Expression of the *Yersinia enterocolitica* pYV-Encoded Type III Secretion System Is Modulated by Lipopolysaccharide O-Antigen Status[▽]

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We show that the expression of a *Yersinia enterocolitica* O:8 pYV-encoded type III secretion system was altered in a rough mutant (YeO8-R) due to elevated levels of FlhDC. H-NS might underlie *flhDC* upregulation in YeO8-R, and the data suggest a relationship between the absence of O antigen and the expression of H-NS.

The type III secretion system (TTSS) is an export system used by several pathogens to secrete effector proteins into the extracellular milieu or to inject them into the membranes or cytosol of eukaryotic cells (for reviews, see references 10 and 14). *Yersinia enterocolitica* is a human pathogen causing a broad range of gastrointestinal disorders (6). This pathogen harbors a plasmid-encoded TTSS that is required for virulence. A set of virulence factors, called Yops, are secreted by this system and enable *Y. enterocolitica* to multiply extracellularly in lymphoid tissues (for reviews, see references 10 and 11). YopH, YopE, YopT, and YopO/YpkA disturb cytoskeletal dynamics, thereby inhibiting phagocytosis by polymorphonuclear leukocytes and macrophages (for a review, see reference 9). YopP induces apoptosis of macrophages and inhibits the activation of NF- κ B, thereby downregulating the secretion of proinflammatory mediators by eukaryotic cells (9). YopM is another effector protein, although at present its cellular function is not clear (9, 18). Nevertheless, YopM is an important virulence factor in *Y. enterocolitica*: a *yopM* mutant is unable to establish a systemic infection (30).

Yops are indispensable when bacteria meet the host immune cells. To cause a disease, however, bacteria need several plasmid- and chromosomally encoded virulence factors. The latter include invasins (Inv) (23, 24), phospholipase A (YplA) (26), and iron-sequestering proteins (7), and their role in the virulence of *Y. enterocolitica* has been established. Our group has demonstrated the importance of lipopolysaccharide (LPS) O antigen in virulence in different animal models (1, 21, 33). The O-antigen mutant (referred to below as YeO8-R) used in these studies was isolated as a spontaneous mutant resistant to the *Y. enterocolitica* O:8 bacteriophage ϕ 80-18 (33). This mutant did not express any intracellular O antigen and was complemented in *trans* with plasmid pLZ116, which harbors genes *hemH* to *gne* of the *wb* cluster (GenBank accession number U46859)

(33) and which restored the virulence of YeO8-R (33). However, the exact role of O antigen in *Y. enterocolitica* virulence remains elusive. O antigen could play a direct role in virulence by protecting bacteria from host defense mechanisms. In other pathogens, O antigen is involved in the resistance to complement and antimicrobial peptides (28, 29). The current data, however, suggest that this is not the case for YeO8 (C. Pérez and J. A. Bengoechea, unpublished data).

In a recent study, we presented evidence suggesting that the expression of O antigen is coordinated with and affects the expression of other *Yersinia* virulence factors (1). Supporting this hypothesis, *inv* expression is downregulated, whereas the expression of *flhDC*, the flagellar master regulatory operon, is upregulated, with a concomitant increase in the flagellar TTSS-mediated secretion of YplA, in YeO8-R (1). At present we can only speculate about the effect of increased *flhDC* expression on *Yersinia* virulence. This is even more difficult because the nature of the flagellar TTSS is poorly understood, and in addition, it seems that *flhDC* may regulate systems other than the flagellum regulon (20).

In order to determine whether other virulence-related systems are affected in YeO8-R, we analyzed the expression and functionality of the plasmid-encoded TTSS.

At 37°C in Trypticase soy broth (TSB) supplemented with 20 mM sodium oxalate and 20 mM MgCl₂ (TSBox), YeO8 secreted larger amounts of Yops to the culture supernatant than YeO8-R (Fig. 1A). Complementation of YeO8-R with plasmid pLZ116 restored Yop secretion to wild-type levels (Fig. 1A). YeO8::pRVddhB is a defined rough mutant constructed by insertion mutagenesis (1). In this strain, the suicide vector pRVddhB, which contains a 0.6-kb fragment of the *ddhB* gene from the *wb* cluster, is inserted into the genome by homologous recombination (1). Like YeO8-R, YeO8::pRVddhB secreted smaller amounts of Yops to the culture supernatant than YeO8 (Fig. 1A). No difference in the growth rate was observed between YeO8, YeO8-R, and YeO8::pRVddhB either at room temperature (RT), at 37°C, or under calcium restriction conditions (data not shown). Analysis by Western blotting revealed that the amount of YopE in the bacterial pellets was greater in YeO8 than in YeO8-R (Fig. 1B, upper panel), and this correlated with decreased secretion of YopE

