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YtxR, a Conserved LysR-Like Regulator That Induces Expression of Genes Encoding a Putative ADP-Ribosyltransferase Toxin Homologue in *Yersinia enterocolitica*▽

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Yersinia enterocolitica **causes human gastroenteritis, and many isolates have been classified as either "American" or "non-American" strains based on their geographic prevalence and virulence properties. In this study we describe identification of a transcriptional regulator that controls expression of the *Y. enterocolitica ytxAB* genes. The *ytxAB* genes have the potential to encode an ADP-ribosylating toxin with similarity to pertussis toxin. However, a *ytxAB* null mutation did not affect virulence in mice. Nevertheless, the *ytxAB* genes are conserved in many *Y. enterocolitica* strains. Interestingly, American and non-American strains have different *ytxAB* alleles encoding proteins that are only 50 to 60% identical. To obtain further insight into the *ytxAB* locus, we investigated whether it is regulated as part of a known or novel regulon. Transposon mutagenesis identified a LysR-like regulator, which we designated YtxR. Expression of *ytxR* from a nonnative promoter increased Φ(*ytxA-lacZ*) operon fusion expression up to 35-fold. YtxR also activated expression of its own promoter. DNase I footprinting showed that a His_6-YtxR fusion protein directly interacted with the *ytxA* and *ytxR* control regions at similar distances upstream of their probable transcription initiation sites, identified by primer extension. Deletion analysis demonstrated that removal of the regions protected by His_6-YtxR in vitro eliminated YtxR-dependent induction in vivo. The *ytxAB* locus is not present in most *Yersinia* species. In contrast, *ytxR* is conserved in multiple *Yersinia* species, as well as in the closely related organisms *Photorhabdus luminescens* and *Photorhabdus asymbiotica*. These observations suggest that YtxR may play a conserved role involving regulation of other genes besides *ytxAB*.**

Three of the species that make up the genus *Yersinia* are widely accepted as organisms that are pathogenic to humans. *Y. pestis* is the etiological agent of plague, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* usually cause intestinal disease. *Y. enterocolitica* is the species most frequently isolated from humans (6, 7), and infections are commonly acquired through ingestion of contaminated food or water (4). During a typical *Y. enterocolitica* infection the bacteria travel to the terminal ileum and penetrate the M cells overlaying the Peyer's patches. They multiply within the Peyer's patches before draining into and infecting the mesenteric lymph nodes. Disease usually manifests as self-limiting gastroenteritis and mesenteric lymphadenitis but can progress to septicemia, especially in patients with complicating conditions (6, 9).

Pathogenic *Y. enterocolitica* strains have been divided into two broad groups, based on serological typing and pathogenicity (7). The high-pathogenicity, so-called "American" strains are associated with large-scale outbreaks and more severe disease than their low-pathogenicity "non-American" counterparts (6). The variable pathogenicity of *Y. enterocolitica* is probably attributable to multiple factors, including the high-pathogenicity island that encodes an iron acquisition system unique to American serotypes (for a review, see reference 5).

An approximately 70-kb virulence plasmid is common to the three pathogenic *Yersinia* species (36). This plasmid encodes the Ysc type III secretion system and the Yop effector proteins that it exports, which disarm some features of the host innate immune response (8). This plasmid is necessary but not sufficient for virulence (7, 21). Chromosomal loci important for invasion of epithelial cells (48, 50), a stress response (11), and an additional type III secretion system (20) also play roles in virulence (for a review, see reference 37). There may be additional chromosomally encoded virulence factors that can be characterized.

Relatively common virulence factors of enteric pathogens are enterotoxins, which fall into two classes. Heat-stable enterotoxins are small peptides that induce fluid secretion from host cells (32). Heat-labile enterotoxins also play a role in inducing fluid secretion and are exemplified by cholera toxin of *Vibrio cholerae* and the heat-labile toxins of *Escherichia coli* (45). Each toxin consists of two different proteins associated in an A_1B_5 stoichiometry. The B pentamer binds to the host cell and triggers endocytic uptake of the complex. The A subunit is responsible for enzymatic modification of host cell proteins. The A subunits of both cholera toxin and the heat-labile toxins of *E. coli* are ADP-ribosyltransferases that modify the α subunit of a subset of heterotrimeric G proteins. This causes an increase in intracellular cyclic AMP levels, ultimately resulting in increased fluid secretion into the intestinal lumen (45).

Many bacterial genes are tightly regulated to ensure that they are expressed only in appropriate environments. This is especially true for virulence factors. For example, the *V. cholerae ctxAB* operon, which encodes cholera toxin, is subject to complex regulation in concert with several other members of overlapping regulons (for reviews, see references 35 and 43).

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In *Yersinia* species the Ysc-Yop regulon is also regulated by several proteins, some of which control many other genes (e.g., YmoA [23]).

Virulent *Y. enterocolitica* strains produce a heat-stable enterotoxin (Yst) that has been implicated as the cause of diarrhea in a rabbit model of infection (13, 14) but whose role in pathogenesis remains controversial (41). To date, genes with the potential to encode a heat-labile enterotoxin have not been described for *Y. enterocolitica*. Here we describe the *ytxAB* genes, which are conserved in several *Y. enterocolitica* strains and could encode a heat-labile enterotoxin. The role of these genes remains unknown, but we found that a previously uncharacterized member of the LysR family of transcriptional regulators, which we designated YtxR, positively regulates the *ytxA* promoter by direct interaction. The *ytxR* gene is conserved in many *Yersinia* species and in at least two members of the closely related genus *Photorhabdus*. In contrast, the *ytxAB* genes are not present in most *Yersinia* species or in any other genus. This suggests that YtxR regulates other genes besides *ytxAB*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and routine growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Y. enterocolitica* strains were routinely grown at 26°C in Luria-Bertani (LB) (Miller) broth or on LB agar plates (29). Antibiotics were used as described previously (27).

Southern hybridization analysis. Chromosomal DNA was digested with HindIII, resolved by electrophoresis on a 0.8% agarose gel, and transferred to nitrocellulose by the method of Southern (44). Approximately 300-bp "*ytxA*" probe fragments were generated by PCR using primers that annealed to the central region of *ytxA* from strains JB580v and MC22. Labeling, hybridization, and detection were done with the ECL direct nucleic acid labeling and detection system (GE Healthcare Life Sciences).

PCR amplification of the *sapA-pspF* intergenic region. The following primers annealed to the 5′ end of *sapA* and the 3′ end of *pspF*, incorporating BamHI and XbaI sites, respectively (underlined): 5′-CGCGGATCCCCACTGACACAATA GACAAAACCGCGCTGAC (*sapA* primer) and 5′-GGCTCTAGAATTGGCT GCATAATAGTGAATATCAGATGCT (*pspF* primer).

The primers were used in PCRs with chromosomal DNA from various *Y. enterocolitica* strains. The products were cloned into plasmid pHG329, and their DNA sequences were determined.

Transposon mutagenesis. Transposon mutagenesis of *Y. enterocolitica* strain YVM619 was performed exactly as described previously (27). Mutants with increased Φ(*ytxA-lacZ*) expression were identified as described in the Results. Southern blotting was done to ensure that each mutant contained a single transposon insertion, and the transposon-chromosome junctions were isolated and their DNA sequences were determined as described previously (27).

