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 His6-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 His6-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 A1B5 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).
In *Yersinia* species the Ysc-Yop regulon is also regulated by several proteins, some of which control 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 ytaAB 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 ytaA 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 ytaAB genes are not present in most *Yersinia* species or in any other genus. This suggests that YtxR regulates other genes besides ytaAB.

**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-μg ytaA probe fragments were generated by PCR using primers that annealed to the central region of ytaA 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 sap1-pspF intergenic region.** The following primers annealed to the 5’ end of sap1 and the 3’ end of pspF, incorporating BamH I and XbaI sites, respectively (underlined): 5’-CCGGATCCCATGGATGCT (sap1 primer) and 5’-GGCTCTAGAATTGGCT (pspF primer).

**Purification of His6-YtxR.** A 1-liter culture of *E. coli* strain BL21-CodonPlus containing plasmid pAD769 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 NaH2PO4, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM MgCl2 (pH 7.5) containing 1× complete protease inhibitor (Roche) and 1.25 mM mg lysomyme. Cells were incubated on ice for 20 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-nitrotriacetic acid-agarose (QIAGEN) and kept on ice for 1 h and then poured into a column. The column was washed with 20 ml of a solution containing 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM MgCl2 (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 $A_{270}$ measurement and a bovine serum albumin standard in the Hi5_YtxR elution buffer.

**Preparation of probes for DNase I footprinting.** The ytxA control region preparation was generated from plasmid pAD610 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 EcoRI and cleaned again 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 reamplified with 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-His6-ytxR expression plasmid, a ytxR fragment was amplified by PCR and cloned into plasmid pQE30 (QIAGEN Inc.). It was then excised as an EcoRI-Sall fragment and cloned into plasmid pBAD18-Km to obtain plasmid pAD676.

**β-Galactosidase assays.** To determine the effect of transposon insertions on $\Phi^{(ytaA-lacZ)}$ 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* strain with a single-copy chromosomal $\Phi^{(ytaA-lacZ)}$ operon fusion (AJD1299) or a $\Phi^{(ytxR-lacZ)}$ operon fusion (AJD1300) and araBp-ytxR plasmid pAD604. Cultures were grown as described above for the β-galactosidase assays and were harvested for RNA extraction exactly as described in the Results. Total RNA was isolated using an RNeasy mini kit (QIAGEN). End labeling of the oligonucleotide primer and extension reactions were done with the Primer Extension System avian myeloblastosis virus reverse transcriptase (Promega). The primer used was 5’-TCATCGGTGTGCGATCCGA, which corresponds to a region in the template strand 60 bp downstream of the cloning site in the lacZ fusion plasmid pAD905. The primer was labeled at the 5’ end with $[32P]ATP$ and used in reverse transcription reactions with 5 μg of total RNA. To generate size markers, the same primer was used in DNA sequencing reactions with the pAD1062 (ytaA) or pAD1065 (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 His6-YtxR.** A litter culture of E. coli strain BL21-CodonPlus containing plasmid pAD879 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 NaH2PO4, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM MgCl2 (pH 7.5) containing 1× complete protease inhibitor (Roche) and 1.25 mM mg lysomyme. Cells were incubated on ice for 20 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-nitrotriacetic acid-agarose (QIAGEN) and kept on ice for 1 h and then poured into a column. The column was washed with 20 ml of a solution containing 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM MgCl2 (pH 7.5). His6_YtxR protein was eluted with 10 ml of a solution containing 50 mM NaH2PO4, 300 mM NaCl, 200 mM imidazole, 5 mM β-mercaptoethanol, 0.1% Tween 20, and 5 mM MgCl2 (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 $A_{270}$ measurement and a bovine serum albumin standard in the Hi5_YtxR elution buffer.

