INFECTION AND IMMUNITY, Mar. 1999, p. 1533–1537 Vol. 67, No. 3
0019-9567/99/$04.00+0

Protective Efficacy of Recombinant *Yersinia* Outer Proteins against Bubonic Plague Caused by Encapsulated and Nonencapsulated *Yersinia pestis*

G. P. ANDREWS,* S. T. STRACHAN, G. E. BENNER, A. K. SAMPLE, G. W. ANDERSON, JR.,†
J. J. ADAMOVICZ, S. L. WELKOS, J. K. PULLEN, AND A. M. FRIEDLANDER*

Division of Bacteriology, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702-5011

Received 8 September 1998/Returned for modification 5 October 1998/Accepted 21 December 1998

To evaluate the role of *Yersinia* outer proteins (Yops) in conferring protective immunity against plague, six *yop* loci from *Yersinia pestis* were individually amplified by PCR, cloned, and expressed in *Escherichia coli*. The recombinant proteins were purified and injected into mice. Most Yop-vaccinated animals succumbed to infection with either wild-type encapsulated *Y. pestis* or a virulent, nonencapsulated isogenic variant. Vaccination with YpkA significantly prolonged mean survival time but did not increase overall survival of mice infected with the nonencapsulated strain. The only significant protection against death was observed in YopD-vaccinated mice challenged with the nonencapsulated strain.

Yersinia outer proteins (Yops) are virulence determinants synthesized by the *Yersinia* species pathogenic for humans, including *Y. pestis*, the causative agent of plague (29). The Yop proteins are encoded on a 75-kb plasmid, and in vitro expression from these genes and subsequent secretion and translocation by a Type III secretion system are regulated by temperature, calcium, and eukaryotic cell contact (11, 13, 18, 32, 34). There are various functions known for some of the Yops, including a cytotoxic effect on the host cell, with depolymerization of actin microfilaments (YopE [31, 32]); dephosphorylation of host cell proteins (YopH [2, 6]); serine/threonine kinase activity (YpkA [16, 17, 20]); translocation and sensor functions (YopB/D and YopN [7, 14, 19–21, 28]); inhibition of platelet aggregation (YopM [23, 30]); and control of Yop translocation, as well as establishment of a systemic infection (YopK [22, 23, 35]).

Previous studies showed that antibodies to some Yops are present in convalescent-phase serum from patients infected with *Y. pestis*, as well as in rodent serum after experimental *Y. pestis* infection (5, 8, 12, 25, 27), which suggests that Yops are antigenic. Furthermore, vaccination with Yop-containing culture supernatants from growth-restricted *Yersinia enterocolitica* protected mice from a lethal intraperitoneal (i.p.) dose of virulent *Y. pestis* (26). Interpretation of the latter results, however, is complicated by the likely presence of V antigen in the crude supernatants, as V is known to be a protective antigen (1).

To define further the role of individual Yops in plague immunity, we evaluated six recombinant *Y. pestis* Yop products, YopD, YopE, YopH, YopK, YopN, and YpkA, for their ability to elicit a protective immune response against subcutaneous (s.c.) plague infection in an animal model. Wild-type *Y. pestis*, CO92, was obtained from the laboratory of T. Quan, Centers for Disease Control, Ft. Collins, Colo., and an attenuated pigmentation-negative (Pgm^-) derivative of this strain was isolated by serial passage on Congo red medium (36). The variant was used to obtain template plasmid DNA for amplifying *yopD*, *E*, *H*, *K*, and *N* and *ypkA* by PCR. The specific sequences used for amino- (11 to 13 bases 5′ to 3′ from the ATG start codon) and carboxy- (11 to 13 bases 3′ to 5′ from the stop codon) terminal oligonucleotide primers for the *yop* open reading frames (ORFs) were identical to those sequences published for *Y. pseudotuberculosis* or *Y. pestis* (9, 14–17, 19, 23). PCRs were performed with purified plasmid DNA combined with each primer pair by use of the Perkin Elmer GeneAmp reagent kit with AmpliTaq polymerase (Roche Molecular Systems, Inc., Branchburg, N.J.) according to the manufacturer's procedures. The PCR products were ligated in frame into the expression vector pET19b (Novagen, Inc., Madison, Wis.). The resulting recombinant plasmids encoded gene fusions with a 10-histidine amino terminus followed by a porcine enterokinase site immediately upstream of the ATG start codon of the respective *yop* ORF. Standard genetic manipulations (33) were used to ligate, transform, and select for positive recombinant clones in *Escherichia coli* DH5-α (Bethesda Research Laboratories, Gaithersburg, Md.). Recombinant plasmids, containing *yop* inserts, were then transferred into the protease-deficient (*ompT lon*) *E. coli* strain BL21(DE3) or BL21(DE3)pLysS (Novagen), which contains a copy of the T7 polymerase gene under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter.