Strain and plasmid construction. To construct a *ytxAB* deletion mutant, two DNA fragments were amplified from *Y. enterocolitica* strain JB580v chromosomal DNA by PCR. One fragment had a BglII site followed by the first 10 codons of *ytxA* and approximately 1 kb of upstream DNA. The other fragment had a BglII site followed by the last 22 codons of *ytxB* and approximately 1 kb of downstream DNA. These fragments were ligated at the BglII site and cloned into plasmid pEP185.2. The BamHI kanamycin resistance gene fragment from mini-Tn*5* Km2 (15) was then cloned into the unique BglII site. The resulting plasmid, pAJD213, was integrated into the JB580v chromosome, and Km^r Cm^s exconjugants were isolated. The Δ*ytxAB*::*kan* mutation was confirmed by Southern hybridization analysis (data not shown).

To construct Φ(*ytxA-lacZ*) and Φ(*ytxR-lacZ*) single-copy operon fusion strains, *ytxA* or *ytxR* control region fragments were amplified from strain JB580v chromosomal DNA by PCR. The fragments were cloned into plasmid pFUSE or pKN8 and integrated into the chromosome by homologous recombination (2) or were cloned into plasmid pAJD905 and integrated into the *ara* locus exactly as described previously (28).

A Δ*ytxR* in-frame deletion mutant (AJD239) was constructed with the λ Red recombinase gene replacement system (12), adapted for use in *Y. enterocolitica* (27). Briefly, a Δ*ytxR*::*kan* mutation was made using allelic exchange mediated by Red recombinase. The kanamycin resistance gene was removed by FLP recombinase-mediated excision, and the in-frame deletion was confirmed by Southern hybridization, colony PCR, and DNA sequencing (data not shown).

araBp-ytxR expression plasmids were constructed by amplifying fragments from *Y. enterocolitica* strain JB580v genomic DNA and cloning them into pBAD18-Km or pBAD33. To construct an *araBp*-His_6-*ytxR* expression plasmid, a $ytxR^+$ fragment was amplified by PCR and cloned into plasmid pQE30 (QIAGEN Inc.). It was then excised as an EcoRI-SalI fragment and cloned into pBAD18-Km to obtain plasmid pAJD679.

β-Galactosidase assays. To determine the effect of transposon insertions on Φ(*ytxA-lacZ*) expression, saturated cultures were diluted into 4 ml of LB broth containing appropriate antibiotics in 18-mm-diameter test tubes so that the optical density at 600 nm was approximately 0.08. Cultures were grown on a roller drum at 26°C for 3 h. Then 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the cultures were returned to the roller drum for an additional 2 h.

To determine the effects of an *araBp-ytxR*$^+$ plasmid, saturated cultures were diluted as described above into 4 ml of LB broth containing appropriate antibiotics. The cultures were grown on a roller drum at 26°C for 2 to 3 h (optical density at 600 nm, approximately 0.2 to 0.4), and then 0.2% (final concentration) arabinose was added. Cells were then grown for an additional 2 to 2.5 h at 26°C.

β-Galactosidase activity was determined at room temperature (approximately 22°C) using permeabilized cells (26). Activities were expressed in arbitrary units, which were determined using the formula described by Miller (29). Individual cultures were assayed in duplicate, and the activities reported below are the averages from three independent cultures.

RNA isolation and primer extension analysis. Total RNA was isolated from *Y. enterocolitica* strains with a single-copy chromosomal Φ(*ytxA-lacZY*) operon fusion (AJD1299) or a Φ(*ytxR-lacZY*) operon fusion (AJD1300) and *araBp-ytxR*$^+$ plasmid pAJD654. Cultures were grown as described above for the β-galactosidase assay experiments to determine the effect of an *araBp-ytxR*$^+$ plasmid. RNA was isolated using an RNeasy mini kit (QIAGEN). End labeling of the oligonucleotide and primer extension reactions were done with the Primer Extension System avian myeloblastosis virus reverse transcriptase (Promega). The primer used was 5′-TCATCGGTTGTCGGATCGGA, which corresponds to a region in the template strand 60 bp downstream of the cloning site in the *lacZ* fusion plasmid pAJD905. The primer was labeled at the 5′ end with [γ-^{32}P]ATP and used in extension reaction mixtures containing 5 μg of RNA. To generate size markers, the same primer was used in DNA sequencing reactions with the pAJD1062 (*ytxAp*) or pAJD1065 (*ytxRp*) template using the *fmol* DNA cycle sequencing system (Promega). Samples were resolved by denaturing 8% polyacrylamide–urea electrophoresis and visualized by autoradiography.

Purification of His_6-YtxR. A 1-liter culture of *E. coli* strain BL21-CodonPlus containing plasmid pAJD679 was grown at 30°C to an optical density at 600 nm of approximately 0.9. Arabinose (final concentration, 0.2%) was added, and the culture was incubated for an additional 3 h. Bacterial cells were collected by centrifugation, frozen at −20°C, and then resuspended in 20 ml of a solution containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM $MgCl_2$ (pH 7.5) containing 1× Complete protease inhibitor (Roche) and 1.25 mg/ml lysozyme. Cells were incubated on ice for 30 min and disrupted by sonication. The soluble and insoluble fractions were separated by centrifugation, and the soluble extract (supernatant) was mixed with 4 ml Ni-nitrilotriacetic acid-agarose (QIAGEN) for 1 h at 4°C and then poured into a column. The column was washed with 20 ml of a solution containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM $MgCl_2$ (pH 7.5). His_6-YtxR protein was eluted with 10 ml of a solution containing 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM $MgCl_2$ (pH 7.5) and collected in 1-ml fractions, which were used directly in DNase I footprinting assays. Protein concentrations were estimated using NanoDrop ND-1000 spectrophotometer A_{280} measurement and a bovine serum albumin standard in the His_6-YtxR elution buffer.

Preparation of probes for DNase I footprinting. The *ytxA* control region fragment was generated from plasmid pAJD610 using M13 reverse primer and a primer that annealed approximately 450 bp upstream of *ytxA* position +1 and incorporated an EcoRI site. The product was digested with BamHI, which cleaved downstream of *ytxA* position 1, and was dephosphorylated with calf intestinal alkaline phosphatase (Promega). The bottom (template) strand was labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Unincorporated [γ-^{32}P]ATP was removed with the Promega Wizard SV gel and PCR cleanup system. To eliminate any label from the other end of the DNA fragment, the product was digested with EcoRI and cleaned again with the Promega Wizard SV gel and PCR cleanup system.

A *ytxR* control region fragment was generated from plasmid pAJD1252 using a primer that annealed to the cloning site of the plasmid (downstream of *ytxRp* position +1) and a primer that annealed approximately 550 bp upstream of *ytxR*