**Preparation of probes for DNase I footprinting.** The ytaA control region preparation was generated from plasmid pAD610 using M13 reverse primer and a primer that annealed approximately 450 bp upstream of ytaA position +1 and incorporated an EcoRI site. The product was digested with EcoRI and cleaned again 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 reamplified with FLP recombinase-medi­ated excision, and the in-frame deletion was confirmed by Southern hybridization, colony PCR, and DNA sequencing (data not shown).
TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or features</th>
<th>Source or reference</th>
</tr>
</thead>
</table>
| **Yersinia enterocolitica 8081 strains**
  (serogroup O:8, American strains)
  JB808b | ΔytxR (Δ-ytxR) pYV<sup>a</sup> | 24 |
| YVM619 | ΔytxR (Δ-ytxR) pYV<sup>a</sup> | This study |
| YVM707 | ΔytxR<sup>b</sup> | This study |
| AJD239 | ΔytxR<sup>b</sup> | This study |
| AJD199 | ΔytxR<sup>b</sup> | This study |
| AJD200 | ΔytxR<sup>b</sup> | This study |
| AJD254 | ΔytxR<sup>b</sup> | This study |
| AJD378 | ΔytxR<sup>b</sup> | This study |
| AJD1296 | ΔytxR ΔaraGFB [Δ(ytxA-lacZY)] (Δ110 construct) | This study |
| AJD1297 | ΔytxR ΔaraGFB [Δ(ytxA-lacZY)] (Δ150 construct) | This study |
| AJD1295 | ΔytxR ΔaraGFB [Δ(ytxA-lacZY)] (Δ21 construct) | This study |
| AJD1300 | ΔytxR ΔaraGFB [Δ(ytxA-lacZY)] (Δ500 construct) | This study |
| AJD1303 | ΔytxR ΔaraGFB [Δ(ytxA-lacZY)] (Δ52 construct) | This study |
| AJD1304 | ΔytxR ΔaraGFB [Δ(ytxA-lacZY)] (Δ552 construct) | This study |
| **Y. enterocolitica CDC reference strains**
  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:4,32, non-American strain | CDC |
  661-83 | Serogroup O:27, non-American strain | CDC |
| **Y. enterocolitica clinical isolates**
  MC5 | Biogroup 1, serogroup O:3, 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:2, 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<sup>a</sup> | 18 |
  Y. pseudotuberculosis K286 | Clinical isolate | 30 |
  Y. kristensenii | Clinical isolate (chronic diarrhea, weight loss) | Walter Hill, FDA<sup>d</sup> |
  Y. frederikensenii | Clinical isolate (chronic diarrhea, weight loss) | Walter Hill, FDA<sup>d</sup> |
  Y. rohdei 3022-83 | Clinical isolate | CDC |
  Y. rohdei 3435-85 | Clinical isolate | CDC |
  Y. aldovae 670-83 | Isolated from water | CDC |
  Y. intermedia | Clinical isolate | Walter Hill, FDA<sup>d</sup> |

