorography revealed the proteins from the culture supernatant (*lower panel*). V denotes the V antigen; M and N designate polypeptides that comigrated with Yops M and N, respectively.

data suggest that the intracellular environment may be an important niche in vivo that supports Yop expression by yersiniae.

An obvious major unanswered question about the Yops is their role in pathogenesis. They are highly heterogeneous with respect to both molecular weight and isoelectric point (figure 2). This heterogeneity suggests the possibility of multiple functions. It is possible that Yops apparently unique to *Y. pestis* (K, L, and M) specifically mediate pathogenic properties unique to *Y. pestis*. Clearly, in future work we need to consider the properties and functions of the individual Yops.

Why are the Yops in separate operons? This finding suggests the possibility of independent regulation and might be anticipated if the Yops do confer more than one property on *Y. pestis*.

Another question is whether the Yops actually function in pathogenesis as outer-membrane proteins. We find Yops in the cytoplasm of *Y. pestis* (unpublished data) but not in outer membranes prepared by isopycnic centrifugation of sonicated sphero-

plasts. Culture supernatants of *Y. pestis* KIM5 analyzed at various times during growth and restriction at 37°C in the absence of Ca^{++} contain a discrete set of proteins and abundant low-molecular-weight material suggestive of degradation products (unpublished data) (figure 2). Two-dimensional electrophoresis of a mixture of this supernatant material with Yops expressed in membranes of *Y. pseudotuberculosis* showed that two of these proteins are Yops M and N (figure 2). We do not know at present whether Yops M and N differ functionally from other Yops. We also do not know if the proteolysis evident in vitro also takes place in vivo. Yops E and K, which are necessary for virulence of *Y. pestis* and are encoded by genes that are transcribed actively in macrophages, are not found in the culture supernatant. Perhaps conditions in vivo permit these (and others) to be released intact or expressed on the bacterial surface.

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