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or features	Source or reference
Escherichia coli B BL21-CodonPlus	*ompT gal* [*dcm*] [*lon*] *hsdSB* (r_B^- m_B^-) λDE3 lysogen pRIL	Stratagene
Yersinia enterocolitica 8081 strains (serogroup O:8, American strains)[a]		
JB580v	Δ*yenR* (r^- m^+) pYV^+	24
YVM619	Φ(*ytxA-lacZYA*)	This study
YVM707	Δ*ytxAB*::*kan*	This study
AJD239	Δ*ytxR*	This study
AJD199	Φ(*ytxA-lacZYA*) Φ([Tn*Mod*-RKm′-*lacI*^{q}*tacp*]-*ytxR*)	This study
AJD200	Φ(*ytxA-lacZYA*) Φ([Tn*Mod*-RKm′-*lacI*^{q}*tacp*]-*ytxR*)	This study
AJD254	Δ*ytxR* Φ(*ytxA-lacZYA*)	This study
AJD378	Δ*ytxR* Φ(*ytxR-lacZYA*)	This study
AJD1296	Δ*ytxR* Δ*araGFB*::[Φ(*ytxA-lacZY*)] (Δ110 construct)	This study
AJD1297	Δ*ytxR* Δ*araGFB*::[Φ(*ytxA-lacZY*)] (Δ52 construct)	This study
AJD1299	Δ*ytxR* Δ*araGFB*::[Φ(*ytxA-lacZY*)] (Δ485 construct)[b]	This study
AJD1295	Δ*ytxR* Δ*araGFB*::[Φ(*ytxR-lacZY*)] (Δ150 construct)	This study
AJD1300	Δ*ytxR* Δ*araGFB*::[Φ(*ytxR-lacZY*)] (Δ500 construct)[b]	This study
AJD1303	Δ*ytxR* Δ*araGFB*::[Φ(*ytxR-lacZY*)] (Δ86 construct)	This study
AJD1304	Δ*ytxR* Δ*araGFB*::[Φ(*ytxR-lacZY*)] (Δ21 construct)	This study
Y. enterocolitica CDC reference strains[c]		
657-83	Serogroup O:20, American strain	CDC
658-83	Serogroup O:21, American strain	CDC
655-83	Serogroup O:18, American strain	CDC
634-83	Serogroup O:4,32, American strain	CDC
637-83	Serogroup O:5,27, non-American strain	CDC
661-83	Serogroup O:27, non-American strain	CDC
Y. enterocolitica clinical isolates		
MC5	Biogroup 1, serogroup O:6,30, Crohn's disease	M. Cafferkey
MC7	Biogroup 1, serogroup O:9, colitis with perforation	M. Cafferkey
MC8	Biogroup 1, serogroup O:9, septicemia	M. Cafferkey
MC17	Biogroup 1, serogroup O:3, acute diarrhea	M. Cafferkey
MC22	Biogroup 3, serogroup O:3, acute appendicitis	M. Cafferkey
MC33	Biogroup 3, serogroup O:3, acute colitis	M. Cafferkey
MC28	Biogroup 4, serogroup O:3, acute diarrhea	M. Cafferkey
MC6	Biogroup 4, serogroup O:3, mesenteric adenitis	M. Cafferkey
MC51	Biogroup 4, serogroup O:3, acute terminal ileitis	M. Cafferkey
Other *Yersinia* strains		
Y. pseudotuberculosis YPIII	pYV^+	18
Y. pseudotuberculosis K286	Clinical isolate	30
Y. kristensenii		Walter Hill, FDA[d]
Y. frederikensenii		Walter Hill, FDA
Y. frederikensenii MC31	Clinical isolate (chronic diarrhea, weight loss)	M. Cafferkey
Y. rohdei 3022-83	Dog stool isolate	CDC
Y. rohdei 3435-85	Human stool isolate	CDC
Y. aldovae 670-83	Isolated from water	CDC
Y. intermedia		Walter Hill, FDA
Plasmids		
pFUSE	Cm^r, *mob*$^+$ (RP4), R6K *ori*, *lacZYA*$^+$ operon fusion vector	2
pKN8	BglII linker in SmaI site of pFUSE	17
pBAD18-Km	Km^r, *araBp* expression vector, Col E1 *ori*	19
pBAD33	Cm^r *araBp* expression vector, p15A *ori*	19
pEP185.2	Cm^r, *mob*$^+$ (RP4), R6K *ori*	24
pWSK129	Km^r, low-copy-number cloning vector, pSC101 *ori*	49
pHG329	Amp^r, cloning vector, pBR322 *ori*	46
pQE30	Ap^r, Col E1 *ori*, T5*p* expression vector for His_6 fusion proteins	QIAGEN
pAJD213	Δ*ytxAB*::*kan* in pEP185.2	This study
pAJD593	*araBp-ytxR* in pBAD18-Km	This study
pAJD610	*ytxA* full-length control region in pHG329	This study
pAJD654	*araBp-ytxR* in pBAD33	This study
pAJD679	*araBp*-His_6-*ytxR* in pBAD18-Km	This study
pAJD905	Cm^r, R6K *ori*, *mob*$^+$ (RP4), *sacB1*$^+$, *lacZY* operon fusion vector	28
pAJD1060	Δ110 *ytxAp* fragment in pAJD905	This study
pAJD1061	Δ53 *ytxAp* fragment in pAJD905	This study
pAJD1062	Δ485 (full length) *ytxAp* fragment in pAJD905	This study
pAJD1065	Δ500 (full length) *ytxRp* fragment in pAJD905	This study
pAJD1057	Δ150 *ytxRp* fragment in pAJD905	This study
pAJD1058	Δ110 *ytxRp* fragment in pAJD905	This study
pAJD1059	Δ21 *ytxRp* fragment in pAJD905	This study
pAJD1252	*ytxR* control region (positions −552 to 40) in pWSK129	This study

[a] All *Y. enterocolitica* 8081 strains are derivatives of strain JB580v.
[b] The Φ(*ytxA-lacZY*) Δ485 and Φ(*ytxR-lacZY*) Δ500 constructs represent the full-length control regions (all noncoding upstream DNA) upstream of the *lacZY* operon.
[c] CDC, Centers for Disease Control and Prevention.
[d] FDA, Food and Drug Administration.

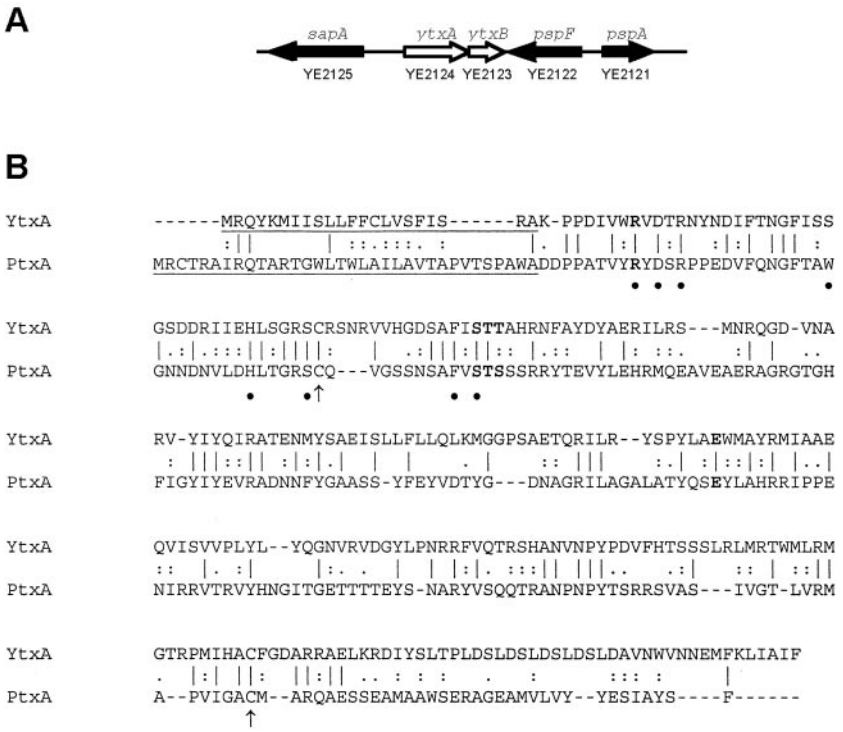

FIG. 1. *ytxAB* locus encodes a putative ADP-ribosylating toxin. (A) Diagram of the arrangement of the *ytxAB* genes in the *sapA-pspF* intergenic region of *Y. enterocolitica* strain JB580v (8081). (B) CLUSTALW alignment of the YtxA protein with the catalytic subunit of pertussis toxin (PtxA; GenBank accession number P04947). The signal sequences (predicted for YtxA) are underlined. Identical, strongly similar, and similar residues are indicated by vertical lines, colons, and periods, respectively, between the sequences. Three conserved sequences characteristic of ADP-ribosyltransferases, including the catalytic glutamic acid residue, are indicated by boldface type (10, 16, 33). Residues important for full ADP-ribosyltransferase activity of PtxA are indicated by bullets below the sequence. Cysteine residues that form a disulfide bond in PtxA are indicated by arrows below the sequence.

position +1 and incorporated an XbaI site. The product was digested with BamHI, which cleaved downstream of *ytxR* position +1, and dephosphorylated. The bottom (template) strand was labeled as described above, except that an XbaI digest was used to eliminate any label from the other end of the DNA fragment.

