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Heterogeneity in Levels of Vacuolating Cytotoxin Gene (*vacA*) Transcription among *Helicobacter pylori* Strains

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Broth culture supernatants from Tox$^+$ *Helicobacter pylori* strains induce vacuolation of HeLa cells in vitro and contain VacA in concentrations that are higher than those found in supernatants from Tox$^-$ *H. pylori* strains. To investigate the basis for this phenomenon, we analyzed the transcription of the vacuolating cytotoxin gene (*vacA*) in eight Tox$^+$ strains (each with a type s1/m1 *vacA* genotype) and nine Tox$^-$ strains (each with a type s2/m2 *vacA* genotype). Most of the Tox$^+$ and Tox$^-$ strains tested used the same *vacA* transcriptional start point, but Tox$^+$ strains yielded significantly stronger primer extension signal intensities than did Tox$^-$ strains (mean densitometry values of 15.8 ± 1.9 versus 8.9 ± 1.7, $P = 0.0016$). Correspondingly, when we introduced *vacA*::*xylE* transcriptional fusions into the chromosomes of a Tox$^+$ strain (60190) and a Tox$^-$ strain (86-313), the level of XylE activity in 60190 *vacA*::*xylE* was about 30-fold higher than that in 86-313 *vacA*::*xylE*. Sequence analysis and promoter exchange experiments indicated that the different levels of *vacA* transcription in these two strains cannot be explained solely by a difference in promoter strength. These data indicate that Tox$^+$ and Tox$^-$ *H. pylori* strains typically differ not only in the VacA amino acid sequence but also in the level of *vacA* transcription.

Helicobacter pylori organisms are curved, gram-negative bacteria found associated with the gastric epithelia of humans and other primates. Colonization of the human stomach with *H. pylori* consistently results in the development of gastric mucosal inflammation and is a risk factor for the development of peptic ulcer disease and gastric adenocarcinoma (7, 17, 21). One putative virulence determinant of *H. pylori* is a unique toxin (VacA) that induces vacuolation of epithelial cells (5, 22). VacA is initially translated as a 140-kDa protoxin, which subsequently undergoes both N-terminal and C-terminal processing to yield an ~90-kDa mature secreted toxin (10, 23–25). Deep-etch electron microscopic analysis indicates that VacA forms large, six- or seven-sided complexes comprised of 12 or 14 subunits (9, 20).

Considerable variation exists among different *H. pylori* strains in the production of vacuolating cytotoxin activity. Thus, broth culture supernatants from some *H. pylori* strains (designated Tox$^+$) induce vacuolation of HeLa cells in vitro, whereas other *H. pylori* strains (designated Tox$^-$) lack detectable vacuolating activity in this assay (2, 8, 18). In previous studies, it has been shown that all *H. pylori* isolates hybridize with *vacA* probes (2, 10, 24, 25), but the *vacA* alleles in Tox$^+$ strains are typically considerably different from those in Tox$^-$ strains (2, 10). A system for classifying *vacA* alleles has been developed in which specific families of *vacA* alleles are associated with the production of detectable vacuolating cytotoxin activity (2). Specifically, most *H. pylori* strains with a type s1 *vacA* signal sequence and a type m1 *vacA* midregion induce prominent cell vacuolation, whereas strains with a type s2 signal sequence and type m2 midregion consistently fail to induce cytotoxic effects (2). In addition to these *vacA* sequence differences, there is also evidence that concentrations of VacA are higher in broth culture supernatants from Tox$^+$ strains than in supernatants from Tox$^-$ strains (6, 8).

In this report, we demonstrate that *vacA* is transcribed in both Tox$^+$ and Tox$^-$ strains, but transcription typically occurs at higher levels in Tox$^+$ strains than in Tox$^-$ strains. This variation is not attributable to differences in *vacA* transcriptional start points and is not due solely to differences in *vacA* promoter strength. Heterogeneity in *vacA* transcription levels among *H. pylori* strains may be a factor that contributes to different vacuolating cytotoxin phenotypes.

MATERIALS AND METHODS

Bacteria and culture conditions. *H. pylori* strains were cultured at 37°C in ambient air containing 5% CO_2. The wild-type *H. pylori* strains used in this study are listed in Table 1. The *vacA* genotypes of all strains were determined by a PCR-based typing method as previously described (2). Complete or partial *vacA* sequences from several of these strains have been reported previously (Table 1).