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFUSE</td>
<td>Cm&lt;sup&gt;b&lt;/sup&gt;, mob&lt;sup&gt;b&lt;/sup&gt; (RP4), R6K ori, lacZYA&lt;sup&gt;b&lt;/sup&gt; operon fusion vector</td>
</tr>
<tr>
<td>pKN8</td>
<td>Km&lt;sup&gt;c&lt;/sup&gt;, araBp expression vector, Col El ori</td>
</tr>
<tr>
<td>pBAD18-Km</td>
<td>Km&lt;sup&gt;c&lt;/sup&gt;, araBp expression vector, p15A ori</td>
</tr>
<tr>
<td>pEP158.2</td>
<td>Km&lt;sup&gt;c&lt;/sup&gt;, mob&lt;sup&gt;b&lt;/sup&gt; (RP4), R6K ori</td>
</tr>
<tr>
<td>pWSK129</td>
<td>Km&lt;sup&gt;c&lt;/sup&gt;, low-copy-number cloning vector, pSC101 ori</td>
</tr>
<tr>
<td>pHG329</td>
<td>Amp&lt;sup&gt;c&lt;/sup&gt;, cloning vector, pBR322 ori</td>
</tr>
<tr>
<td>pQE30</td>
<td>Amp&lt;sup&gt;c&lt;/sup&gt;, Col El ori, Tsp expression vector for H&lt;sub&gt;is&lt;/sub&gt; fusion proteins</td>
</tr>
<tr>
<td>pADJ213</td>
<td>ΔytxA::kan in pEP158.2</td>
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<tr>
<td>pADJ395</td>
<td>araBp-ytxR in pBAD18-Km</td>
</tr>
<tr>
<td>pADJ610</td>
<td>ytxA full-length control region in pHG329</td>
</tr>
<tr>
<td>pADJ654</td>
<td>araBp-ytxR in pBAD33</td>
</tr>
<tr>
<td>pADJ679</td>
<td>araBp-H&lt;sub&gt;is&lt;/sub&gt;ytxR in pBAD18-Km</td>
</tr>
<tr>
<td>pADJ9905</td>
<td>Cm&lt;sup&gt;c&lt;/sup&gt;, R6K ori, mob&lt;sup&gt;b&lt;/sup&gt; (RP4), lac&lt;sup&gt;c&lt;/sup&gt;B&lt;sup&gt;c&lt;/sup&gt;, lacZYA&lt;sup&gt;b&lt;/sup&gt; operon fusion vector</td>
</tr>
<tr>
<td>pADJ1060</td>
<td>Δ110 ytxRp fragment in pADJ905</td>
</tr>
<tr>
<td>pADJ1061</td>
<td>Δ53 ytxRp fragment in pADJ905</td>
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<tr>
<td>pADJ1062</td>
<td>Δ85 (full length) ytxRp fragment in pADJ905</td>
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<td>pADJ1065</td>
<td>Δ500 (full length) ytxRp fragment in pADJ905</td>
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<tr>
<td>pADJ1057</td>
<td>Δ150 ytxRp fragment in pADJ905</td>
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<tr>
<td>pADJ1058</td>
<td>Δ110 ytxRp fragment in pADJ905</td>
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<tr>
<td>pADJ1059</td>
<td>Δ21 ytxRp fragment in pADJ905</td>
</tr>
<tr>
<td>pADJ1292</td>
<td>ytxR control region (positions −552 to 40) in pWSK129</td>
</tr>
</tbody>
</table>

<sup>a</sup> All Y. enterocolitica 8081 strains are derivatives of strain JB808b.

<sup>b</sup> 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.

<sup>c</sup> CDC, Centers for Disease Control and Prevention.

<sup>d</sup> FDA, Food and Drug Administration.
position +1 and incorporated an XbaI site. The product was digested with BamHI, which cleaved downstream of start 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 His6-YtxR protein in a buffer containing 400 μM Hepes (pH 7.5), 50 mM (NH₄)₂SO₄, 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 CaCl₂, 10 mM MgCl₂ 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 (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.

**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 ytxA or ytxR fragments. XbaI and BamHI restriction digestion sites were incorporated for the ytxR 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 BLAST search revealed homology between YtxA and 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 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.
6- to 7-week-old female BALB/c mice essentially as described previously (34). The 50% lethal doses of \( \text{ytxAB} / \text{H11001} \) and \( \text{ytxAB} \) 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 \( \text{ytxAB} \) 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 \( \text{YtxA} \) 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 \( \text{ytxAB} \) alleles. The \( \text{ytxAB} \) locus might have been acquired recently because its \( \text{G} / \text{H11001} \text{C} \) content (39%) is much lower than the average \( \text{G} / \text{H11001} \text{C} \) content of the chromosome (47%) (http://www.sanger.ac.uk/Projects/Y_enterocolitica/). Therefore, to investigate \( \text{ytxAB} \) conservation, a Southern hybridization experiment was done using a probe that encoded the central region of the \( \text{Y. enterocolitica} \) strain JB580v \( \text{ytxA} \) gene.