DNA of the *yop* gene fusion isolates was sequenced with purified recombinant plasmid double-stranded template DNA by use of a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) following the instructions of the manufacturer. Sequencing analysis of positive *yopD*, *yopE*, *yopK*, and *ypkA* fusions revealed that the sequences of the cloned PCR products were identical to published sequences for the four genes.

Y. pestis yopH and *yopN* loci were PCR amplified, cloned into pET21a (Novagen), and sequenced under contract by PerImmune, Inc, Rockville, Md. The sequences were identical

* Corresponding author. Mailing address for G. P. Andrews: Department of Pathology & Area Laboratory Services, Landstuhl Regional Medical Center, D-66849 Landstuhl, Germany. Mailing address for A. M. Friedlander: Division of Bacteriology, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702-5011. Phone: (301)-619-7341. Fax: (301)-619-2152. E-mail: friedlan@ncifcrf.gov.

† Present address: Southern Research Institute, Frederick, MD 21701-4756.

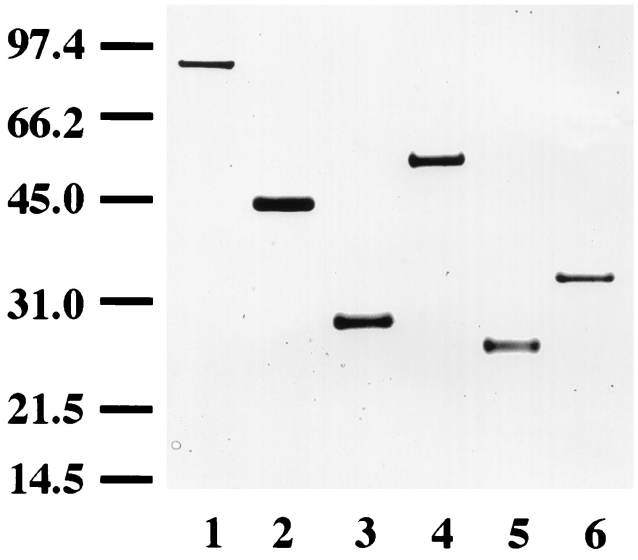

FIG. 1. SDS-PAGE analysis of purified recombinant Yops. Lanes: 1, YpkA; 2, YopD; 3, YopE; 4, YopH; 5, YopK; 6, YopN. The positions of molecular mass standards (in kilodaltons) are indicated on the left axis.

to published sequences except for a silent mutation in *yopH*, in which a T was replaced by a C at base 1237 of the ORF. These constructs expressed full-length recombinant products without a histidine tag (YopH and YopN) from *E. coli* BL21(DE3) pLysS when induced with 1 mM IPTG.

Histidine-tagged YopD, YopE, YopK, and YpkA from recombinant strains were expressed and purified by IPTG induction of batch cultures and Ni^{2+} chelation chromatography under denaturing conditions (6 M urea) according to the procedures specified by the manufacturer (Novagen). YopH was purified by high-performance liquid chromatography (HPLC) cation exchange and size-exclusion chromatography under nondenaturing conditions, and YopN was purified by HPLC anion exchange and size-exclusion chromatography, after solubilization in 6 M urea (PerImmune, Inc.). The urea-extracted proteins were step dialyzed against 3, 1, and 0.5 M urea and phosphate-buffered saline.

Shown in Figure 1 are samples of post-chromatographed, dialyzed YpkA, YopD, YopE, YopH, YopK, and YopN run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue (33). The proteins were greater than 90% pure, with only minor contaminating species present. The molecular masses of YpkA (85 kDa), YopD (36 kDa), YopE (26 kDa), and YopK (21 kDa) agreed with the predicted molecular masses of the histidine-tagged recombinant species. Additionally, the masses of the YopH and YopN products (50 and 27 kDa, respectively) matched the masses of the respective species predicted by the sequence.