Analysis of VacA production. *H. pylori* strains were cultured in sulfite-free brucella broth containing 5% fetal bovine serum (FBS) for approximately 24 h and harvested after reaching an optical density at 600 nm (OD_{600}) of about 0.5. After centrifugation of the cultures, the supernatants were concentrated by ultrafiltration and tested for vacuolating cytotoxin activity by adding serial dilutions to HeLa cells in tissue culture medium containing 10 mM ammonium chloride as described previously (8). The broth culture supernatants were immunoblotted with rabbit anti-VacA serum prepared by immunizing a rabbit with purified, denatured VacA from *H. pylori* 60190 as described previously (6). As another approach for analyzing concentrations of VacA in culture supernatants, *H. pylori* 60190, 86-338, and 86-313 were grown in sulfite-free brucella broth containing 0.5% activated charcoal, and oligomeric VacA was purified from the broth culture supernatants as described previously (9). Yields of purified VacA were assessed by measuring the OD_{280} of VacA-containing fractions and by semiquantitative analysis of the density of VacA bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining.

Molecular biology methods. To prepare genomic DNA from *H. pylori*, cells were suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and lysed by the addition of sodium dodecyl sulfate and proteinase K (final concentrations of 0.5% and 0.1 mg/ml, respectively) at 37°C for 45 min. Sodium chloride was then added to a final concentration of 0.7 M, and a solution of 10% hexadecyltrimethylammonium bromide–0.7 M sodium chloride was added to yield a final hexadecyltrimethylammonium bromide concentration of 1%. Cell lysates were incubated at 65°C for 10 min. Following the addition of an equal volume of

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TABLE 1. Vacuolating cytotoxin activities and *vacA* transcriptional activities of *H. pylori* strains used in this study

Strain	*vacA* genotype	*cagA*	Vacuolating activity for HeLa cells[e]	*vacA* primer extension signal intensity[f]
60190 (ATCC 49503)	s1a/m1[a]	+	160	25.0
84-183 (ATCC 53726)	s1b/m1[b]	+	80	11.9
87-33	s1b/m1	+	20	12.9
87-81	s1b/m1[b]	+	320	20.1
92-25	s1b/m1	+	20	16.3
92-29	s1b/m1	+	80	15.8
92-26	s1b/m1	+	20	6.8
87-199	s1a/m1	+	320	17.4
86-338	s2/m2	−	<10	19.1
Tx30a (ATCC 51932)	s2/m2[c]	−	<10	8.3
86-313	s2/m2[b]	−	<10	8.9
87-75	s2/m2	−	<10	4.8
87-203	s2/m2[d]	−	<10	8.9
92-28	s2/m2	−	<10	8.6
87-90	s2/m2	−	<10	3.3
87-230	s2/m2	−	<10	9.6
92-20	s2/m2	−	<10	0

[a] GenBank accession no. U05676 (10).
[b] Partial *vacA* sequence from this strain has been reported previously (2).
[c] GenBank accession no. U29401 (2).
[d] GenBank accession no. U05677 (10).
[e] Reciprocal titer of the maximum supernatant dilution that produced vacuolation of HeLa cells.
[f] Quantified by laser densitometry (OD per square millimeter).

chloroform, cell debris was cleared by centrifugation for 10 min at 10,000 × *g*. Supernatants were then extracted sequentially with equal volumes of chloroform and phenol-chloroform (1:1) and precipitated with isopropanol (3).

All PCRs were carried out in 100-μl volumes with 1.5 mM magnesium chloride and 200 μM dATP, dCTP, dGTP, and dTTP. AmpliTaq DNA polymerase (Perkin-Elmer) was added to a final concentration of 2.5 U/100 μl. Primers were used at a concentration of 1 μM. The template DNA concentration was 100 ng of chromosomal DNA per reaction or 25 to 100 ng of plasmid DNA per reaction. Denaturation was uniformly at 94°C for 1 min, and annealing temperatures were 5°C below the melting temperature of the primers. Extension at 72°C was for 1 min/kb of amplification product.

Inverse PCR was performed as described above but by using oppositely oriented primers with *Bgl*II restriction sites incorporated at their 5′ ends. After completion of thermal cycling, the template DNA was eliminated by *Dpn*I digestion. The sample then was digested with *Bgl*II and purified by phenol-chloroform extraction and ethanol precipitation. Inverse PCR products were recircularized with T4 DNA ligase and transformed into *Escherichia coli* DH5α.

