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DNA Adenine Methylase Overproduction in *Yersinia pseudotuberculosis* Alters YopE Expression and Secretion and Host Immune Responses to Infection

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Yersinia pseudotuberculosis **mutants that overproduce the DNA adenine methylase (Dam) are highly attenuated, confer fully protective immune responses, and secrete several *Yersinia* virulence proteins (*Yersinia* outer proteins [Yops]) under conditions that are nonpermissive for secretion in wild-type strains. We examined here the effects of Dam overproduction on *Yersinia* virulence determinant expression and secretion, as well as the host immune response to *Yersinia* antigens. Western blot analysis with convalescent antisera identified several low-calcium-responsive antigens whose synthesis was affected by Dam overproduction. One of these antigens was shown to be the type III secretion effector protein, YopE, a cytotoxin involved in antiphagocytosis. Dam overproduction disrupted both the thermal and calcium regulation of YopE synthesis and relaxed the thermal but not the calcium dependence of YopE secretion. Altered expression and/or secretion of *Yersinia* proteins in Dam-overproducing strains may contribute to the decreased virulence and heightened immunity observed in vaccinated hosts and may provide a means by which to deliver heterologous antigens and/or immune modulators of the inflammatory response.**

Yersinia spp. are human and animal pathogens with a clear tropism for lymphoid tissue. *Yersinia pestis* is usually transmitted by fleas and is the causative agent of plague, which is often fatal (4, 5). *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogens causing self-limiting infections in humans, including gastroenteritis and mesenteric adenitis. *Yersinia* spp. pathogenesis is dependent on virulence proteins called Yops (for *Yersinia* outer proteins) (7, 9, 11, 30) which, upon host contact, are injected directly into the host cell cytoplasm via type III secretion machinery, where they act as effectors to inhibit phagocytosis and proinflammatory cytokine release (3, 5, 6, 8, 12, 25, 26, 29, 31, 35). The secretion of Yops is under strict regulatory control by the low calcium response, whereby Yop secretion only occurs in vitro under conditions of low calcium (Ca^{2+}) and high temperature (37°C [32, 33]). We recently showed that overproduction of Dam in *Y. pseudotuberculosis* relaxed the temperature but not the low calcium dependence of Yop secretion (18). Moreover, such Dam-overproducing *Yersinia* strains were avirulent and elicited protective immune responses in vaccinated mice. Here we examined the effects of Dam overproduction on protein expression and secretion, as well as the humoral response to *Yersinia* antigens.

Yersinia **spp. overproducing Dam efficiently colonize mucosal but not systemic tissues.** To understand the mechanism by which Dam-overproducing *Yersinia* spp. are attenuated for virulence yet elicit protective immune responses, the survival rates of wild-type (Dam^+) and Dam-overproducing yersiniae were compared in mouse tissue sites after oral infection. Dam-overproducing yersiniae survive near wild-type levels in Peyer's patches of the mouse small intestine and mesenteric lymph nodes for at least 24 h. However, at day 5, $>10^5$-fold fewer Dam-overproducing yersiniae were observed in the Peyer's patches and mesenteric lymph nodes, and 10^3- to 10^6-fold fewer Dam-overproducing yersiniae were observed in the liver and spleen, respectively, compared to Dam^+ bacteria (Fig. 1). These data suggest that Dam-overproducing yersiniae are proficient in the targeting and colonization of mucosal but not deep systemic tissues, which may result in the elicitation of host immune responses without acute disease manifestations.

Dam-overproducer ***Y. pseudotuberculosis*** **synthesizes and secretes YopE under conditions nonpermissive for the wild type.** Recently, we showed that the strict regulatory control of Yop secretion is disrupted in Dam-overproducing *Yersinia* mutants (15). These mutants secrete Yops at low Ca^{2+} and low temperature, which are nonpermissive conditions for Yop secretion in wild-type *Yersinia*. Here we wanted to test whether the synthesis and cellular localization of Yops was also disrupted in Dam overproducer conditions. For these experiments, we focused our efforts on YopE, a 23-kDa *Yersinia* cytotoxin that is secreted under low-calcium conditions (1, 2, 34) and is also known to be antigenic (16, 20).