For vaccination, the purified proteins were mixed with an equal volume of the adjuvant R-730 emulsion (Ribi ImmunoChem Research, Inc., Hamilton, Mont.) to give a final protein concentration of 100 μg/ml. Thirty micrograms of each Yop-adjuvant mixture was next administered s.c. to two groups each of 8 to 14 female, 8-week-old, Hsd:ND4 Swiss Webster outbred mice (Harlan Sprague Dawley, Indianapolis, Ind.), followed by one boost s.c. of 30 μg at 30 days post-primary vaccination. Serum samples from each Yop-vaccinated mouse were analyzed for antibody to individual Yops by an enzyme-linked immunosorbent assay (ELISA) with urea-solubilized Yops as the coating antigens on glutaraldehyde-pretreated microtiter plates (24). After two doses, all Yops except YopE induced high antibody titers in vaccinated mice (data not shown). Except as indicated, mice in all groups were subsequently boosted with 30 μg of each antigen (15 μg s.c. and 15 μg i.p.) on day 60. The YopE mice were boosted once more s.c. and i.p. on day 90. In the YpkA experiment and in a second YopD experiment (Tables 1 and 2), mice were vaccinated with only two doses. The mice were then retested for Yop antibody titers. A control group was vaccinated with the adjuvant R-730 emulsion alone. Mice in additional control groups were each given 0.1 ml of the killed whole-cell plague vaccine licensed for human use

TABLE 1. Survival of Yop-vaccinated Hsd:ND4 mice after lethal s.c. challenge with encapsulated *Y. pestis* CO92

Trial	Treatment group	LD_{50}[a]	No. of survivors/total no. (%)	*P* value[b]	MST ± SE (days)[c]	*P* value[d]
1	YopD	140	3/9 (33)	0.206	13.0 ± 3.9	0.025
	YopH	140	1/9 (11)	1.000	7.4 ± 2.7	0.300
	F1	140	9/10 (90)	<0.001	27.1 ± 0.9	<0.001
	Plague Vaccine USP	140	7/9 (78)	0.002	24.7 ± 3.1	<0.001
	R-730 control	140	0/8 (0)		4.3 ± 0.5	
2	YopD[e]	140	0/10 (0)	1.000	5.2 ± 0.4	0.936
	R-730 control	140	0/10 (0)		5.2 ± 0.5	
3	YopK	78	0/10 (0)	1.000	5.4 ± 0.4	0.50
	R-730 control	78	0/10 (0)		5.4 ± 0.2	
4	YopM	55	0/10 (0)	1.000	5.2 ± 0.5	0.206
	R-730 control	55	0/10 (0)		5.2 ± 0.2	
5	YopN	150	0/10 (0)	1.000	4.6 ± 0.5	0.466
	R-730 control	150	0/10 (0)		4.1 ± 0.4	

[a] Multiples of the LD_{50} administered.
[b] Fisher's exact test (two-tailed) compared with the trial control.
[c] MST, mean survival time. Day 28, the last day of observation, was used in the calculation for the mice that survived.
[d] Log-rank statistic compared with the trial control.
[e] Mice in this group received two doses of vaccine.

TABLE 2. Survival of Yop-vaccinated Hsd:ND4 mice after lethal s.c. challenge with nonencapsulated *Y. pestis* C12

Trial	Treatment group	LD_{50}[a]	No. of survivors/total no. (%)	*P* value[b]	MST ± SE (days)[c]	*P* value[d]
1	YopD	187	7/9 (78)	0.057	22.4 ± 3.1	0.006
	YopH	187	2/9 (22)	1.000	12.9 ± 3.5	0.820
	F1	187	0/9 (0)	0.471	7.7 ± 1.4	0.621
	Plague vaccine USP	187	2/9 (22)	1.000	11.2 ± 3.3	0.878
	R-730 control	187	2/9 (22)		10.8 ± 3.4	
2	YopD[e]	74	9/10 (90)	0.001	26.2 ± 1.7	<0.001
	R-730 control	74	1/10 (10)		10.4 ± 2.2	
3	YpkA[e]	195	3/10 (30)	0.211	14.1 ± 3.4	0.022
	YopE	195	4/14 (29)	0.114	11.8 ± 2.9	0.149
	R-730 control	195	0/10 (0)		5.4 ± 0.5	
4	YopK	101	1/10 (10)	1.000	10.0 ± 2.3	0.52
	R-730 control	101	0/10 (0)		7.9 ± 0.4	
5	YopM	73	2/10 (20)	0.474	10.4 ± 3.0	0.217
	R-730 control	73	0/10 (0)		6.9 ± 1.4	
6	YopN	124	0/9 (0)	1.000	8.0 ± 1.7	0.091
	R-730 control	124	0/10 (0)		5.4 ± 0.4	

