INFECTION AND IMMUNITY, Nov. 2004, p. 6707–6710 Vol. 72, No. 11
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.11.6707–6710.2004
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LcrV Synthesis Is Altered by DNA Adenine Methylase Overproduction in *Yersinia pseudotuberculosis* and Is Required To Confer Immunity in Vaccinated Hosts

Golnaz Badie, Douglas M. Heithoff, and Michael J. Mahan*

Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, California

Received 3 June 2004/Returned for modification 14 July 2004/Accepted 29 July 2004

Yersinia pseudotuberculosis **mutants that overproduce the DNA adenine methylase (Dam^{OP}** ***Yersinia*****) are attenuated, confer robust protective immune responses, and synthesize or secrete several** ***Yersinia*** **outer proteins (Yops) under conditions that are nonpermissive for synthesis and secretion in wild-type strains. To understand the molecular basis of immunity elicited by Dam^{OP}** ***Yersinia*****, we investigated the effects of Dam overproduction on the synthesis and localization of a principal** ***Yersinia*** **immunogen, LcrV, a low-calcium-responsive virulence factor involved in Yop synthesis, localization, and suppression of host inflammatory activities. Dam overproduction relaxed the stringent temperature and calcium regulation of LcrV synthesis. Moreover, the LcrV-dependent synthesis and localization of the actin cytotoxin, YopE, were shown to be relaxed in Dam^{OP} cells, suggesting that the synthesis and localization of Yops can occur via both LcrV-dependent and -independent mechanisms. Last, the immunity conferred by Dam^{OP}** ***Yersinia*** **was strictly dependent on the presence of LcrV, which may result from its role (i) as an immunogen, (ii) as an immunomodulator of host anti-inflammatory activities, or (iii) in the altered synthesis and localization of Yops that could contribute to immunogen repertoire expansion.**

Yersinia pestis is the causative agent of human plague (5, 7), whereas enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are the causative agents of mesenteric lymphadenitis and gastroenteritis, respectively (6). The pathogenicity of *Yersinia* is dependent on the presence of a virulence plasmid, pCD in *Y. pestis* or pYV in enteropathogenic species, that encodes a type III secretion apparatus and antihost effector proteins, termed *Yersinia* outer proteins (Yops) (6, 9, 24). Upon host cell contact, the effectors are injected by the type III secretion apparatus into the host cytoplasm of target cells, where they inhibit phagocytosis and engage in anti-inflammatory activities (6, 8, 10, 15, 42, 49). The secretion of Yops is normally under strict regulatory control by the low-calcium response, whereby Yop secretion maximally occurs under conditions of low calcium (Ca^{2+}) and high temperature (37°C) in vitro (12, 45).

Alteration of DNA adenine methylase (Dam) activity has been shown to attenuate the virulence of several pathogens and confer protective immune responses in vaccinated animals (13, 17, 18, 26). The molecular basis of virulence attenuation and protection conferred in Dam mutant strains appears to involve ectopic gene expression and the resultant elaboration of an expanded repertoire of antigens. In *Y. pseudotuberculosis*, Dam overproduction has been shown to attenuate virulence, confer protective immune responses, cause the secretion of several Yops under conditions that are nonpermissive for secretion in wild-type strains, and alter host immune responses to *Yersinia* antigens (22, 23). One of the low-calcium-responsive *Yersinia* antigens whose synthesis is affected by Dam overproduction is YopE, a 23-kDa actin cytotoxin involved in antiphagocytosis that is secreted under low-calcium conditions (1, 3, 48). Dam overproduction relaxed the high-temperature and low-calcium dependence of YopE synthesis and relaxed the high-temperature but not the low-calcium dependence of YopE secretion (22, 23). Such patterns of altered expression and secretion may contribute to the attenuated virulence and robust immunity observed in vaccinated animals.

Dam overproduction in *Yersinia* relaxes the temperature and calcium dependence of LcrV synthesis. Here we examined the effect of Dam overproduction on the synthesis, localization, and secretion of LcrV, a low-calcium-responsive *Yersinia* virulence protein involved in Yop expression (30), Yop translocation (38), and the suppression of host inflammatory activities (6, 33, 43). LcrV is also a principal *Yersinia* immunogen, as robust levels of protection are conferred when LcrV is delivered as a subunit vaccine (7, 25, 32); additionally, administration of antibodies directed against LcrV epitopes confers passive immunity (reviewed in reference 6). Dam^{+} and Dam^{OP} *Yersinia* (Table 1; Fig. 1) were grown under conditions permissive for LcrV synthesis (low calcium, high temperature) and conditions nonpermissive for LcrV synthesis (high calcium, low temperature; high calcium, high temperature; and low calcium, low temperature). Whole-cell, membrane, and supernatant fractions were analyzed by immunoblotting using anti-LcrV antibody. Dam overproduction relaxed the temperature or calcium dependence of LcrV synthesis under all three nonpermissive conditions tested (Fig. 1, whole-cell fraction). Thus, Dam overproduction disrupts both the temperature and calcium control of LcrV synthesis in a manner similar to what has been observed for YopE (23).