In order to characterize the expression, localization, and secretion profiles of YopE in response to Dam overproduction, whole-cell, membrane, and supernatant fractions of Dam^+ and Dam-overproducing cells grown under Yop-inducing and non-inducing conditions were analyzed by immunoblotting. In contrast to the wild type, Dam-overproducing *Yersinia* strains synthesized YopE under all three nonpermissive conditions (high calcium and low temperature, high calcium and high temperature, and low calcium and low temperature) (Fig. 2, whole-

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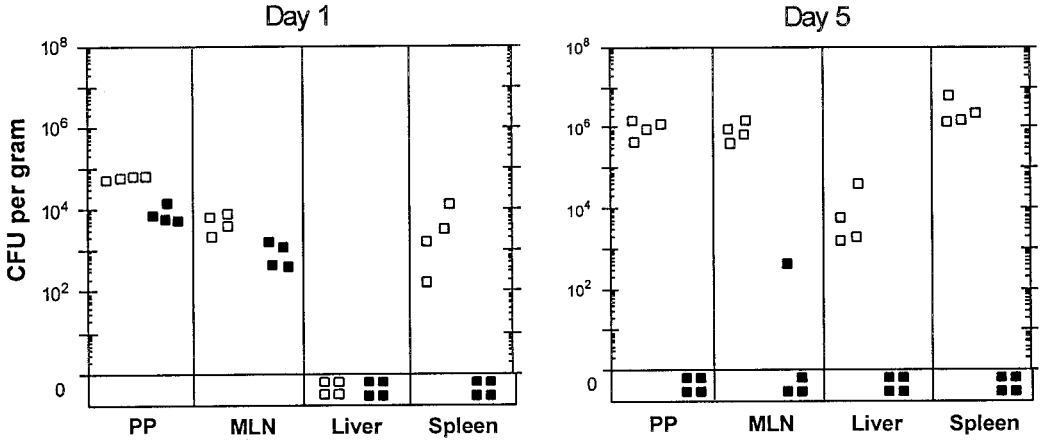

FIG. 1. Colonization of mouse tissue sites by Dam-overproducing *Y. pseudotuberculosis*. Six- to eight-week-old BALB/c mice were infected via gastrointubation at a dose of ca. 2.5×10^{10} Dam^+ (□) or Dam-overproducing (■) *Y. pseudotuberculosis*. At 1 or 5 days postinfection, mice were sacrificed and bacteria were recovered from the host tissues indicated. Abbreviations: PP, Peyer's patches (the four Peyer's patches proximal to the ileal-cecal junction); MLN, mesenteric lymph nodes.

cell fraction). However, the localization of YopE to the membrane or supernatant fractions required low calcium at either permissive or nonpermissive temperatures (Fig. 2). Thus, YopE is synthesized under all nonpermissive conditions in Dam-overproducing strains, but its export from the cytoplasm still requires a low calcium signal for secretion. These data suggest that Dam overproduction disrupts both thermal and calcium regulation of YopE synthesis and, in addition, relaxes the thermal but not the calcium dependence of YopE secretion. Alternatively, Dam overproduction may lead to YopE overexpression coupled with increased YopE secretion (only at low Ca^{2+} and a low temperature) simply as a consequence of an increased amount of protein in the cell. The proposed altered expression or secretion of YopE and the possible ectopic expression or secretion of other bacterial antigens may contribute to the heightened immune response in hosts vaccinated with Dam-overproducing *Yersinia* mutants.

The effect of Dam overproduction on YopE synthesis is comparable to the phenotype of *Yersinia* mutants defective in LcrQ, a negative regulator of Yop synthesis (27). Wild-type *Yersinia* secretes LcrQ out of the bacterial cell under the permissive conditions of low calcium and high temperature, resulting in a decreased intracellular concentration of LcrQ and increased expression of Yops. LcrQ mutants are disrupted for the normally strict thermal and calcium regulation of Yop synthesis; however, low calcium is still required as a signal for the type III secretion-dependent delivery of most Yops (28). Similarly, Dam overproduction relaxes the temperature and calcium regulation of YopE synthesis, but export of YopE from the cytoplasm remains dependent on the low-calcium secretion signal.