[a] Multiples of the LD_{50} administered.
[b] Fisher's exact test (two-tailed) compared with the trial control.
[c] MST, mean survival time. Day 28, the last day of observation, was used in the calculation for the mice that survived.
[d] Log-rank statistic compared with the trial control.
[e] Mice in these groups received two doses of vaccine.

(Plague Vaccine USP, lot number 10K01A; Miles, Inc.; Cutter Biological, Elkhart, Ind.) or recombinant *Y. pestis* F1 capsular antigen in R-730 emulsion (10 μg/dose) in a three-dose s.c./i.p. vaccination regimen. The recombinant F1 capsular antigen was produced from *E. coli* and purified from cells as described previously (3). During all vaccinations and subsequent challenges, animals were provided with fresh water and feed ad libitum. Additionally, all experiments were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (10).

After the three- to four-dose vaccination regimen, all Yop-vaccinated animal groups possessed high titers of antibody to the recombinant proteins, while mice in the control group, given R-730, possessed negligible anti-Yop antibody titers. The reciprocal geometric mean endpoint titers were greater than 275,000 for all the Yops except YopE, for which it was 50,686. Additionally, serum samples from the control animal group vaccinated with purified *Y. pestis* recombinant F1 capsular antigen in R-730 did not cross-react with any of the Yops in an ELISA (data not shown).

The vaccinated mice were challenged s.c. 4 weeks after the last dose with multiples of the dose lethal for 50% of nonvaccinated animals (LD_{50}) of either wild-type *Y. pestis* CO92 or a nonencapsulated, virulent isogenic derivative, C12 (38). The s.c. LD_{50} is 1.9 CFU for CO92 (37) and 9.1 CFU for C12 (38). The organisms were grown and the CFU were measured as previously described (1).

The effectiveness of vaccination against the encapsulated *Y. pestis* CO92 presented in Table 1 was analyzed by overall survival at 28 days postchallenge by Fisher's exact test and by mean survival time by the log-rank statistic obtained from product-limit survival estimates from the BMDP Lifetest program (BMDP Software, University of California, Los Angeles). None of the Yops tested, including YopD (two experiments), YopH, YopK, YopM, and YopN, significantly protected mice against challenge with encapsulated CO92 (Table 1; $P = 0.206$ to 1.000). The only group showing any protection by the more sensitive indicator of mean survival time was YopD, and we observed this in only one of the two experiments (Table 1; $P = 0.025$ and $P = 0.936$, respectively). As previously described (3), both F1 capsular antigen itself and Plague Vaccine USP provided significant protection against death after CO92 challenge (Table 1; $P < 0.001$ and $P = 0.002$, respectively).

As expected, when F1- or Plague Vaccine USP-vaccinated animals were challenged with the F1-negative nonencapsulated C12 strain, there was no statistically significant protection observed (Table 2; $P = 0.471$ and $P = 1.000$, respectively). It is possible that the F1 capsule could mask some surface-exposed Yop proteins and thus prevent protection by antibodies to such antigens. If this was the case, then it should be easier to protect against capsule-negative strains than against capsule-positive strains. Table 2 shows that vaccination with YopH, YpkA, YopE, YopK, YopM, or YopN did not protect mice challenged with the nonencapsulated C12 strain against death (0 to 30% survival; $P = 0.114$ to 1.0). In these groups, only the YpkA-vaccinated mice had a significantly increased mean survival time, 14.1+/−3.4 days, compared with controls (Table 2, $P = 0.022$). While YopD-vaccinated animals were not protected against challenge with encapsulated *Y. pestis*, as noted above, they were protected against challenge with the virulent, nonencapsulated C12, with a statistically significant increase in survival (Table 2; experiment 2, 90%, $P = 0.001$) and a significant increase in mean survival time (26.2+/−1.7 days, $P < 0.001$) in one experiment. We observed protection against a higher-dose C12 challenge in a second experiment with 78% survival of YopD-vaccinated mice. While the increased survival observed in the second experiment did not reach statistical significance (Table 2; experiment 1, $P = 0.057$), the mean

survival time was again highly significant (22.4+/−3.1 days, $P = 0.006$).