In addition to chromosomal DNA of the strain that it was derived from (serotype O:8), the \( \text{ytxA} \) probe hybridized to chromosomal DNA of \( \text{Y. enterocolitica} \) 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 \( \text{Y. enterocolitica} \) strain (Fig. 2) or to DNA from any other \( \text{Yersinia} \) species listed in Table 1 (data not shown). Furthermore, a BLAST search of \( \text{Y. pestis} \) genomes, which were not included in this hybridization experiment, did not reveal any homology to \( \text{ytxAB} \). Therefore, it appeared that the \( \text{ytxA} \) gene (and presumably \( \text{ytxB} \)) is present only in some American strains of \( \text{Y. enterocolitica} \).

To confirm the absence of \( \text{ytxAB} \) from non-American \( \text{Y. enterocolitica} \) 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 \( \text{ytxAB} / \text{H11001} \) fragment from the chromosome of \( \text{Y. enterocolitica} \) strain JB580v (data not shown). Unexpectedly, the non-American \( \text{Y. enterocolitica} \) 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 \( \text{YtxA} \) and \( \text{YtxB} \) proteins, respectively, of strain JB580v (data not shown). Despite the significant divergence of these \( \text{ytxAB} \) genes, residues predicted to be important for ADPRT activity of \( \text{YtxA} \) were conserved. Strikingly, although there was such a major difference between the \( \text{ytxAB} \) coding regions, the same was not true for the noncoding DNA sequences extending 200 bp upstream of the American and non-American \( \text{ytxA} \) initiation codons, which were 97% identical (data not shown).

The Southern hybridization experiment was repeated with a non-American strain \( \text{ytxA} \) probe. This probe hybridized to DNA from \( \text{Y. enterocolitica} \) 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 \( \text{Y. enterocolitica} \) strain that did not hybridize to the \( \text{ytxA} \) probe from American strain JB580v (serotype O:18) (Fig. 2). The probe did not hybridize to DNA from any other \( \text{Yersinia} \) species listed in Table 1 (data not shown).

*FIG. 2.* Southern hybridization analysis of \( \text{ytxA} \) conservation in \( \text{Y. enterocolitica} \). Chromosomal DNA from various \( \text{Y. enterocolitica} \) 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 \( \text{ytxA} \) fragment from American strain JB580v and with a labeled \( \text{ytxA} \) 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.
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 ytxAB 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 cassette was inserted 186 bp (AJD199) and 156 bp (AJD200) upstream of the ytxA start codon, ytxB stop codon, and *pspF* stop codon (complementary strand) are labeled and underlined.

![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 (serotype O:9, no 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.](http://jb.asm.org/)

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Taken together, all of these data show that expression of ytxR from its own promoter under standard laboratory conditions. When ytxR expression from a nonnative promoter is sufficient to induce mutation. When ytxR expression.

This suggests that the YtxR is an autoregulator. Most LTTRs act as autoregulators, enhancing or repressing their own transcription (40). To view, see reference 40). We designated YE2253 the ytxR gene.

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 ytxR-lacZ operon fusion was constructed in a strain with a lacZ control region.

A single-copy ytxR-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 ytxR-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 ytxR-lacZ expression.

YtxR interacts with defined regions upstream of ytxA 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 ytxA-lacZ and 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 ρ70-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 (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 His6-YtxR interaction with the ytxA and ytxR control regions. The simplest hypothesis to explain how ytxR overexpression induces ytxA-lacZ and ytxR-lacZ expression is that YtxR directly binds to the ytxA and ytxR control regions. To test this hypothesis, a His6-YtxR fusion protein was purified (we first confirmed that production of His6-YtxR was able to induce ytxA-lacZ expression in vivo (data not shown) (40). An alignment of the protected regions revealed significant sequence similarity (Fig. 7). Similar concentrations of the His6-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 His6-

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 His6-YtxR in vitro were required for

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 laco transposon insertions upstream of ytxR (AJD199 and AJD200). (B) Expression of Φ(ytxA-lacZ) in ytxR+ (YVM619) or ΔytxR 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.

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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.