Analysis of the effect of Dam on expression of *Y. pseudotuberculosis* antigens. To begin to characterize the humoral response conferred by *Yersinia* Dam-overproducing strains, we examined protein expression profiles of Dam^+ and Dam-overproducing strains (Table 1). Proteins derived from Dam^+ and Dam-overproducing strains grown under laboratory conditions (in vitro) were subjected to Western analysis with convalescent-phase antisera derived from mice infected with either wild-type (Fig. 3A) or Dam-overproducing (Fig. 3B) *Y. pseudotuberculosis*.

At least three groups of antigens were identified. Group 1 antigens were produced by wild-type and Dam-overproducing strains in vitro and were recognized by convalescent-phase

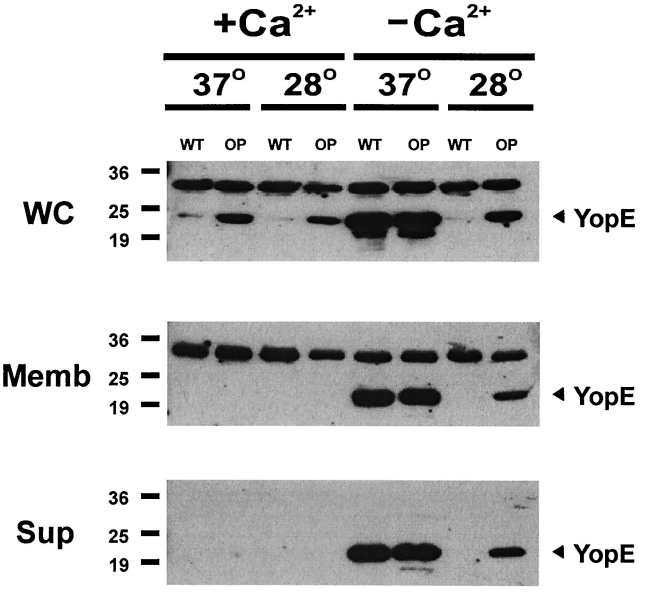

FIG. 2. Dam overproduction results in YopE synthesis and secretion under conditions nonpermissive for the wild type. Whole-cell (WC), membrane (Memb), and supernatant (Sup) fractions were prepared from wild-type (WT) and Dam-overproducing (OP) *Y. pseudotuberculosis* grown under the indicated conditions. For each growth condition, total protein corresponding to 2.0×10^6 cells was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and probed with convalescent-phase antisera derived from BALB/c mice infected with wild-type *Y. pseudotuberculosis* as a source of anti-YopE antibody. Western analysis with YopE antibody confirmed that the 23-kDa low-calcium-responsive protein was YopE. Numbers refer to protein sizes (in kilodaltons).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
Y. pseudotuberculosis		
YPIIIpYV	Wild type	Stanley Falkow
MT2294	*dam*::Kn + pTP166-Cm	15
MT2365	Δ*yopE* (in-frame deletion)	This work
MT2366	Δ*yopE dam*::Kn + pTP166-Cm	This work
Plasmid pTP166-Cm	*E. coli dam* under *tac* promoter control; chloramphenicol-resistant derivative of pTP166	15, 19

antisera derived from mice infected with wild type or Dam-overproducing strains infected mice (Fig. 3, arrows labeled 1). Group 2 antigen was produced by wild-type and Dam-overproducing strains in vitro and was preferentially recognized by convalescent antisera derived from wild type-infected mice (Fig. 3, arrows labeled 2). Group 3 antigens were expressed under Yop inducing conditions (low Ca^{2+} and high temperature) and were often preferentially expressed by either wild-type or Dam-overproducing strains in vitro (Fig. 3, arrows labeled 3).