On the basis of our results, humoral immunity to most of the individual Yops tested was not sufficient to afford good protection against *Y. pestis* infection introduced by the s.c. route. We make this conclusion with the caveat that four of the recombinant antigens which did not protect (YopE, YopK, YopN, and YpkA) were denatured before their use as immunogens. Thus, the manipulation of these proteins during extraction and purification may have destroyed their protective epitopes, if they exist. Some evidence, however, does suggest that some of the urea-solubilized recombinant Yops do retain some epitopes present on the native proteins. In preliminary experiments, mouse hyperimmune serum generated with recombinant YopD, YopE, YopH, and YopN, but not with YpkA, reacted with the respective native proteins by a whole-cell surface-labeling immunoassay technique (4) (data not shown). Conversely, in a separate study (5) some animals that survived experimental plague infection produced antibodies during the course of infection that reacted against urea-solubilized YopK with the same ELISA methodology described in the present report.

Of interest, vaccination with YopN, which is thought to function as a surface-located sensor for low-calcium- or eucaryotic-cell-contact-induced Yop secretion (7, 14), prolonged mean survival time only slightly after challenge with nonencapsulated C12 compared with the control group (Table 2; 8.0+/−1.7 days versus 5.4+/−0.4 days), but this difference did not reach statistical significance ($P = 0.091$). In regard to YpkA, vaccination did not affect overall survival but did result in a statistically significant increase in mean survival time of mice challenged with the nonencapsulated C12 strain (Table 2, $P = 0.022$). It is possible that YpkA has an even greater protective effect when purified in its native (active) form. However, as suggested by a model of vectorial translocation of the effector Yops, YpkA, as well as YopH and YopE, may remain intracellular in the bacteria until eucaryotic cell contact, after which they are delivered to the host cell cytosol (14, 24). Thus, it is possible that antibody to these Yops in the vaccinated host would not have access to the antigens. The failure of YpkA, YopE, and YopH antibody to protect mice against *Y. pestis* challenge in our experiments is consistent with this model.

A study by Nemeth and Straley (27) also showed that mice vaccinated with purified native *Y. pestis* YopM, an alpha-thrombin-binding protein which is also translocated to the host cell cytosol (7, 27), were not protected against an intravenous challenge with the Pgm^- *Y. pestis* strain KIM5. We obtained similar results when mice vaccinated with identical preparations of YopM (kindly provided by S. Straley, University of Kentucky, Louisville) were challenged s.c. with the fully virulent *Y. pestis* strains CO92 (Table 1) and C12 (Table 2). These results supported the hypothesis that some Yops may not be accessible for neutralization by antibody in the *Y. pestis*-infected host.

The ability of YopD to protect mice against nonencapsulated *Y. pestis* C12 strongly suggests that at least one of the Yops is important in eliciting a protective immune response against lethal *Y. pestis* s.c. challenge. Given the accessory function of YopD in facilitating delivery of other Yops into their eukaryotic targets, it is possible that antibody to YopD interferes with this function. The failure of YopD to protect against encapsulated organisms to the same degree as it protects against the nonencapsulated strain may be the result of a masking effect of the F1 capsule on secreted YopD which blocks the antibody-antigen interaction at the surface of the bacterium. Experiments are currently being conducted in our laboratory to examine these hypotheses.

We are grateful for the excellent technical assistance of Gerald Howe in the cloning of the *Y. pestis* YopD and YopE genes, Steve Tobery for animal studies, Chris Bolt for serology, Gene Nelson and Paul Gibbs for review of statistical methods, Kathy Kenyon for editorial assistance, and Marcia Baker for secretarial help.

Purified native *Y. pestis* YopM was provided by Susan Straley, University of Kentucky, under the Department of Army, USAMRDC, Collaborative Agreement DAMD1794-J-4013.