DNase I footprinting assays. Labeled *ytxA* or *ytxR* control region probes (approximately 2 nM) were mixed with His_6-YtxR protein in a buffer containing 400 μg/ml salmon sperm DNA (Sigma-Aldrich), 100 mM HEPES (pH 7.6), 50 mM $(NH_4)_2SO_4$, 5 mM dithiothreitol, 1% (vol/vol) Tween 20, and 150 mM KCl (total reaction volume, 50 μl). The reaction mixtures were incubated at 32°C for 15 min, and then 53 μl of a solution containing 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.005 U/μl DNase I was added. Then the mixtures were incubated for 2 min, and digestion was stopped by adding 25 μl of a solution containing 2 M ammonium acetate, 250 mM EDTA, 100 μg/ml salmon sperm DNA, and 1 mg/ml glycogen. The DNA was precipitated with ethanol and resuspended in formamide loading dye. To generate a size marker, the pAJD610 (*ytxAp*) or pAJD1252 (*ytxRp*) plasmid was used in DNA sequencing reactions with the *fmol* DNA cycle sequencing system (Promega). The sequencing primers annealed downstream of the position +1 sites and had 5′ ends that corresponded exactly to the labeled ends of the fragments used in the footprint reactions. Samples were resolved by denaturing 8% polyacrylamide–urea electrophoresis and visualized by autoradiography.

Control region deletion analysis. Truncated *ytxA* and *ytxR* control region fragments were generated by PCR using a common downstream primer that annealed within the 5′ ends of the coding regions and primers that annealed at various distances upstream. XbaI and BglII restriction sites were incorporated for the *ytxAp* fragments. XbaI and BamHI restriction digestion sites were incorporated for the *ytxRp* fragments. The fragments were cloned into pAJD905, and the DNA sequences were confirmed. The operon fusions were integrated into the *ara* locus and confirmed by colony PCR as described previously (28).

Nucleotide sequence accession numbers. The nucleotide sequence data generated in this study have been assigned the following GenBank accession numbers: AY008264 for the *ytxAB* locus from *Y. enterocolitica* strain 8081(serotype O:8) and AY183120 for the *ytxAB* locus from *Y. enterocolitica* strain MC22 (serotype O:3).

RESULTS

Description of the *ytxAB* locus. During characterization of the *Y. enterocolitica* phage shock protein (*psp*) locus (11) we identified two adjacent open reading frames (*ytxAB*) (Fig. 1). A BLASTP search revealed homology between YtxA and the catalytic subunit of pertussis toxin (PtxA; E = 1e-16). Notably, YtxA has the three conserved motifs characteristic of bacterial ADP-ribosylating toxins (ADPRT) (10, 16, 33), including the catalytic glutamic acid residue (Fig. 1). Other amino acids important for full catalytic activity of pertussis toxin (for a review, see reference 25) are also mostly conserved in YtxA (Fig. 1). Therefore, *ytxA* might encode an ADPRT. Two cysteine residues that form an important PtxA disulfide bond (31) are also present in YtxA, and YtxA is predicted to have a *sec*-dependent signal sequence.

A BLASTP search with YtxB revealed no significant homology (E < 10) to previously characterized proteins. YtxB is small, as are the B subunits of cholera toxin and the heat-labile enterotoxins of *E. coli*, and alignment of YtxB with the B subunit of cholera toxin revealed some similarities (data not shown). YtxB is also predicted to have a *sec*-dependent signal sequence.

A *ytxAB* deletion mutant was assessed using oral infection of

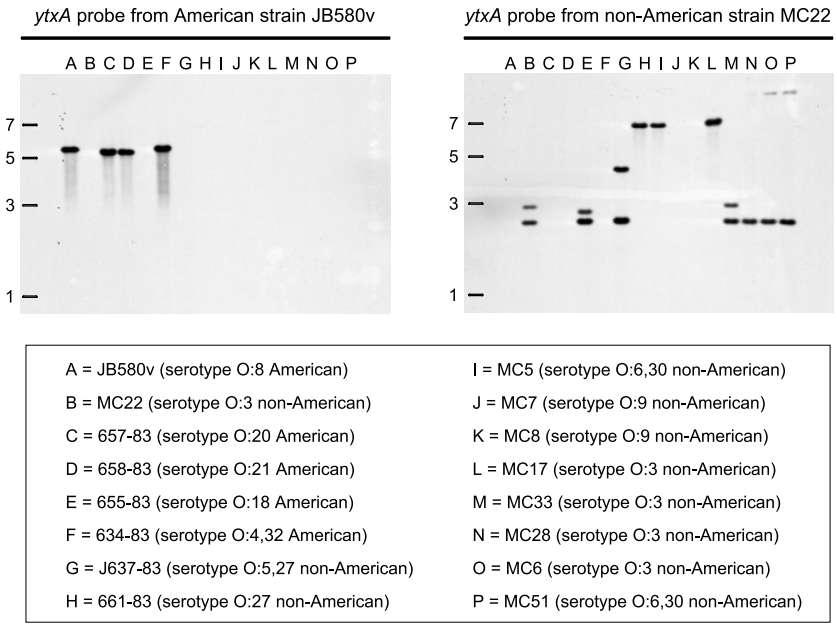

FIG. 2. Southern hybridization analysis of *ytxA* conservation in *Y. enterocolitica*. Chromosomal DNA from various *Y. enterocolitica* strains was digested with HindIII, separated by electrophoresis on a 0.8% agarose gel, and transferred to nitrocellulose. In separate experiments the same nitrocellulose membrane was hybridized with a labeled *ytxA* fragment from American strain JB580v and with a labeled *ytxA* fragment from non-American strain MC22. The approximate positions (in kb) of size markers in the original agarose gel are indicated on the left. The lower panel shows the lane assignments for the chromosomal DNA samples.

6- to 7-week-old female BALB/c mice essentially as described previously (34). The 50% lethal doses of *ytxAB*$^+$ and *ytxAB* null strains were indistinguishable, as were the bacterial loads of these strains in different tissues over time (data not shown). From these experiments, we concluded that the *ytxAB* locus is not required for virulence in an adult mouse model of acute infection. This does not rule out a role for this putative toxin, perhaps a role that is limited to the intestinal stage of disease and/or is host species specific. We also attempted to overexpress the YtxA protein. However, the overexpressed protein was completely insoluble (data not shown), and we were unable to detect ADP-ribosyltransferase activity.

American and non-American strains have divergent *ytxAB* alleles. The *ytxAB* locus might have been acquired recently because its G+C content (39%) is much lower than the average G+C content of the chromosome (47%) (http://www.sanger.ac.uk/Projects/Y_enterocolitica/). Therefore, to investigate *ytxAB* conservation, a Southern hybridization experiment was done using a probe that encoded the central region of the *Y. enterocolitica* strain JB580v *ytxA* gene.