Primer extension analysis. Seventeen different *H. pylori* strains were inoculated into sulfite-free brucella broth containing 5% FBS such that the initial OD_{600} was approximately 0.05. Cultures were harvested when the OD_{600} reached approximately 0.5. Total cellular RNA was extracted from the bacterial pellets by using the hot phenol method (12). Standardized (40-μg) RNA samples from each strain were heated to 90°C for 2 min in a buffer consisting of 20 mM Tris (pH 8.0), 100 mM sodium chloride, 0.1 mM EDTA, and 20 ng of a ^{32}P-end-labeled oligonucleotide (5′ TTTTTGCACAAAGGGTGCGAC). Following primer annealing at 50°C for 3 h, extension of the labeled primer was accomplished by incubation in 50 mM Tris (pH 8.2)–6 mM $MgCl_2$–10 mM dithiothreitol–0.2 mM deoxynucleoside triphosphates–5 U of avian myeloblastosis virus reverse transcriptase (Promega) for 1 h at 42°C. Primer extension products and sequencing reaction ladders generated by using the same primer were analyzed on 7 M urea–8% polyacrylamide gels. Signal intensities were quantified by densitometry using a GS-670 densitometer and Molecular Analyst version 1.4.1 software (Bio-Rad).

Construction of a *vacA*::*xylE* transcriptional fusion in Tox$^+$ *H. pylori* 60190. The promoterless *xylE* gene, encoding *Pseudomonas putida* catechol 2,3-dioxygenase, was fused upstream of a kanamycin resistance gene (hereafter designated *km*) such that these two genes were transcribed in the same direction and the kanamycin resistance gene retained its native promoter sequence (16). This *xylE*/*km* cassette was cloned into the unique *Bgl*II site within pCTB6, which contains a *vacA* gene fragment of *H. pylori* 60190 (10). The resultant plasmid construct, pCTB6::*xylE*/*km*, was used to introduce the *xylE*/*km* cassette into *vacA* of *H. pylori* 60190 by natural transformation and allelic exchange (10). Transformants were selected on brucella agar plates supplemented with 5% FBS and kanamycin (40 μg/ml). The orientation of the cassette in the transformants was determined by PCR with a *xylE*-specific forward primer (5′ CATGACGTCAC CTCTTCATAG) and a *vacA*-specific reverse primer (5′ GCCTTTTTTACAAC CGTGATC). The resultant Tox$^+$ *vacA* reporter strain, with *xylE* in the same orientation as *vacA* transcription, is designated 60190 VX-1 (see Fig. 4A).

Construction of a *vacA*::*xylE* transcriptional fusion in Tox$^-$ *H. pylori* 86-313. A 1.3-kb internal fragment of *vacA* from Tox$^-$ strain 86-313 was PCR amplified by using primers 5′ CCCACGCAAGTCATTGATGG 3′ and 5′ GGTATTATTTT TTCGCACCAC 3′ (2) and cloned into pT7 Blue (Novagen), resulting in pA144. The *xylE*/*km* cassette described above was cloned into the unique *Eco*RV site within this *vacA* sequence. The resulting plasmid, pA144::*xylE*/*km*, was introduced into strain 86-313 via natural transformation, and kanamycin-resistant colonies were selected. The resulting 86-313 *vacA*::*xylE* reporter strain, with *xylE* in the same orientation as *vacA* transcription, is designated 86-313 VX-1 (see Fig. 3A).