The LcrV dependence of YopE synthesis and localization is relaxed under Dam^{OP} conditions. Since LcrV is directly involved in Yop translocation (38) and indirectly involved in the

* Corresponding author. Mailing address: Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, CA 93106. Phone: (805) 893-7160. Fax: (805) 893-4724. E-mail: mahan@lifesci.lscf.ucsb.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference(s)
Y. pseudotuberculosis strain		
YPIIIpYV	Wild type	Stanley Falkow
MT2294	*dam*::Kn + pTP166-Cm	22
MT2394	Δ*lcrV*	This work
MT2395	Δ*lcrV dam*::Kn + pTP166-Cm	This work
Plasmid		
pTP166-Cm	*E. coli dam* under *tac* promoter control; chloramphenicol-resistant derivative of pTP166	22, 29

positive control of Yop expression (30), we examined the effects of the presence and absence of LcrV on YopE synthesis and localization under Dam^{OP} conditions. LcrV is encoded on the *Yersinia* pYV virulence plasmid, within the *lcrGVH-yopBD* operon (2, 37). To assess the contribution of LcrV to Yop synthesis and localization under Dam^{OP} conditions, a nonpolar deletion was constructed in *lcrV* according to standard methods (18). Briefly, a PCR-based strategy was implemented such that 879 bp (293 codons) within *lcrV* were removed (bp 28 to 906 out of a total of 981 bp); the native *lcrV* reading frame was confirmed to be intact by DNA sequencing. The deletion strain (MT2394) showed no LcrV expression as assessed by Western analysis utilizing anti-LcrV antibodies (data not shown). This LcrV null mutant was used to discern whether Dam overproduction enabled *Yersinia* to override the strict LcrV dependence of Yop production and translocation (38).

Although the lack of LcrV resulted in a considerable reduction in YopE synthesis and localization to extracytosolic fractions in Dam^{OP} *Yersinia*, significantly more was observed under permissive conditions compared to Dam^{+} *Yersinia* (Fig. 2A and B). Further, when grown under nonpermissive conditions, the absence of LcrV did not abrogate the Dam^{OP}-mediated ectopic synthesis and localization of YopE (Fig. 2B). Taken together, these data suggest that the ectopic Yop synthesis and localization observed in Dam^{OP} cells (22, 23) can occur via LcrV-dependent and -independent mechanisms.

The protection conferred by DamOP *Yersinia* is dependent on the presence of the LcrV antigen. LcrV is a principal *Yersinia* immunogen as potent levels of immunity to *Yersinia* infection are conferred when LcrV is delivered as a subunit vaccine (7, 32). Thus, we examined whether LcrV is required for the heightened immunity observed in animals vaccinated with Dam^{OP} *Yersinia* (22, 23) by comparing the protection conferred by LcrV^{-} Dam^{OP} *Yersinia* to that conferred by LcrV^{+} Dam^{OP} *Yersinia*. Table 2 shows that the protection conferred by Dam^{OP} *Yersinia* is highly dependent on the presence of LcrV, as BALB/c mice orally immunized with LcrV^{-} Dam^{OP} *Y. pseudotuberculosis* were not protected against a challenge with the virulent strain at more than 700 or 7,000 times the 50% lethal dose, whereas LcrV^{+} Dam^{OP} *Yersinia* elicited complete protection at these challenge doses. Additionally, the time of death following virulent challenge was similar in LcrV^{-} Dam^{OP} vaccinated mice and control (nonvaccinated) mice, indicating that the immune protection conferred by Dam^{OP} *Yersinia* requires LcrV (data not shown). Such dependence on LcrV may be due to its role as a principal immunogen and/or its role in the synthesis and localization of Yops, which may also contribute to the immunity observed in Dam^{OP} *Yersinia*-vaccinated hosts.