In addition to chromosomal DNA of the strain that it was derived from (serotype O:8), the *ytxA* probe hybridized to chromosomal DNA of *Y. enterocolitica* strains belonging to serotypes O:20, O:21, and O:4,32 (Fig. 2). These strains are all American strains (note that the probe did not hybridize to DNA from one American strain, a serotype O:18 strain). The probe did not hybridize to DNA from any non-American *Y. enterocolitica* strain (Fig. 2) or to DNA from any other *Yersinia* species listed in Table 1 (data not shown). Furthermore, a BLAST search of *Y. pestis* genomes, which were not included in this hybridization experiment, did not reveal any homology to *ytxAB*. Therefore, it appeared that the *ytxA* gene (and presumably *ytxB*) is present only in some American strains of *Y. enterocolitica*.

To confirm the absence of *ytxAB* from non-American *Y. enterocolitica* strains, we amplified the *sapA-pspF* intergenic region (Fig. 1) of one of them by PCR. As a control, we also amplified an approximately 2.3-kb *ytxAB*$^+$ fragment from the chromosome of *Y. enterocolitica* strain JB580v (data not shown). Unexpectedly, the non-American *Y. enterocolitica* strain (strain MC22 [Table 1]) produced a PCR product that was a similar size (data not shown). The DNA sequence of this fragment revealed genes that encoded proteins with 53% and 62% identity to the YtxA and YtxB proteins, respectively, of strain JB580v (data not shown). Despite the significant divergence of these *ytxAB* genes, residues predicted to be important for ADPRT activity of YtxA were conserved. Strikingly, although there was such a major difference between the *ytxAB* coding regions, the same was not true for the noncoding DNA sequences extending 200 bp upstream of the American and non-American *ytxA* initiation codons, which were 97% identical (data not shown).

The Southern hybridization experiment was repeated with a non-American strain *ytxA* probe. This probe hybridized to DNA from *Y. enterocolitica* strains belonging to serotypes O:5,27, O:27, O:6,30, and O:3 (Fig. 2), all of which were non-American strains (note that the probe did not hybridize to DNA from two non-American strains, both belonging to serotype O:9). The probe also hybridized to DNA from the only American *Y. enterocolitica* strain that did not hybridize to the *ytxA* probe from American strain JB580v (serotype O:18) (Fig. 2). The probe did not hybridize to DNA from any other *Yersinia* species listed in Table 1 (data not shown).

FIG. 3. Comparison of *sapA-pspF* intergenic region DNA sequences from *Y. enterocolitica* strains JB580v (serotype O:8, *ytxAB*$^+$) and MC7 (serotype O:9, no *ytxAB* locus). Only part of each intergenic region is shown. The DNA sequence in boldface type is unique to strain JB580v (*ytxAB* gene sequences were omitted for clarity). Bullets above the sequences indicate differences between the regions conserved in both strains. The *ytxA* start codon, *ytxB* stop codon, and *pspF* stop codon (complementary strand) are labeled and underlined.

These data indicate that there are two versions of *ytxAB* in *Y. enterocolitica*. One is present only in American strains, and the other is present primarily in non-American strains.

DNA sequence analysis identified a *ytxAB* cassette. Two clinical isolates belonging to non-American *Y. enterocolitica* serotype O:9 (strains MC7 and MC8 [Table 1]) are the only *Y. enterocolitica* strains tested that do not have a *ytxAB* locus. This conclusion was based on the failure of chromosomal DNA from these strains to hybridize to either probe (Fig. 2) and on the small size of their *sapA-pspF* intergenic region PCR fragments (data not shown).

The DNA sequence of the relatively small *sapA-pspF* intergenic region PCR fragment from one of these *Y. enterocolitica* serotype O:9 strains was compared to that from the *ytxAB*$^+$ strain JB580v (Fig. 3). This revealed the extent of the unique region present in the *ytxAB*$^+$ strain. In addition to the *ytxAB* genes and the 48-bp intergenic region (not shown in Fig. 3), there are 207 bp of upstream DNA and 52 bp of downstream DNA. The non-American *Y. enterocolitica* strain MC22 has a region that is a similar length and has a similar sequence upstream of its *ytxAB* genes (data not shown). Therefore, strains with any version of the *ytxAB* locus probably contain a unique region that includes approximately 200 bp of noncoding upstream DNA. We hypothesized that this region probably contains the *ytxA* promoter and any important regulatory sequences. This hypothesis was investigated in the series of experiments described below.

A LysR-like transcriptional regulator induces *ytxAB* expression. Understanding whether *ytxAB* expression is regulated and, if it is, the underlying mechanism(s) might provide insight into its role. To begin to investigate this, a single-copy Φ(*ytxA-lacZ*) operon fusion strain was constructed. The level of β-galactosidase activity expressed from this fusion was relatively low at 26°C or 37°C (less than 100 Miller units), suggesting that *ytxA* was poorly expressed under standard laboratory conditions. We hypothesized that *ytxAB* might be expressed only under specific conditions and that control is mediated by a regulatory protein interacting with the unique region upstream of *ytxA* (Fig. 3). Therefore, a genetic screen was devised to identify regulatory proteins, even if they were poorly expressed in our standard growth conditions.

The screen relied on a transposon encoding the *E. coli lac* repressor (*lacI*) and an outward-facing *tac* promoter (27). This transposon causes null mutations by insertion (e.g., a *ytxA* repressor) and/or IPTG-dependent overexpression of downstream genes (e.g., a *ytxA* activator). Approximately 40,000 transposon mutants of a Φ(*ytxA-lacZ*) operon fusion strain were screened after growth at 26°C in the presence of IPTG on LB indicator agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Mutants with increased Φ(*ytxA-lacZ*) expression were identified as dark blue colonies and were later confirmed by β-galactosidase assays (data not shown). Any mutants with transposon insertions immediately upstream of Φ(*ytxA-lacZ*) were identified by Southern hybridization analysis and eliminated from further analysis.

The screen did not identify any mutants with an IPTG-independent increase in Φ(*ytxA-lacZ*) expression. However, we identified six mutants that exhibited severalfold IPTG-dependent increases in Φ(*ytxA-lacZ*) expression, suggesting that this expression was caused by overexpression of a gene downstream of the transposon. Southern hybridization analysis indicated that all six mutants had a single transposon insertion in the same chromosomal region (data not shown). Two of these mutants (designated strains AJD199 and AJD200) were randomly selected for further analysis. These mutants exhibited 14- to 20-fold IPTG-dependent induction of Φ(*ytxA-lacZ*) expression (Fig. 4).