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FIG. 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining of proteins from the supernatants of Ca^{2+} -deprived cultures. Proteins were precipitated overnight with ammonium sulfate (47.5%, wt/vol). (B) Immunoblot analysis of the amounts of YopE present in bacterial pellets and culture supernatants of Ca^{2+} -deprived cultures after 1 h of incubation. In panels A and B, the number of bacteria corresponding to the loaded volume of supernatant is given below each lane. (C) Actin disruption by *Yersinia* infection. HeLa cells (monolayer with 80% confluence) were infected with either YeO8, YeO8-R, or YeO8-R/pLZ116 (MOI, 100:1) for 1 h. After fixing and permeabilization of cells, actin was stained with phalloidin-fluorescein isothiocyanate, and cells were analyzed by fluorescence microscopy. (D) Translocation of YopE into HeLa cells by YeO8, YeO8-R, or YeO8-R/pLZ116 (MOI, 100:1; 1 h of infection). After protease protection and digitonin extraction (22), aliquots corresponding to approximately 6×10^4 infected HeLa cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using a rabbit polyclonal antiserum raised against YopE (1:2,000 dilution). Results in all panels are representative of four independent experiments.

to the culture supernatant (Fig. 1B, lower panel). Next, we determined the minimal length of O antigen required for normal secretion of Yops. Strain YeO8-WbcEGB expresses one single O unit in the LPS, since it has a nonpolar mutation in the *wzy* gene, coding for the O-antigen polymerase (2). The *wzy* mutant secreted similar amounts of Yops as YeO8, indicating that the presence of one O unit is sufficient for the wild-type secretion of Yops (Fig. 1A). This may explain why we always see almost 100% substitution of the LPS core with at least one O unit in YeO8, even though the overall O-antigen expression is downregulated at 37°C (3).

One putative explanation for our results could be that the expression of the *yop* virulon is downregulated in YeO8-R. The expression of the *yop* genes is thermoregulated, and the ex-

pression of many is reinforced by the transcriptional regulator VirF (8, 27). We studied whether the expression of *virF* and *yopE* is altered in YeO8-R by constructing transcriptional reporter fusions where a promoterless *cat* gene was under the control of the promoter of *virF* or *yopE*. Briefly, the promoter regions of *virF* (P_{virF}) and *yopE* (P_{yopE}) were amplified by PCR with primers *virF*Pf (5'-CGGATCCCCCTCTCTTTTCCAGAGCGAG-3' [BamHI site underlined]) and *virF*Pr (5'-CCCAAGCTTGGCAAAAGAATATATAGGCCATCTTG-3' [HindIII site underlined]) and primers *YopE*Pf (5'-CGGATCCGGGTAAACATTAATATTTGCCCGAC-3' [BamHI site underlined]) and *YopE*Pr (5'-CCCAAGCTTGGCTGTGAGACTGAGCGCCAG-3' [HindIII site underlined]), respectively, using YeO8 DNA as a template and *Vent* DNA polymerase (New England

Biolabs). The PCR fragments (P_{virF} [342 bp] and P_{yopE} [442 bp]) were gel purified, digested with BamHI and HindIII, and cloned into the BamHI-HindIII sites of pKK232.8 (Amersham Pharmacia Biotech) to obtain pKKPvirF and pKKPyopE. The cloned fragments were sequenced to ensure that no mistakes were introduced during amplification. Plasmids were electroporated into YeO8 and YeO8-R, and the chloramphenicol acetyltransferase activity in cellular extracts was measured as previously described (13). The P_{virF} -cat was expressed to a higher level in YeO8 than in YeO8-R (248.0 ± 106.3 versus 94.0 ± 12.4 U/ μ g of protein [$P < 0.05$ by the two-tailed t test]). In good agreement, the expression of the P_{yopE} -cat fusion was also significantly higher in YeO8 than in YeO8-R (435.8 ± 54.2 versus 15.7 ± 0.9 U/ μ g of protein [$P < 0.05$ by the two-tailed t test]). We conclude from these results that the absence of O antigen acts negatively on the transcription of elements of the Yop virulon.

The injection of YopE into the cytosol of HeLa cells by wild-type bacteria induces disruption and condensation of the actin microfilament structure of the cells (25). We studied whether YeO8-R infection of HeLa cells would trigger similar cytoskeleton disturbances. Figure 1C shows that HeLa cells infected with YeO8-R displayed intact actin microfilaments. This correlated with the lack of YopE translocation by YeO8-R (Fig. 1D). Plasmid pLZ116 complemented all these phenotypes (Fig. 1).