REFERENCES

1. **Anderson, G. W., Jr., S. E. C. Leary, E. D. Williamson, R. W. Titball, S. L. Welkos, P. L. Worsham, and A. M. Friedlander.** 1996. Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of *Yersinia pestis*. Infect. Immun. **64:**4580–4585.
2. **Andersson, K., N. Carballeira, K.-E. Magnusson, C. Persson, O. Stendhal, H. Wolf-Watz, and M. Fallman.** 1996. YopH of *Yersinia pseudotuberculosis* interrupts early phosphotyrosine signalling associated with phagocytosis. Mol. Microbiol. **20:**1057–1069.
3. **Andrews, G. P., D. G. Heath, G. W. Anderson, Jr., S. L. Welkos, and A. M. Friedlander.** 1996. Fraction 1 capsular antigen (F1) purification from *Yersinia pestis* CO92 and an *Escherichia coli* recombinant strain and efficacy against lethal plague challenge. Infect. Immun. **64:**2180–2187.
4. **Andrews, G. P., A. E. Hromockyj, C. Coker, and A. T. Maurelli.** 1991. Two novel virulence loci, *mxiA* and *mxiB*, in *Shigella flexneri* 2a facilitate excretion of invasion plasmid antigens. Infect. Immun. **59:**1997–2005.
5. **Benner, G. E., G. P. Andrews, W. R. Bryne, S. D. Strachan, A. K. Sample, D. G. Heath, and A. M. Friedlander.** Immune response to *Yersinia* outer proteins (Yops) and other virulence determinants after experimental plague infection in mice. Infect. Immun., in press.
6. **Black, D. S., and J. B. Bliska.** 1997. Identification of $p130^{Cas4}$ as a substrate of *Yersinia* YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. EMBO J. **16:**2730–2744.
7. **Boland, A., M.-P. Sory, M. Iriate, C. Kerbourch, P. Wattiau, and G. R. Cornelis.** 1996. Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y. enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. EMBO J. **15:**5191–5201.
8. **Bolin, I., D. Portnoy, and H. Wolf-Watz.** 1985. Expression of the temperature-inducible outer membrane proteins of yersiniae. Infect. Immun. **48:**234–240.
9. **Bolin, I., and H. Wolf-Watz.** 1988. The plasmid-encoded Yop2b protein of *Yersinia pseudotuberculosis* is a virulence determinant regulated by calcium and temperature at the level of transcription. Mol. Microbiol. **2:**237–245.
10. **Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.** 1985. Guidelines for the care and use of laboratory animals, revised. National Institutes of Health publication no. 86-23. National Institutes of Health, Bethesda, Md.
11. **Cornelis, G. R., and H. Wolf-Watz.** 1997. The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. Mol. Microbiol. **23:**861–867.
12. **Drobkov, V. I., I. V. Marakulin, I. P. Pogorelsky, I. V. Darmov, and E. V. Smirnov.** 1996. Antibody spectrum after the inoculation of *Yersinia pestis* and *Yersinia pseudotuberculosis* to susceptible animals. Zh. Mikrobiol. (Moscow) **2:**81–85. (In Russian.)
13. **Forsberg, A., R. Rosqvist, and H. Wolf-Watz.** 1994. Regulation and polarized transfer of the *Yersinia* outer proteins (Yops) involved in antiphagocytosis. Trends Microbiol. **2:**14–19.
14. **Forsberg, A., A.-M. Viitanen, M. Skurnik, and H. Wolf-Watz.** 1991. The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. Mol. Microbiol. **5:**977–986.
15. **Forsberg, A., and H. Wolf-Watz.** 1990. Genetic analysis of the *yopE* region of *Yersinia* spp.: identification of a novel conserved locus, *yerA*, regulating *yopE* expression. J. Bacteriol. **172:**1547–1555.
16. **Galyov, E. E., S. Hakansson, A. Forsberg, and H. Wolf-Watz.** 1993. A secreted protein kinase of *Yersinia pseudotuberculosis* is an indispensable virulence determinant. Nature **361:**730–732.
17. **Galyov, E. E., S. Hakansson, and H. Wolf-Watz.** 1994. Characterization of the operon encoding the YpkA Ser/Thr protein kinase and the YopJ protein of *Yersinia pseudotuberculosis*. J. Bacteriol. **176:**4543–4548.
18. **Goguen, J. D., J. Yother, and S. C. Straley.** 1984. Genetic analysis of the low calcium response in *Yersinia pestis* Mu d1(Ap lac) insertion mutants. J. Bacteriol. **160:**842–848.
19. **Hakansson, S., T. Bergman, J.-C. Vanooteghem, G. Cornelis, and H. Wolf-Watz.** 1993. YopB and YopD constitute a novel class of *Yersinia* Yop proteins. Infect. Immun. **61:**71–80.
20. **Hakansson, S., E. E. Galyov, R. Rosqvist, and H. Wolf-Watz.** 1996. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to