The role of Dam in virulence and in the elicitation of protective immune responses may rely on its capability as a global regulator of gene expression (18, 26, 28, 36). Elucidation of the possible mechanisms by which Dam regulates gene expression comes from genetic analysis of the *Escherichia coli* pyelone-

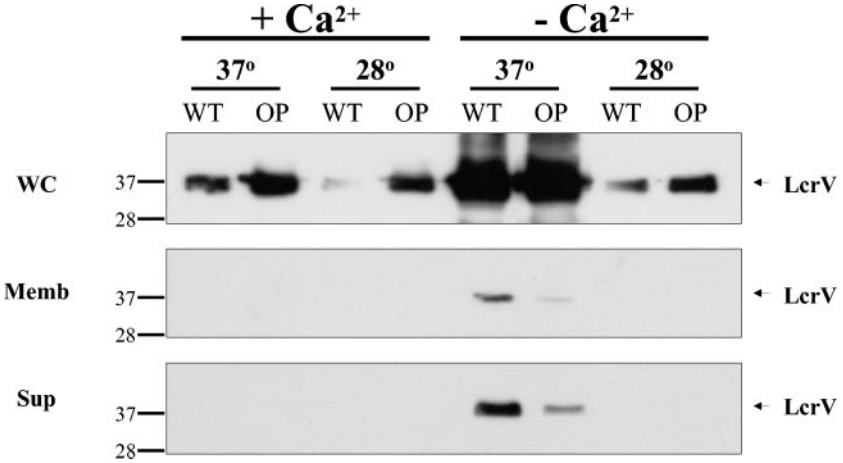

FIG. 1. The high-temperature and low-calcium dependence of LcrV synthesis is relaxed in Dam-overproducing *Y. pseudotuberculosis*. Whole-cell (WC), membrane (Memb), and supernatant (Sup) fractions (12) were prepared from wild-type (WT) and Dam-overproducing (OP) *Y. pseudotuberculosis* grown under the indicated conditions according to methods described previously (12, 44, 45). For each growth condition, total protein extracts corresponding to 2.0×10^6 cells (~20 μg of protein/well) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Pierce), and probed with mouse anti-LcrV monoclonal antibodies (1:4,000 dilution). Peroxidase-conjugated sheep anti-mouse immunoglobulin G (1:40,000 dilution; Amersham Biosciences), was used as the secondary antibody, and hybridization was detected by chemiluminescence using Supersignal West Femto Maximum Sensitivity Substrate (Pierce) followed by a 2-min exposure to film. Inspection of corresponding Coomassie-stained gels showed similar band intensities of nonregulated proteins under all conditions tested (data not shown). Western analysis of *lcrV*$^{+}$ and *lcrV*$^{-}$ strains confirmed that the 37-kDa protein was LcrV (data not shown).

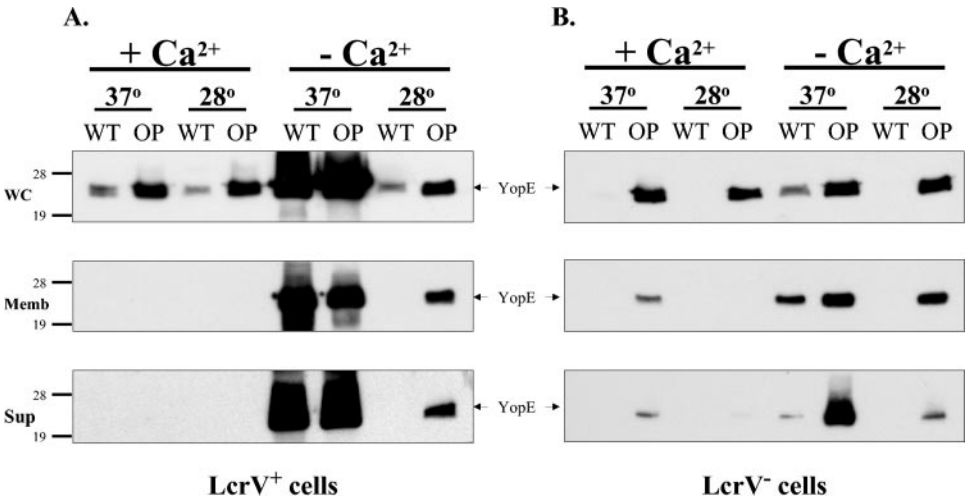

FIG. 2. The LcrV dependence of YopE synthesis and localization is relaxed under Dam-overproducing conditions in *Y. pseudotuberculosis*. Whole-cell (WC), membrane (Memb), and supernatant (Sup) fractions (12) were prepared from *dam* wild-type (WT) and Dam-overproducing (OP) *Y. pseudotuberculosis* containing (A) or lacking (B) the *lcrV* gene. For each growth condition (12, 44, 45), total protein extracts corresponding to 2.0×10^6 cells (~20 μg of protein/well) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Pierce), and probed with rabbit anti-YopE polyclonal antibodies (1:50,000 dilution). Peroxidase-conjugated donkey anti-rabbit immunoglobulin G was used as the secondary antibody (1:20,000 dilution; Amersham Biosciences), and hybridization was detected by chemiluminescence using Supersignal West Femto Maximum Sensitivity Substrate (Pierce) followed by a 30-s (A) or 2-min (B) exposure to film. Inspection of corresponding Coomassie-stained gels showed similar band intensities of nonregulated proteins under all conditions tested (data not shown). Western analysis of *yopE*$^+$ and *yopE*$^-$ strains confirmed that the 23-kDa protein was YopE (23).