These data show that Dam overproduction affects the humoral response but not the in vitro synthesis of the group 2 antigen. This suggests that, although the group 2 antigen was produced at wild-type levels in Dam-overproducing cells in vitro, it is not produced in sufficient quantity or for sufficient duration, nor is it presented to the appropriate immune cells, during Dam-overproducing infection. Alternatively, Dam-overproducing cells may inhibit the humoral response to the group 2 antigen. Such differential expression of an in vivo-induced antigen or altered immune response to an in vivo expressed antigen can have profound effects on the immunity conferred by Dam-overproducing *Yersinia* strains.

The role of Dam in virulence and in the elicitation of protective immune responses may be attributed to its capacity as a global regulator of gene expression (10, 13, 14, 17–19). Overproduction of Dam activity in *Yersinia* strains alters the expres-

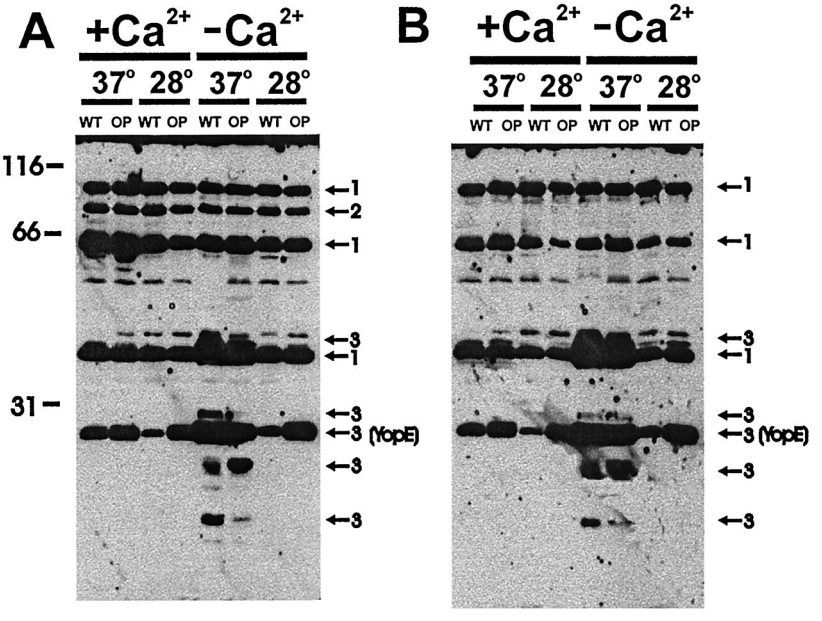

FIG. 3. Mice vaccinated with Dam-overproducing *Yersinia* strains show an altered humoral response compared to mice infected with wild-type *Yersinia* strains. Whole-cell protein extracts from wild-type (WT) and Dam-overproducing (OP) *Y. pseudotuberculosis* grown under the indicated temperature and calcium conditions served as the antigen source. Whole-cell protein extracts derived from 2.0×10^6 cells (~20 μg of protein/well) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Pierce), and probed with Dam^+ (A) or Dam-overproducing (B) pooled convalescent antisera at a 1/15,000 or a 1/7500 dilution of antibody, respectively. Peroxidase-conjugated sheep anti-mouse immunoglobulin G (Amersham Life Sciences) was used as the secondary antibody at 1/40,000 dilution, and hybridization was detected by using Supersignal West Femto Maximum Sensitivity Substrate (Pierce). Group 1 antigens were produced by wild-type and Dam-overproducing strains in vitro and recognized by both wild-type and Dam-overproducing convalescent-phase antisera (arrows 1). Group 2 antigen was produced by wild-type and Dam-overproducing strains in vitro and preferentially recognized by wild-type convalescent-phase antisera (arrows 2). Group 3 antigens were expressed under Yop inducing conditions (low Ca^{2+} and high temperature) and were preferentially produced by either Dam^+ or Dam-overproducing cells in vitro (arrows 3). Numbers refer to protein sizes in kilodaltons. To generate the wild-type and Dam-overproducing convalescent-phase sera, 6- to 8-week-old BALB/c mice were gastrointubated with 2.5×10^7 wild-type *Y. pseudotuberculosis* (the lethal dose required to kill 50% of the animals [21]) or 2.5×10^{10} Dam-overproducing *Y. pseudotuberculosis* bacteria. At 5 weeks postimmunization, whole blood from four to six mice was collected by cardiac puncture, and the sera were pooled and stored in 20% glycerol at −20°C.