The DNA sequences of the transposon-chromosome junctions from the two mutants revealed that the transposon had inserted 186 bp (AJD199) and 156 bp (AJD200) upstream of the same open reading frame (YE2253), in an orientation that would direct its expression from the *tac* promoter of the transposon. YE2253 is located on the complementary strand between nucleotides 2461446 and 2462306 of the *Y. enterocolitica*

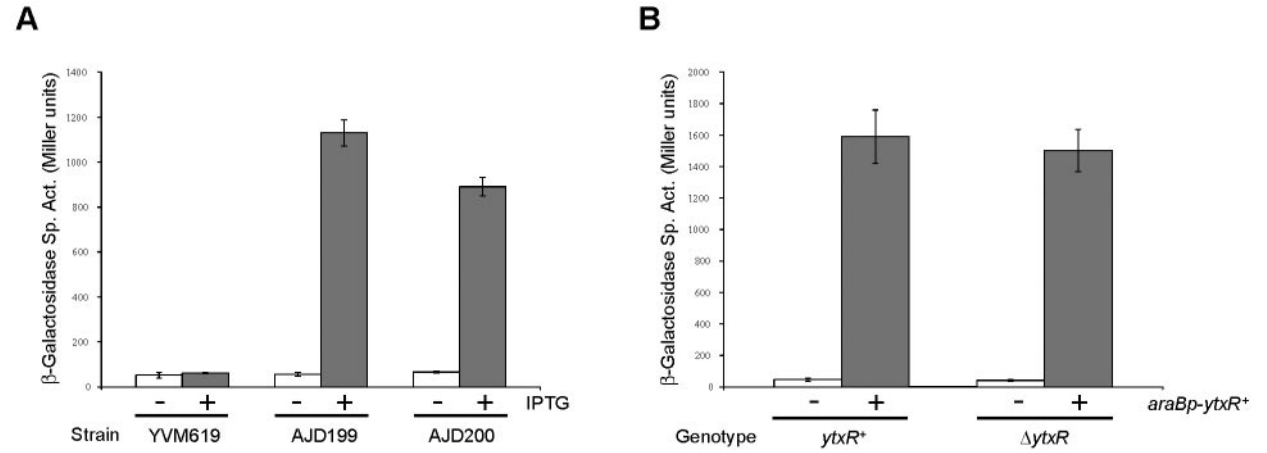

FIG. 4. Increased *ytxR* expression induces a Φ(*ytxA-lacZ*) operon fusion. (A) Expression of Φ(*ytxA-lacZ*) in the presence (+) or absence (−) of IPTG in a strain without a transposon (YVM619) or two mutants with *tacp* transposon insertions upstream of *ytxR* (AJD199 and AJD200). (B) Expression of Φ(*ytxA-lacZ*) in *ytxR*$^+$ (YVM619) or Δ*ytxR* null (AJD254) strains with either *araBp-ytxR*$^+$ expression plasmid pAJD593 (+) or the pBAD18-Km control vector (−). Cultures were grown and β-galactosidase activities were determined as described in Materials and Methods. The data are averages from three independent cultures, and the error bars indicate the standard deviations from the means. Sp. Act., specific activity.

chromosome (http://www.sanger.ac.uk/Projects/Y_enterocolitica/). It is predicted to encode an uncharacterized member of the family of LysR-type transcriptional regulators (LTTRs) (for a review, see reference 40). We designated YE2253 the *ytxR* gene.

Finally, we checked whether *ytxR* overexpression alone was responsible for increasing Φ(*ytxA-lacZ*) expression. The *ytxR* gene was cloned into the *araBp* expression plasmid, pBAD18-Km. Expression of *ytxR* from this plasmid induced Φ(*ytxA-lacZ*) expression approximately 35-fold (Fig. 4). This did not occur for unrelated *lacZ* operon fusions studied in our laboratory (data not shown). A *ytxR* in-frame deletion mutant was also constructed. However, there was no difference in Φ(*ytxA-lacZ*) expression between *ytxR*$^+$ and *ytxR* null strains (Fig. 4). This suggests that the *ytxR* gene is not significantly expressed from its own promoter under standard laboratory conditions. Taken together, all of these data show that expression of *ytxR* from a nonnative promoter is sufficient to induce Φ(*ytxA-lacZ*) expression.

YtxR is an autoregulator. Most LTTRs act as autoregulators, enhancing or repressing their own transcription (40). To test whether this is the case for YtxR, a single-copy Φ(*ytxR-lacZ*) operon fusion was constructed in a strain with a Δ*ytxR* mutation. When *ytxR* was expressed from *araBp* expression plasmid pAJD593, it induced Φ(*ytxR-lacZ*) expression approximately 100-fold (data not shown). This indicates that YtxR is a positive autoregulator.

Determination of *ytxA* and *ytxR* 5′ mRNA ends. Next we wanted to characterize the *ytxA* and *ytxR* promoters and their control by YtxR at the molecular level. An important first step was to locate the 5′ ends of the *ytxA* and *ytxR* mRNAs. Therefore, RNA was isolated separately from Φ(*ytxA-lacZ*) and Φ(*ytxR-lacZ*) strains containing an *araBp-ytxR*$^+$ expression plasmid and analyzed by primer extension (see Materials and Methods).

A single *ytxA* 5′ end was detected that corresponded to 50 nucleotides upstream of the probable *ytxA* ATG start codon (Fig. 5 and data not shown). This result was confirmed by 5′ rapid amplification of cDNA ends using a different *ytxA* template (data not shown). This 5′ mRNA end may have originated from a σ^{70}-dependent promoter because putative −10 and −35 sequences were identified upstream (Fig. 5).

In the case of *ytxR* the 5′ mRNA end corresponded to 237 nucleotides upstream of the probable ATG start codon (Fig. 5). No other smaller products of the primer extension reaction were detected (Fig. 5 and data not shown). Therefore, *ytxR* has an unusually long 5′ untranslated region. However, this is not unprecedented, even for genes that encode LTTRs (39). Once again, sequences with some similarity to −10 and −35 elements were detected upstream of the position corresponding to the 5′ mRNA end.

DNase I footprint analysis of His$_6$-YtxR interaction with the *ytxA* and *ytxR* control regions. The simplest hypothesis to explain how *ytxR* overexpression induces Φ(*ytxA-lacZ*) and Φ(*ytxR-lacZ*) expression is that YtxR directly binds to the *ytxA*/*ytxR* control regions. To test this hypothesis, a His$_6$-YtxR fusion protein was purified [we first confirmed that production of His$_6$-YtxR was able to induce Φ(*ytxA-lacZ*) in vivo (data not shown)]. His$_6$-YtxR protected nucleotides in both the *ytxA* and *ytxR* control regions from DNase I cleavage (Fig. 6). In both cases the protected regions were centered at approximately position −75. This is in good agreement with the binding site locations of other members of the LTTR family (40). An alignment of the protected regions revealed significant sequence similarity (Fig. 7). Similar concentrations of the His$_6$-YtxR protein produced clearly observable DNase I footprints of the *ytxA* and *ytxR* control regions, suggesting that the binding affinities were comparable. These data demonstrate that His$_6$-YtxR interacts with defined regions upstream of *ytxA* and *ytxR* in vitro. Therefore, YtxR probably activates *ytxA* and *ytxR* transcription directly in vivo. Further support for this conclusion came from the set of experiments described below.