Construction of chimeric strains with alternate *vacA* promoters. To place the *vacA*::*xylE* transcriptional fusion in strain 86-313 VX-1 under the control of a Tox$^+$ promoter, a 1.3-kb fragment was amplified from Tox$^+$ 60190 genomic DNA by using primers 5′ AATTACTTGCTAGGGGTGCATTAT 3′ and 5′ ATCAGCACTATCCTTATAGCTTG 3′. This fragment contains 519 bp from the 3′ end of *cysS*, the *cysS*-*vacA* intergenic region, and 548 bp from the 5′ end of *vacA* and was cloned into pT7Blue (Novagen) to yield pBW5. The chloramphenicol acetyltransferase (*cat*) gene of *Campylobacter coli* (26) was then cloned into the *Hin*dIII site at the 3′ terminus of *cysS*, in the orientation opposite to that of *vacA* to yield pBW5*cat* (see Fig. 3B). This plasmid was used to introduce the Tox$^+$ *vacA* promoter and adjacent sequences into the Tox$^-$ reporter strain 86-313 VX-1 described above. Transformants were screened on brucella agar containing 5% FBS and chloramphenicol (10 μg/ml). The extent of replacement of 86-313 sequences with 60190 sequences in the resulting Km^r Cm^r 86-313 VX-1 transformants was determined by PCR amplification and sequencing of the *cysS*-*vacA* intergenic region. The chimeric strain shown in Fig. 3B is designated 86-313 VXC-1.

To place the *vacA*::*xylE* transcriptional fusion in strain 60190 VX-1 under the control of a Tox$^-$ promoter, a DNA fragment was amplified from Tox$^-$ strain 86-313 by using primers 5′ GAAGAACTGCTTGGCATCGGG 3′ and 5′ ATT CCATTTTCTTCCTTTC 3′. This fragment contains 221 bp of *cysS*, the entire *cysS*-*vacA* intergenic region, and the first 7 bp of the *vacA* structural gene. The resulting PCR product was cloned into pBluescript SK+ to yield pBW3. A unique *Bgl*II site was introduced 3 bp downstream of the stop codon of *cysS* in pBW3 by inverse PCR mutagenesis by using primers with *Bgl*II sites incorporated at the 5′ ends (5′ GAAGATCTAGCTTAAAAAAGCTTCTCCCAAATCGT GCC and 5′ GAAGATCTTCTTTAAATTTTACCTATTTACGCACTC) to yield pBW4. The *cat* gene from *C. coli* (26) was cloned into the *Bgl*II site after ends were made blunt by treatment with Klenow fragment (3). A construct, designated pBW4*cat*, was selected in which *cat* and *vacA* are divergently transcribed (see Fig. 4B). To replace the native *vacA* promoter in Tox$^+$ reporter strain 60190 VX-1 with the *vacA* promoter from Tox$^-$ strain 86-313, strain 60190 VX-1 was transformed with pBW4*cat*. Cm^r Km^r transformants were selected, and the extent of sequence replacement was determined by PCR amplifying and sequencing the entire *cysS*-*vacA* intergenic region of the transformants. The resulting chimeric strain shown in Fig. 4B is designated 60190 VXC-1.

The introduction of heterologous promoter sequences into either of the chimeric reporter strains required the presence of two different selectable markers (described above). To determine whether introduction of the *cat* gene alone altered levels of *vacA* transcription, this gene was introduced into the chromosomes of strains 60190 VX-1 and 86-313 VX-1 in the same orientation and at the same sites as described previously. This was accomplished by transformation of strain 86-313 VX-1 with pBW4*cat* to generate an isogenic Km^r Cm^r Tox$^-$ reporter strain (86-313 VX-1 *cat* control) with a *cat* marker at the 3′ terminus of *cysS* (see Fig. 3C). A similar control for the Tox$^+$ reporter, 60190 VX-1, was generated by transformation with pCTB2*cat* to yield the isogenic Km^r Cm^r reporter strain 60190 VX-1 *cat* control (see Fig. 4C).

Assay for XylE activity. XylE activity was assessed qualitatively at the colony level by spraying colonies grown on brucella agar–5% FBS–kanamycin (40 μg/ml) with 20 mM catechol in distilled water and visually examining colonies for the yellow reaction product 2-hydroxymuconic semialdehyde. For quantitative assays, cells were harvested from broth cultures by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 7.5), and the cell density was quantified and standardized by measuring OD_{600}. Catechol was added to a final concentration of 3 mM, and enzyme specific activities were determined spectrophotometrically in a Beckman DU 7400 spectrophotometer at 375 nm (11, 27). One unit of XylE activity corresponds to the formation at 22°C of 1 mmol of 2-hydroxymuconic semialdehyde/min (molar extinction coefficient, 4.4×10^4).