sion and/or secretion of low-calcium-responsive proteins (Fig. 2 and 3) (15) and possibly other virulence functions required for pathogenesis. Additionally, Dam overproduction may contribute to the elicitation of protective responses by the elaboration of an expanded repertoire of antigens and/or immune modulators of host inflammatory activities. Although not known to be subject to Dam regulation, LcrV is a *Yersinia* virulence protein responsive to low calcium that suppresses inflammatory cytokines during infection (23, 24) and induces high levels of protection when delivered as a subunit vaccine (5, 22). Similarly, dysregulation of Dam activity may result in altered expression and/or secretion of functions required for virulence, immune modulation, and the elicitation of protection immune responses. Such a delivery strategy may also provide a means to deliver heterologous antigens and modulators of the inflammatory response (e.g., for suppression of inflammatory cytokines).

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REFERENCES

1. **Andor, A., K. Trulzsch, M. Essler, A. Roggenkamp, A. Wiedemann, J. Heesemann, and M. Aepfelbacher.** 2001. YopE of *Yersinia*, a GAP for Rho GTPases, selectively modulates Rac-dependent actin structures in endothelial cells. Cell. Microbiol. **3:**301–310.
2. **Black, D. S., and J. B. Bliska.** 2000. The RhoGAP activity of the *Yersinia pseudotuberculosis* cytotoxin YopE is required for antiphagocytic function and virulence. Mol. Microbiol. **37:**515–527.
3. **Bleves, S., and G. R. Cornelis.** 2000. How to survive in the host: the *Yersinia* lesson. Microbes Infect. **2:**1451–1460.
4. **Brubaker, R. R.** 1991. Factors promoting acute and chronic diseases caused by yersiniae. Clin. Microbiol. Rev. **4:**309–324.
5. **Brubaker, R. R.** 2000. *Yersinia pestis* and bubonic plague. Springer-Verlag, New York, N.Y. [Online.]
6. **Cheng, L. W., and O. Schneewind.** 2000. Type III machines of gram-negative bacteria: delivering the goods. Trends Microbiol. **8:**214–220.
7. **Cornelis, G. R.** 2000. Molecular and cell biology aspects of plague. Proc. Natl. Acad. Sci. USA **97:**8778–8783.
8. **Cornelis, G. R.** 2000. Type III secretion: a bacterial device for close combat with cells of their eukaryotic host. Philos. Trans. R. Soc. London B Biol. Sci. **355:**681–693.
9. **Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier.** 1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol. Mol. Biol. Rev. **62:**1315–1352.
10. **Dueger, E. L., J. K. House, D. M. Heithoff, and M. J. Mahan.** 2001. *Salmonella* DNA adenine methylase mutants elicit protective immune responses to homologous and heterologous serovars in chickens. Infect. Immun. **69:**7950–7954.
11. **Guan, K. L., and J. E. Dixon.** 1990. Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia.* Science **249:**553–556.
12. **Haller, J. C., S. Carlson, K. J. Pederson, and D. E. Pierson.** 2000. A chromosomally encoded type III secretion pathway in *Yersinia enterocolitica* is important in virulence. Mol. Microbiol. **36:**1436–1446.
13. **Heithoff, D. M., E. Y. Enioutina, R. A. Daynes, R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 2001. *Salmonella* DNA adenine methylase mutants confer cross-protective immunity. Infect. Immun. **69:**6725–6730.
14. **Heithoff, D. M., R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 1999. An essential role for DNA adenine methylation in bacterial virulence. Science **284:**967–970.
15. **Julio, S. M., D. M. Heithoff, D. Provenzano, K. E. Klose, R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 2001. DNA adenine methylase is essential for viability and plays a role in the pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae.* Infect. Immun. **69:**7610–7615.