5′ Deletion analysis of the *ytxA* and *ytxR* control regions. The final series of experiments was designed to test whether the regions protected by His$_6$-YtxR in vitro were required for

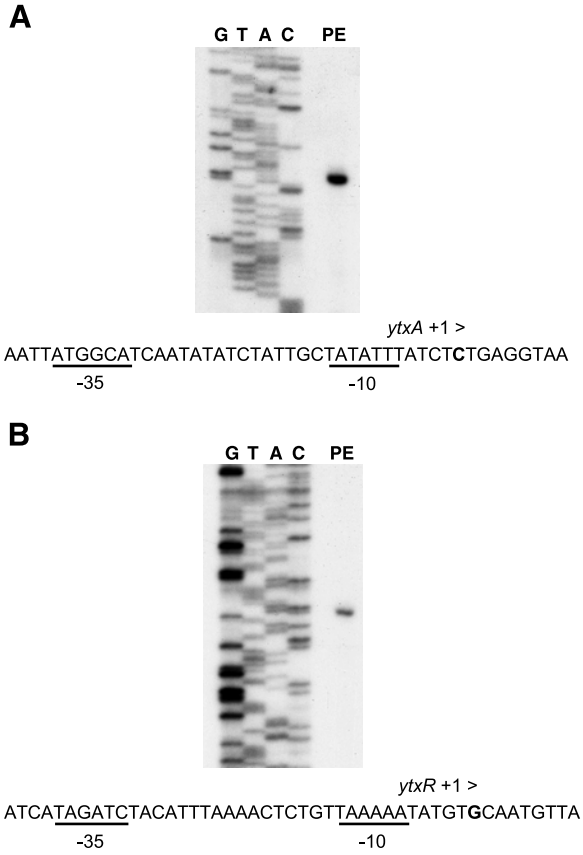

FIG. 5. Primer extension analyses of the *ytxA* and *ytxR* control regions. RNA was extracted from *Y. enterocolitica* strains with either Φ(*ytxA-lacZ*) (A) or Φ(*ytxR-lacZ*) (B) single-copy operon fusions and the *arap-ytxR*$^+$ expression plasmid pAJD654. Lane PE, primer extension reaction; lanes G, T, A, and C, DNA sequencing reactions. The nucleotide sequences surrounding the putative transcription initiation sites are shown below the panels. Nucleotides corresponding to the 5′ mRNA end sites are indicated by boldface type and labeled "+1 >." Putative −10 and −35 elements are underlined and labeled.

YtxR-dependent regulation in vivo. A set of single-copy Φ(*ytxA-lacZ*) and Φ(*ytxR-lacZ*) operon fusion strains was constructed with progressive 5′ deletions of their control regions (Fig. 7). These strains were grown with or without *ytxR* expression from an arabinose-inducible plasmid, and β-galactosidase activities were determined (Table 2).

Deletion of sequences upstream of position −110 did not affect YtxR-dependent induction of Φ(*ytxA-lacZ*) expression (Table 2). However, deletion to position −52 completely eliminated YtxR-induced activity without affecting the basal (YtxR-independent) activity. Therefore, the region between positions −110 and −52 is essential for YtxR-dependent induction in vivo. This is in agreement with the region identified by DNase I footprinting in vitro (Fig. 6 and 7).

In the Φ(*ytxR-lacZ*) deletion analysis, two different phenomena were observed. First, deletion of the region from position −500 to position −150 significantly elevated both YtxR-independent expression and YtxR-dependent expression, while the ability of YtxR to activate expression was maintained (Table 2). The next deletion, from position −150 to position −86, did not have any additional effect on Φ(*ytxR-lacZ*) expression. However, deletion to position −21 eliminated YtxR-dependent induction. Once again, these results are in agreement with the region identified by DNase I footprint analysis.

Conservation of *ytxR*. Southern hybridization analysis (Fig. 2) and BLASTP searches (data not shown) indicated that the *ytxAB* genes are not present in most *Yersinia* species and in all other genera. The *ytxR* gene (YE2253) is not linked to *ytxAB* (YE2124 and YE2123), and so we were interested in investigating *ytxR* conservation. We performed BLASTP searches with the predicted YtxR protein sequence. This analysis revealed that *ytxR* is intact and conserved (more than 90% amino acid identity) in the seven *Yersinia* species whose genome sequences are available (http://www.ncbi.nlm.nih.gov), including several different *Y. pestis* genomes (e.g., YPO2169 in *Y. pestis* CO92 [data not shown]). We confirmed this conservation by successfully amplifying an internal "*ytxR*" fragment from the chromosomes of all the strains tested in the *ytxA* hybridization analysis (Fig. 2) except *Y. aldovae* 670-83 (data not shown). As a negative control, the PCR failed to amplify a fragment from the Δ*ytxR* strain AJD239 (Table 1). Besides *Yersinia*, BLASTP searches also revealed that *ytxR* is conserved in the insect pathogen *Photorhabdus luminescens* and also in *Photorhabdus asymbiotica* (more than 60% amino acid identity and the same chromosomal context). These observations suggest that YtxR probably regulates genes besides *ytxAB* and may play an important conserved role in the closely related genera *Yersinia* and *Photorhabdus*.

DISCUSSION

Multiple *Y. enterocolitica* strains have genes (*ytxAB*) that have the potential to encode an ADPRT. YtxA is a member of a large family of proven and putative bacterial ADPRTs (33), but a *ytxAB* null mutant is virulent in an adult mouse model of acute infection. However, YtxAB could play a role limited to the intestinal stage of disease and/or be host specific. For example, the *Y. enterocolitica* heat-stable enterotoxin Yst had no detectable role in mice (41) or gnotobiotic piglets (38) but did affect diarrhea, weight loss, and death in young rabbits (13). In an attempt to obtain more insight into the *ytxAB* locus, we have begun to investigate the regulation of its expression. Here we report identification of YtxR, an LTTR that induces expression of *ytxAB* and also of its own gene. This regulation is mediated by direct interaction of YtxR with the *ytxA* and *ytxR* control regions.

We discovered two different versions of the *ytxAB* locus in *Y. enterocolitica*. One version is specific to American strains, and the other is specific to non-American strains. An exception is American serotype O:18, which has the non-American version of *ytxAB*. Hybridization analysis with fragments of the *ail* gene also distinguished between American and non-American strains (30). However, the *ail* hybridization pattern of the same O:18 serotype strain placed it with the other American strains. We also found a third version of the *ytxAB* locus in a *Y. intermedia* isolate provided by the Food and Drug Administration (Darwin and Miller, unpublished data). The genome sequence of an American Type Culture Collection *Y. intermedia* strain is also now available (http://www.ncbi.nlm.nih.gov), and the genome contains genes similar to *ytxAB* in the *sapA-pspF* intergenic region. *Y. intermedia*, like several other *Yersinia* spe-