RESULTS AND DISCUSSION

Characterization of VacA production by a panel of *H. pylori* strains. In this study, we analyzed eight *H. pylori* strains with a type s1/m1 *vacA* genotype and nine strains with a type s2/m2 *vacA* genotype (Table 1). Each strain was grown in broth culture until a standardized OD_{600} was reached, and the broth culture supernatants then were concentrated by ultrafiltration and tested in a HeLa cell vacuolation assay. Broth culture

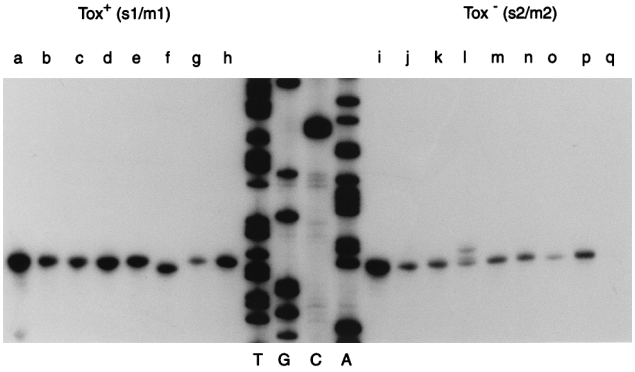

FIG. 1. Primer extension analysis of *vacA* mRNA. *vacA* transcription was analyzed in 17 *H. pylori* strains (8 Tox$^+$ and 9 Tox$^-$) by primer extension analysis using standardized (40-μg) RNA samples from each strain and the primer 5′TTTTTGCACAAAGGGTGCGAC 3′. The sequencing ladder was generated by using the same primer and pCTB2, which contains a partial *vacA* sequence from *H. pylori* 60190, as the template (10). Tox$^+$ strains are shown to the left of the sequencing ladder, and Tox$^-$ strains are shown to the right. Strain designations are as follows: lane a, 60190; lane b, 84-183; lane c, 87-33; lane d, 87-81; lane e, 92-25; lane f, 92-29; lane g, 92-26; lane h, 87-199; lane i, 86-338; lane j, Tx30a; lane k, 86-313; lane l, 87-75; lane m, 87-203; lane n, 92-28; lane o, 87-90; lane p, 87-230; lane q, 92-20. The ninth Tox$^-$ strain (92-20) yielded a weak primer extension product that was detectable with prolonged exposure (data not shown). The signals from Tox$^+$ strains were significantly more intense than signals from Tox$^-$ strains (mean densitometry values of 15.8 ± 1.9 versus 8.9 ± 1.7, P = 0.0016).

supernatants from each of the type s1/m1 strains (Tox$^+$) induced vacuolation of HeLa cells, whereas supernatants from each of the type s2/m2 strains (Tox$^-$) did not (Table 1). Immunoblotting studies with anti-VacA serum indicated that an immunoreactive ~90-kDa band was present in broth culture supernatants from all 17 strains (data not shown). However, when standardized amounts of supernatant protein from different strains were immunoblotted and compared, the VacA bands in Tox$^+$ supernatants tended to be darker than those in Tox$^-$ supernatants. To compare the concentrations of VacA in Tox$^+$ and Tox$^-$ supernatants by another approach, VacA was purified from standardized volumes of culture supernatant from three strains (Tox$^+$ strain 60190, Tox$^-$ strain 86-338, and Tox$^-$ strain 86-313) and the yields of purified VacA were analyzed as described in Materials and Methods. The broth culture supernatant from *H. pylori* 60190 yielded about 10-fold higher quantities of purified oligomeric VacA than did the supernatant from strain 86-338 and >50-fold higher quantities than the supernatant from strain 86-313 (data not shown). These data indicate that all of the *H. pylori* strains tested produce a VacA product, but supernatants from Tox$^+$ strains contain higher concentrations of VacA than do supernatants from Tox$^-$ strains.