16. **Leary, S. E., K. F. Griffin, E. E. Galyov, J. Hewer, E. D. Williamson, A. Holmstrom, Forsberg, and R. W. Titball.** 1999. *Yersinia* outer proteins (YOPS) E, K and N are antigenic but non-protective compared to V antigen, in a murine model of bubonic plague. Microb. Pathog. **26:**159–169.
17. **Low, D. A., N. J. Weyand, and M. J. Mahan.** 2001. Roles of DNA adenine methylation in regulating bacterial gene expression and virulence. Infect. Immun. **69:**7197–7204.
18. **Mahan, M., and D. Low.** 2001. DNA methylation regulates bacterial gene expression and virulence. ASM News **67:**356–361.
19. **Marinus, M. G., A. Poteete, and J. A. Arraj.** 1984. Correlation of DNA adenine methylase activity with spontaneous mutability in *Escherichia coli* K-12. Gene **28:**123–125.
20. **Mazza, G., A. E. Karu, and D. T. Kingsbury.** 1985. Immune response to plasmid- and chromosome-encoded *Yersinia* antigens. Infect. Immun. **48:**676–685.
21. **Monack, D. M., J. Mecsas, D. Bouley, and S. Falkow.** 1998. *Yersinia*-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. J. Exp. Med. **188:**2127–2137.
22. **Motin, V. L., R. Nakajima, G. B. Smirnov, and R. R. Brubaker.** 1994. Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide. Infect. Immun. **62:**4192–4201.
23. **Nakajima, R., and R. R. Brubaker.** 1993. Association between virulence of *Yersinia* pestis and suppression of gamma interferon and tumor necrosis factor alpha. Infect. Immun. **61:**23–31.
24. **Nakajima, R., V. L. Motin, and R. R. Brubaker.** 1995. Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. Infect. Immun. **63:**3021–3029.
25. **Palmer, L. E., S. Hobbie, J. E. Galan, and J. B. Bliska.** 1998. YopJ of *Yersinia* pseudotuberculosis is required for the inhibition of macrophage TNF-alpha production and downregulation of the MAP kinases p38 and JNK. Mol. Microbiol. **27:**953–965.
26. **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.
27. **Pettersson, J., R. Nordfelth, E. Dubinina, T. Bergman, M. Gustafsson, K. E. Magnusson, and H. Wolf-Watz.** 1996. Modulation of virulence factor expression by pathogen target cell contact. Science **273:**1231–1233.
28. **Rimpilainen, M., A. Forsberg, and H. Wolf-Watz.** 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. J. Bacteriol. **174:**3355–3363.
29. **Rosqvist, R., I. Bolin, and H. Wolf-Watz.** 1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. Infect. Immun. **56:**2139–2143.
30. **Rosqvist, R., A. Forsberg, M. Rimpilainen, T. Bergman, and H. Wolf-Watz.** 1990. The cytotoxic protein YopE of *Yersinia* obstructs the primary host defence. Mol. Microbiol. **4:**657–667.
31. **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.
32. **Straley, S. C., and R. D. Perry.** 1995. Environmental modulation of gene expression and pathogenesis in *Yersinia.* Trends Microbiol. **3:**310–317.
33. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. Mol. Microbiol. **8:**1005–1010.
34. **Von Pawel-Rammingen, U., M. V. Telepnev, G. Schmidt, K. Aktories, H. Wolf-Watz, and R. Rosqvist.** 2000. GAP activity of the *Yersinia* YopE cytotoxin specifically targets the Rho pathway: a mechanism for disruption of actin microfilament structure. Mol. Microbiol. **36:**737–748.
35. **Yao, T., J. Mecsas, J. I. Healy, S. Falkow, and Y. Chien.** 1999. Suppression of T and B lymphocyte activation by a *Yersinia pseudotuberculosis* virulence factor, *yopH*. J. Exp. Med. **190:**1343–1350.

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