We aimed to identify the regulatory circuit underlying the downregulation of the *yop* virulon in YeO8-R. Bleves and coworkers demonstrated cross talk between the *yop* virulon and the *flhDC* operon (5). They showed that an *flhDC* mutant of *Y. enterocolitica* serotype O:9 secretes more Yops than the wild type, suggesting that *flhDC* downregulates *yop* expression in the wild-type strain (5). In agreement with this idea, overexpression of *flhDC* in the wild-type strain results in decreased Yop secretion (5). These results and the fact that *flhDC* is overexpressed in YeO8-R (1) may indicate that the upregulation of *flhDC* underlies the downregulation of Yop secretion in YeO8-R. However, Young and Young (31) did not find a regulatory link between *flhDC* and the *yop* virulon in YeO8. To study whether overexpression of *flhDC* in YeO8-R is responsible for the decreased Yop secretion, we analyzed the Yop secretion from a YeO8-R *flhDC* mutant. This strain was constructed by targeted mutagenesis using the suicide vector pKNOCK-FlhDC (1). This mutant secreted higher levels of Yops than YeO8-R, and the amount was similar to that secreted by YeO8 (Fig. 2A). A YeO8 *flhDC* mutant was constructed using the suicide vector pKNOCK-FlhDC. This strain secreted higher levels of Yops than YeO8-R, levels similar to those secreted by YeO8 and the YeO8-R *flhDC* mutant (Fig. 2A). Infection of HeLa cells with YeO8-R *flhDC* mutant triggered a condensation of the actin microfilaments (Fig. 2B). Taking these findings together, we conclude that the elevated levels of FlhDC and/or another protein regulated by FlhDC are responsible for Yop virulon downregulation in YeO8-R.

The question remains of how to explain the regulatory mechanism underlying *flhDC* upregulation in YeO8-R. In *Escherichia coli*, H-NS is a positive regulator of *flhDC* (4), and therefore, we hypothesized that *flhDC* upregulation in YeO8-R could be caused by abnormal levels of H-NS. We first analyzed the expression of the transcriptional fusion

FIG. 2. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining of proteins from the supernatants of Ca^{2+} -deprived cultures. See the legend to Fig. 1 for details. (B) *Yersinia*-induced actin disruption. HeLa cells were infected with YeO8-R::pKNOCK-FlhDC, a YeO8-R *flhDC* mutant (MOI, 100:1), for 1 h. After fixing and permeabilization of cells, actin was stained with phalloidin-fluorescein isothiocyanate, and cells were then analyzed by fluorescence microscopy. Results in panels A and B are representative of three independent experiments.

flhDC::lucFF (pRFlhDCO8 [1]) in isogenic *Escherichia coli* *hns*⁺ and *hns* mutant strains (19). The amount of light was lower in the *hns* mutant strain than in the wild-type strain ($5,367 \pm 656$ versus $221,300 \pm 10,611$ relative light units [RLU] [$P < 0.05$ by the two-tailed t test]), suggesting that H-NS could act as an activator for *Yersinia flhDC*. This prompted us to study whether H-NS overexpression in YeO8 would induce *flhDC* upregulation. To this end, we amplified YeO8 *hns* by PCR using primers YeO8H-NSF (5'-GGAATTCCTGCGTTTATTAGTAGGAAGCAGC-3' [EcoRI site underlined]) and YeO8H-NSR (5'-GGGTACC CTTATCAATTGGGAGGGGAGG-3' [KpnI site under-

lined)). The PCR fragment (687 bp) was gel purified, digested with EcoRI and KpnI, and cloned into the EcoRI-KpnI sites of pBAD30 (16) to yield pBADHNS. The cloned fragment was sequenced to ensure that no mistakes were introduced during amplification. Next we introduced pBADHNS or pBAD30 into YeO8 containing the transcriptional fusion *flhDC::lucFF* (1), and the amount of light was measured. We found that *flhDC* expression was significantly higher ($P < 0.05$ by one-way analysis of variance) in the strain harboring pBADHNS ($30,590 \pm 840$ RLU) than in the strain harboring pBAD30 ($8,473 \pm 208$ RLU) or the strain without any cloning vector ($12,360 \pm 2,413$ RLU). In *Y. enterocolitica*, *flhDC* upregulation has been linked to an increase in motility (1, 32). We then quantified the effect of H-NS on YeO8 migration in motility medium (1% tryptone [1, 32]). YeO8 harboring pBADHNS showed higher motility (3.2 ± 0.3 cm [$P < 0.05$ by one-way analysis of variance]) than the strain harboring pBAD30 (2.0 ± 0.1 cm) or YeO8 without any cloning vector (1.9 ± 0.2 cm). Having established that overexpression of H-NS in YeO8 provokes two of the phenotypes of YeO8-R, i.e., the upregulation of *flhDC* and the elevated motility, we asked whether Yop secretion is altered. The results shown in Fig. 3 demonstrate that YeO8 harboring pBADHNS secreted less Yops than either YeO8 harboring pBAD30 or YeO8 without any cloning vector (Fig. 3A). However, the YeO8 *flhDC* mutant harboring pBADHNS secreted similar amounts of Yops as YeO8 (Fig. 3A), thereby supporting the conclusion that H-NS alters Yop secretion by affecting the expression of FlhDC and/or another protein regulated by FlhDC. On the other hand, these results suggest that H-NS could be overexpressed in YeO8-R. We measured H-NS accumulation by Western blot analysis in YeO8 and YeO8-R under different growth conditions (Fig. 3B). The level of H-NS in YeO8-R was consistently higher than that in YeO8 at RT, at 37°C, and under calcium restriction conditions.