the inner surface of the HeLa cell plasma membrane. Mol. Microbiol. **20:** 593–603.
21. **Hakansson, S., C. Persson, K. Schesser, E. E. Galyov, R. Rosqvist, and H. Wolf-Watz.** 1996. The YopB protein of *Yersinia pseudotuberculosis* is essential for translocation of Yop effector proteins across the target cell plasma membrane and displays contact-dependent membrane-disrupting activity. EMBO J. **15:**5812–5823.
22. **Holmstrom, A., J. Pettersson, R. Rosqvist, S. Hakansson, F. Tafazoli, K.-E. Magnusson, H. Wolf-Watz, and A. Forsberg.** 1997. YopK of *Yersinia pseudotuberculosis* controls translocation of Yop effectors across the eukaryotic cell membrane. Mol. Microbiol. **24:**73–91.
23. **Holmstrom, A., R. Rosqvist, H. Wolf-Watz, and A. Forsberg.** 1995. Virulence plasmid-encoded YopK is essential for *Yersinia pseudotuberculosis* to cause systemic infection in mice. Infect. Immun. **63:**2269–2276.
24. **Klasen, E. A., A. Rigutti, A. Bos, and L. F. Bernini.** 1982. Development of a screening system for detection of somatic mutations. I. Enzyme immunoassay for detection of antibodies against specific hemoglobin determinants. J. Immunol. Methods **54:**241–250.
25. **Mazza, G., A. E. Karu, and D. T. Kingsbury.** 1985. Immune response to plasmid- and chromosome-encoded *Yersinia* antigens. Infect. Immun. **48:** 676–685.
26. **Michel, P., B. Rasoamanana, N. Rasolofonorina, and J. Roux.** 1992. Plague: disease and vaccine. Dakar Med. **37:**2–18.
27. **Nemeth, J., and S. C. Straley.** 1997. Effect of *Yersinia pestis* YopM on experimental plague. Infect. Immun. **65:**924–930.
28. **Persson, C., R. Nordfelth, A. Holmstrom, S. Hakansson, R. Rosqvist, and H. Wolf-Watz.** 1995. Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Mol. Microbiol. **18:**135–150.
29. **Portnoy, D. A., H. Wolf-Watz, I. Bolin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43:**108–114.
30. **Reisner, B. S., and S. C. Straley.** 1992. *Yersinia pestis* YopM: thrombin binding and overexpression. Infect. Immun. **60:**5242–5252.
31. **Rosqvist, R., A. Forsberg, and H. Wolf-Watz.** 1991. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect. Immun. **59:**4562–4569.
32. **Rosqvist, R., K.-E. Magnusson, and H. Wolf-Watz.** 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. **13:**964–972.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51:**445–454.
35. **Straley, S. C., and M. L. Cibull.** 1989. Differential clearance and host-pathogen interactions of $YopE^{-}$ and $YopK^{-}$ *Yersinia pestis* in BALB/c mice. Infect. Immun. **57:**1200–1210.
36. **Surgalla, M. J., and E. D. Beesley.** 1969. Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. Appl. Microbiol. **18:**834–837.
37. **Welkos, S. L., K. M. Davis, L. M. Pitt, P. L. Worsham, and A. M. Friedlander.** 1995. Studies on the contribution of the F1 capsule-associated plasmid pFra to the virulence of *Yersinia pestis*. Contrib. Microbiol. Immunol. **13:**299–305.
38. **Worsham, P. L., M.-P. Stein, and S. L. Welkos.** 1995. Construction of defined F1 negative mutants of virulent *Yersinia pestis*. Contrib. Microbiol. Immunol. **13:**325–328.

Editor: D. L. Burns