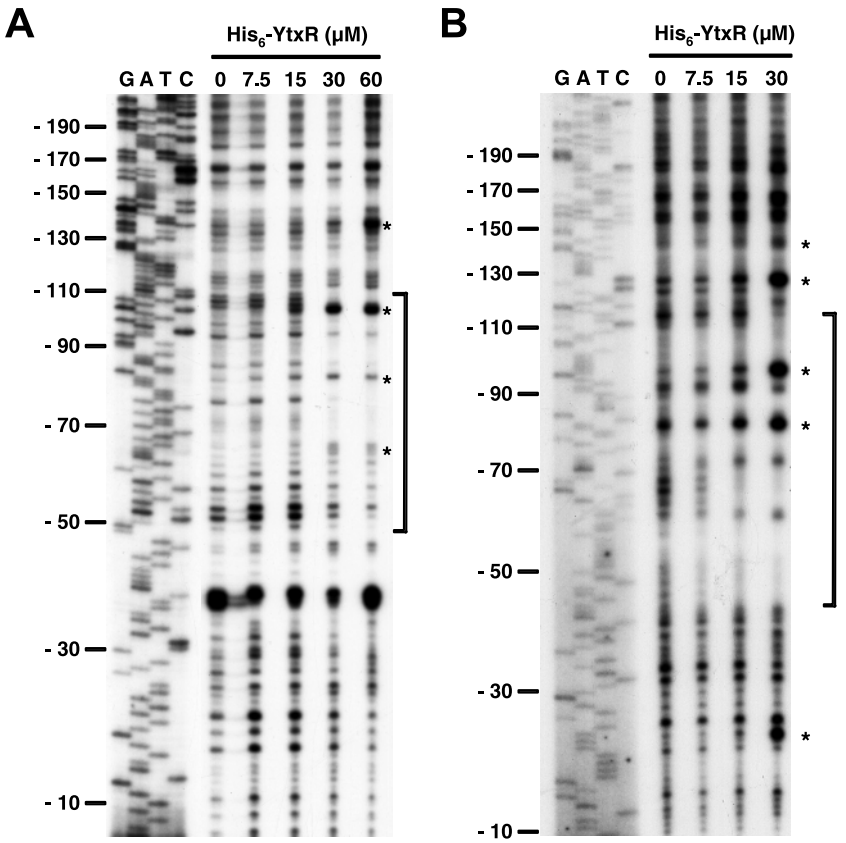

FIG. 6. DNase I footprint analysis of the *ytxA* and *ytxR* control regions. Labeled *ytxA* (A) or *ytxR* (B) control region fragments were incubated with different concentrations of His_6-YtxR protein as indicated above the lanes and then treated with DNase I. Lanes G, A, T, and C show the results for sequencing reactions for each control region and are calibrated with respect to the number of base pairs from the transcription start site. Brackets indicate the approximate region of DNase I protection. Asterisks indicate sites hypersensitive to DNase I cleavage in the presence of His_6-YtxR.

cies, is considered nonpathogenic. However, it has been suggested that some of these species may sometimes cause disease by using uncharacterized virulence proteins (47).

The *ytxAB* locus has a G+C content of 39%, which is much lower than the average G+C content of the chromosome (47%). Perhaps the *ytxAB* locus was acquired by horizontal transfer. Furthermore, genome sequence analysis revealed that in *Y. pestis* CO92 there is an insertion element instead of *ytxAB* between *sapA* and *pspF*. In *Y. pseudotuberculosis* and some other *Yersinia* species there do not appear to be any coding regions between *sapA* and *pspF*. Strikingly, like *ytxAB*, the unlinked *ytxR* gene encoding their regulator also has an extremely low G+C content (33%).

The divergence of the two different versions of *ytxAB* in *Y. enterocolitica* is surprising. The two YtxA versions and two YtxB versions are only 53% and 62% identical, respectively. This contrasts with the 95% amino acid identity between Ail proteins from American and non-American *Y. enterocolitica* strains (3). However, despite the significant dissimilarity between the *ytxA* coding regions of the two American and non-American strains that we studied in detail, the 200 bp of noncoding DNA upstream of their *ytxA* start codons is 97% identical. This suggests that genetic drift may not explain the divergence and that different *ytxAB* cassettes might have been introduced two or more times into the genus *Yersinia*. Of course, we cannot rule out the possibility that a single *ytxAB* locus was acquired by an ancestral strain and strong selective pressures resulted in marked divergence of only the coding sequences.

Many bacterial genes are expressed only weakly in normal laboratory growth conditions. For example, the cholera toxin genes of *V. cholerae* El Tor require highly specialized conditions for expression outside the host (22). Similarly, a Φ(*ytxA-lacZ*) operon fusion was expressed only weakly in the laboratory, which led to the screen that identified *ytxR*. Like the majority of LTTRs, YtxR is an autoregulator. Most LTTRs are negative autoregulators (40), but YtxR falls into a smaller group whose members activate their own expression (for example, see reference 1). Another common feature of most (but not all) LTTRs is regulation of a gene divergently transcribed a short distance immediately upstream (40). The divergently transcribed *xthA* gene (YE2254) is located upstream of *ytxR*. However, it is separated from *ytxR* by almost 800 bp, and a Φ(*xthA-lacZ*) operon fusion is not regulated by YtxR (Axler-DiPerte and Darwin, unpublished data).

Most LTTRs are activated by an interaction with a small coinducer molecule (40). However, our experiments suggested that a coinducer may not be required for YtxR because increased expression of *ytxR* is sufficient to activate its target promoters. The Nac protein of *Klebsiella aerogenes* is an exam-

FIG. 7. Important features of the *ytxA* and *ytxR* control regions. (A) *ytxA* control region. (B) *ytxR* control region. The numbering is relative to the putative transcriptional start sites, indicated by boldface type and labeled "+1>." Putative −10 and −35 elements are underlined and labeled. Regions protected from DNase I cleavage in the presence of His_6-YtxR are double underlined. Deletion endpoints of constructs used in this study are indicated. (C) CLUSTALW alignment of the protected areas of both control regions. Asterisks indicate identical nucleotides.

ple of an LTTR that does not require a coinducer molecule. Like expression of YtxR, increased expression of *nac* from an IPTG-inducible promoter is sufficient to allow it to regulate target promoters (42). If YtxR does not need a coinducer, then activation of the YtxR regulon might be initiated by upregulation of *ytxR* expression. We do not yet know the environmental conditions that allow this to occur. However, we are beginning to obtain some clues about a possible mechanism. First, deletion analysis has suggested that a region far upstream of the *ytxR* promoter negatively regulates its expression (Table 2). Primer extension analysis also revealed that *ytxR* has a long 5′ untranslated region. Therefore, we speculated that *ytxR* regulation is complex and that the untranslated region may play a pivotal role in activation of the YtxR regulon.

TABLE 2. Effects of control region deletions on Φ(*ytxA-lacZ*) and Φ(*ytxR-lacZ*) expression

Control region[a]	β-Galactosidase sp act[b]	
	Without YtxR[c]	With YtxR
Φ(*ytxA-lacZ*)		
Δ485	32 ± 5	950 ± 80
Δ110	40 ± 4	2,120 ± 320
Δ52	47 ± 4	48 ± 6
Φ(*ytxR-lacZ*)		
Δ500	23 ± 6	1,030 ± 340
Δ150	310 ± 120	2,140 ± 560
Δ86	510 ± 60	1,810 ± 610
Δ21	420 ± 32	400 ± 100

[a] Each strain has a Φ(*ytxA-lacZY*) or Φ(*ytxR-lacZY*) fusion integrated on the chromosome with a different amount of DNA upstream of the transcription initiation site, as shown in Fig. 7.

[b] β-Galactosidase specific activity was determined as described in Materials and Methods and is expressed in arbitrary (Miller) units. The values are averages ± standard deviations.

[c] Strains with the *araBp* vector pBAD33 (without YtxR) or the *araBp-ytxR*$^+$ expression plasmid pAJD654 (with YtxR) were grown in the presence of 0.2% arabinose as described in Materials and Methods.

The *ytxR* gene is conserved in all *Yersinia* and *Photorhabdus* species that have been sequenced, most of which do not have the *ytxAB* genes. This strongly suggests that there are probably other unidentified YtxR target promoters. However, we cannot yet ascribe a function to the YtxR regulon. We have not discovered a robust phenotype for a *Y. enterocolitica ytxR* null mutant, including in a mouse model of acute infection (Axler-DiPerte and Darwin, unpublished data). Our favored working hypothesis is that the regulon might be activated in an environment outside the host and then deactivated soon after infection. Therefore, even if the regulon primes *Y. enterocolitica* for stages early in infection, a phenotype in animals would not be apparent if bacteria were not grown in this YtxR-activating environment prior to infection. Another equally plausible possibility is that activation of the YtxR regulon occurs during infection but that it is host specific. Finally, YtxR may play an

environmental role unrelated to host interaction. Uncovering environmental conditions that activate the YtxR regulon, and especially identifying all of the YtxR target promoters, should considerably increase our understanding of the role of the regulon in *Y. enterocolitica* physiology. Addressing these questions will be the major goal of our future investigations. Answering them could provide significant insight into the two very important and well-studied genera *Yersinia* and *Photorhabdus*.

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