Methods).
YtxR-dependent regulation in vivo. A set of single-copy \( \Phi(ytxA-lacZ) \) and \( \Phi(ytxR-lacZ) \) 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 \( \beta\)-galactosidase activities were determined (Table 2).

Deletion of sequences upstream of position −110 did not affect YtxR-dependent induction of \( \Phi(ytxA-lacZ) \) 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 DNease I footprint analysis.

In the \( \Phi(ytxR-lacZ) \) 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 \( \Phi(ytxR-lacZ) \) expression.

However, deletion to position −21 eliminated YtxR-dependent induction. Once again, these results are in agreement with the region identified by DNease 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 YtsR 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 \( \Delta ytxR \) 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 \( ytxA \) locus, we have begun to investigate the regulation of its expression. Here we report identification of YtxR, an LTTR that induces the 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-
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 \textit{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 \textit{ytxAB} locus was acquired by horizontal transfer. Furthermore, genome sequence analysis revealed that in \textit{Y. pestis} CO92 there is an insertion element instead of \textit{ytxAB} between \textit{sapA} and \textit{pspF}. In \textit{Y. pseudotuberculosis} and some other \textit{Yersinia} species there do not appear to be any coding regions between \textit{sapA} and \textit{pspF}. Strikingly, like \textit{ytxAB}, the unlinked \textit{ytxR} gene encoding their regulator also has an extremely low G+C content (33%).

The divergence of the two different versions of \textit{ytxAB} in \textit{Y. enterocolitica} is surprising. The two \textit{YtxA} versions and two \textit{YtxB} versions are only 53% and 62% identical, respectively. This contrasts with the 95% amino acid identity between \textit{Ail} proteins from American and non-American \textit{Y. enterocolitica} strains (3). However, despite the significant dissimilarity between the \textit{ytxA} coding regions of the two American and non-American strains that we studied in detail, the 200 bp of non-coding DNA upstream of their \textit{ytxA} start codons is 97% identical. This suggests that genetic drift may not explain the divergence and that different \textit{ytxAB} cassettes might have been introduced two or more times into the genus \textit{Yersinia}. Of course, we cannot rule out the possibility that a single \textit{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 \textit{V. cholerae} El Tor require highly specialized conditions for expression outside the host (22). Similarly, a \textit{ytxA-lacZ} operon fusion was expressed only weakly in the laboratory, which led to the screen that identified \textit{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 \textit{xthA} gene (YE2254) is located upstream of \textit{ytxR}. However, it is separated from \textit{ytxR} by almost 800 bp, and a \textit{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 \textit{ytxR} is sufficient to activate its target promoters. The Nac protein of \textit{Klebsiella aerogenes} is an exam-

![FIG. 6. DNase I footprint analysis of the \textit{ytxA} and \textit{ytxR} control regions. Labeled \textit{ytxA} (A) or \textit{ytxR} (B) control region fragments were incubated with different concentrations of His\textsubscript{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\textsubscript{6}-YtxR.](image-url)
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.

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

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

<table>
<thead>
<tr>
<th>Control region</th>
<th>β-Galactosidase spec act (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without YtxR</td>
</tr>
<tr>
<td>Φ(ytxA-lacZ)</td>
<td></td>
</tr>
<tr>
<td>ΔA55</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>ΔA110</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>ΔA52</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>Φ(ytxR-lacZ)</td>
<td></td>
</tr>
<tr>
<td>ΔA500</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>ΔA150</td>
<td>310 ± 120</td>
</tr>
<tr>
<td>ΔA86</td>
<td>510 ± 60</td>
</tr>
<tr>
<td>ΔA21</td>
<td>420 ± 32</td>
</tr>
</tbody>
</table>

*a* Each strain has a Φ(ytxA-lacZ) or Φ(ytxR-lacZ) 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.

![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 His6-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.](http://jb.asm.org/.../ytxR.png)
environmental role unrelated to host interaction. Uncovering environmental conditions that activate the YtsR regulon, and especially identifying all of the YtsR 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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