To determine if the expression levels of *hns* correlated with levels of H-NS protein, we constructed an *hns::lucFF* transcriptional fusion. Briefly, the promoter region of *hns* was amplified by PCR with primers ProHNSF (5'-GGAATTCCTTGAGC CATGGGCCCGTAAC-3' [EcoRI site underlined]) and ProHNSRr (5'-CGGATCCGTTCAATTTCTGCTTGCCCT GG-3' [BamHI site underlined]) using YeO8 DNA as a template and *Vent* DNA polymerase (New England Biolabs). The PCR fragment (917 bp) was gel purified, digested with EcoRI, and cloned into the EcoRI-SmaI sites of pGPL01 to obtain pGPLYeProHNS. pGPL01 contains a promoterless *lucFF* gene and an R6K origin of replication (15). The cloned fragment was sequenced to ensure that no mistakes were introduced during amplification. A PstI-HindIII fragment was cloned into PstI-HindIII sites of pUC18 to obtain pUCYeProHNS. This plasmid was introduced into YeO8 and YeO8-R, and the amount of light was measured. The expression of *hns* was not significantly different for YeO8 and YeO8-R grown either at RT ($1,231,000 \pm 210,800$ and $1,275,000 \pm 138,800$ RLU, respectively [$P > 0.05$]), at 37°C ($206,100 \pm 7,398$ and $209,600 \pm 14,990$ RLU, respectively [$P > 0.05$]), or under calcium restriction conditions ($546,800 \pm 73,270$ and $599,900 \pm 64,140$ RLU [$P > 0.05$]). These results indicate that the differences at the protein level between YeO8

FIG. 3. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining of proteins from the supernatants of Ca^{2+} -deprived cultures. See the legend to Fig. 1 for details. Levels of H-NS present in the four strains, which were grown in TSBx at 37°C, are shown below. Whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted. Equal amounts of proteins were normalized to the optical density at 600 nm. The transfer membrane was incubated with an anti-H-NS polyclonal antibody (H200 immunoglobulin G; 1:10,000 dilution), and the bound specific antibodies were detected by peroxidase-conjugated anti-rabbit immunoglobulin antibodies (1:1,000 dilution). Bottom panel shows the levels of a nonspecific protein also detected by the antibody in the whole-cell lysates. (B) Immunoblot analysis of H-NS expression by YeO8 and YeO8-R. Whole-cell lysates of bacteria grown in TSB at 21°C (RT), at 37°C, or in TSBx at 37°C (Ca^{2+} minus) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted. Equal amounts of proteins were normalized to the optical density at 600 nm. Lower panel shows the levels of a nonspecific protein also detected by the antibody in the whole-cell lysates. Note that the protein levels are comparable between lanes, indicating equal loading of protein in each lane. Results in panels A and B are representative of three independent experiments.

and YeO8-R cannot be explained by differences at the level of *hns* transcription, suggesting that absence of O antigen affects H-NS accumulation posttranscriptionally. Analysis of Yop secretion and *flhDC* expression in an *hns* mutant of YeO8-R would be valuable.

However, we could not construct this mutant, since *hns* is an essential gene in *Yersinia* (12, 17; also unpublished data).

In summary, in this work we have presented evidence demonstrating that the expression of the pYV-encoded TTSS was altered in YeO8-R as a consequence of abnormally elevated levels of FlhDC and/or another protein regulated by FlhDC. We also showed that H-NS is an activator of *flhDC* in *Y. enterocolitica* and most likely underlies the *flhDC* upregulation in YeO8-R. The role of H-NS as a global regulator is widely accepted, but the regulation of H-NS itself is still poorly understood. Our data indicate that there is a relationship between the absence of O antigen and the levels of H-NS. Studies to understand this regulatory connection are ongoing.

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