phritis-associated pili (*pap*) operon (20, 26, 47), which encodes adherence factors (pili) that are essential for virulence in monkey and mouse models of pyelonephritis (35, 41). Dam target sites in the *pap* promoter are protected from methylation by the binding of regulatory proteins at or near these sites, forming specific DNA methylation patterns analogous to those exhibited in eukaryotes (4, 14, 19, 40, 46). These DNA methylation patterns regulate gene expression by modulating the binding of regulatory proteins to Dam target sites.

One possible outcome of Dam dysregulation is the production of an expanded repertoire of antigens that contribute to the potent state of immunity observed in vaccinated animals. Additionally, the low-grade persistence of *dam* mutant vaccines in appropriate lymphoid tissues (e.g., Peyer's patches) in *Salmonella* spp. (13, 18) and in *Yersinia* (22) may provide a stable source of antigens in sufficient quantity and duration for the transition to the development of potent adaptive immune responses (11, 26). This suggestion is supported by work with *Salmonella* wherein the loss of Dam function results in a number of changes in the bacterial physiology. *dam*$^-$ mutants appear to express in vitro a number of genes that are normally only produced in vivo during the initiation and progression of bacterial infection (17, 18, 27); additionally, both bacteria-associated and -secreted proteins are affected by the loss of Dam regulation (13, 17, 39).

Similarly, in *Yersinia*, Dam overproduction altered the expression of LcrV (Fig. 1) and the expression and/or secretion of YopE (Fig. 2A) as well as several other low-calcium-responsive *Yersinia* virulence proteins (22, 23). Additionally, since LcrV normally functions by suppressing inflammatory cytokines during infection, altered expression or localization of LcrV and/or Yops may contribute to the elicitation of protective responses by immunogen repertoire expansion and/or by altering pathogen-mediated modulation of host inflammatory activities (6, 21, 33, 34, 43).

TABLE 2. Protective immunity conferred by Dam-overproducing *Y. pseudotuberculosis* is dependent on the presence of the LcrV antigen[a]

Vaccine strain	Relevant genotype[b]	No. of survivors after challenge with indicated no. of organisms/total no. of animals	
		1.8×10^{10}	1.8×10^{11}
None	NA	0/10	0/10
MT2294	Dam^{OP}	11/11	11/11
MT2395	Dam^{OP} Δ*lcrV*	0/12	0/12

[a] Six- to eight-week-old BALB/c mice were perorally immunized via gastrointubation with a dose of 3×10^9 LcrV$^-$ Dam^{OP} or 2×10^9 LcrV$^+$ Dam^{OP} *Y. pseudotuberculosis* organisms (16). Mice were perorally challenged with virulent *Y. pseudotuberculosis* (YPIIIpYV) at the dose indicated 8 weeks postimmunization [the peroral 50% lethal dose of YPIIIpYV is 2.5×10^7 organisms, determined by Monack et al. (31)]. Dam^{OP} *Y. pseudotuberculosis* are cleared from vaccinated animals between day 5 and day 21 (22, 23) postimmunization, and thus Dam^{OP} *Yersinia* were not present at the time of challenge.

[b] Bacterial strains are derivatives of *Y. pseudotuberculosis* YPIIIpYV. Dam^{OP} strains MT2395 and MT2294 contain *E. coli dam* on a chloramphenicol-resistant derivative of the high-copy-number recombinant plasmid pTP166 (22, 29) in *dam* mutant (*dam*::Kn) genetic backgrounds. Since *dam* is essential for viability in *Y. pseudotuberculosis* (22), the loss of the Dam^{OP} plasmids in *dam* mutant backgrounds is lethal for this pathogen. NA, not applicable.

We thank Bob Sinsheimer and Steve Julio for critically reading the manuscript and Robert Brubaker (Michigan State University) and Greg Plano (University of Miami, Miami, Florida) for the generous gifts of LcrV and YopE antibodies, respectively.

This work was supported by the University of California Biotech Program (grants no. 2001-15, 2002-14), the Santa Barbara Cottage Hospital Research Program, USDA grant no. 2000-02539, and the G. Harold & Leila Y. Mathers Foundation (grant to M.J.M).

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Editor: A. D. O'Brien