Primer extension analysis of *vacA* transcription. To investigate a possible basis for the different concentrations of VacA in supernatants from Tox$^+$ and Tox$^-$ strains, we analyzed *vacA* transcription in the panel of 17 *H. pylori* strains by quantitative primer extension analysis (Fig. 1). The primer for these experiments (Fig. 2) was chosen based on the fact that its sequence was 100% complementary to the corresponding *vacA* sequences of the seven different Tox$^+$ and three Tox$^-$ strains sequenced to date (including strains 60190, 84-183, 87-199, Tx30a, 86-313, and 87-203 from the current study), thereby reducing the possibility that varying signal strengths could be due to inefficient primer annealing. As shown in Fig. 1, *vacA* transcription was detected in all 17 strains, and 15 strains (7 Tox$^+$ and 8 Tox$^-$) used the same conserved single transcriptional start point (TSP). This site was located 1 nucleotide downstream from the *vacA* transcriptional start site identified in a previous study (24). The use of a second primer (5′AGA GGGCGATTGATTTTGCGGTGTG), which anneals farther downstream within the *vacA* coding region, confirmed the use of this TSP and failed to demonstrate any alternate start sites (data not shown). A variant Tox$^+$ strain (92-29) appeared to use a TSP located 1 bp closer to the translational start codon (Fig. 1), which could potentially be due to a 1-bp deletion within the 5′ untranslated region of this strain. A variant Tox$^-$ strain (87-75) simultaneously used three different, adjacent nucleotides as TSPs (Fig. 1). The conservation of adenosine as the +1 site for *vacA* transcription in most strains may be important, because it has been demonstrated that the identity of the +1 site can affect transcriptional efficiency (19). Although most strains used the same *vacA* TSP, there was considerable variation in the intensity of primer extension signals (Fig. 1). Primer extension signals from eight Tox$^+$ strains were significantly more intense than signals from nine Tox$^-$ strains (relative densitometry OD values [mean ± the standard error of the mean] of 15.8 ± 1.9 versus 8.9 ± 1.7, P = 0.0016) (Fig. 1), although there were outliers in both groups. These data indicate that Tox$^+$ and Tox$^-$ *H. pylori* strains differ in the level of *vacA* transcription.

Introduction of *vacA*::*xylE* transcriptional fusions into *H. pylori* 60190 and 86-313. To investigate further the apparent differences among strains in the level of *vacA* transcription, we introduced *vacA*::*xylE* transcriptional fusions into the chromosomes of two *H. pylori* strains, Tox$^+$ strain 60190 and Tox$^-$ strain 86-313. These two strains were chosen based on the primer extension data, which indicated a difference in the level

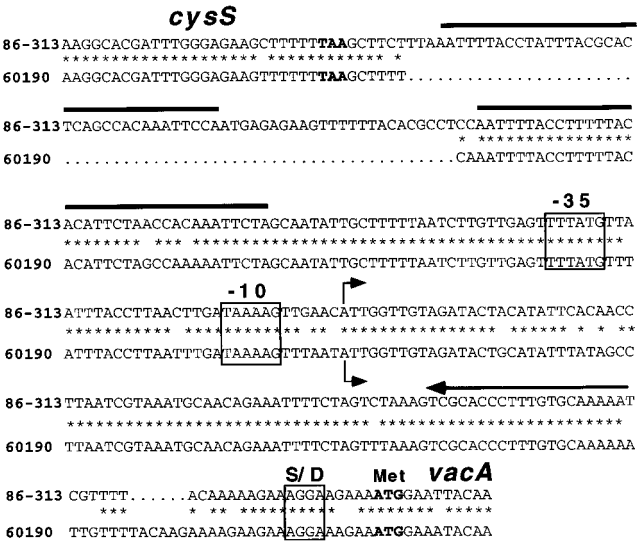

FIG. 2. Comparison of *cysS-vacA* intergenic regions in *H. pylori* 86-313 (Tox$^-$) and 60190 (Tox$^+$). The *cysS-vacA* intergenic region from *H. pylori* 86-313 was PCR amplified and sequenced as described previously (14), and the sequence of the corresponding region from *H. pylori* 60190 has been reported previously (10). Analysis of the aligned sequences demonstrated a 63-bp insertion in the *cysS-vacA* intergenic region of Tox$^-$ strain 86-313. The corresponding absence of this sequence in Tox$^+$ *H. pylori* 60190 is denoted by dots. Positions of nucleotide identity are denoted by asterisks. A 36-bp sequence and its direct repeat are indicated by solid bars. The *vacA* transcriptional start points (determined by primer extension analysis [Fig. 1]) are indicated by the bent arrows. The putative Shine-Dalgarno (S/D) sequence and putative −10 and −35 hexamers are boxed. The stop codon (TAA) of *cysS* and the start codon (ATG) of *vacA* are in boldface. The primer used to determine *vacA* transcriptional start points (Fig. 1) is indicated by an arrow over the complementary sequences.

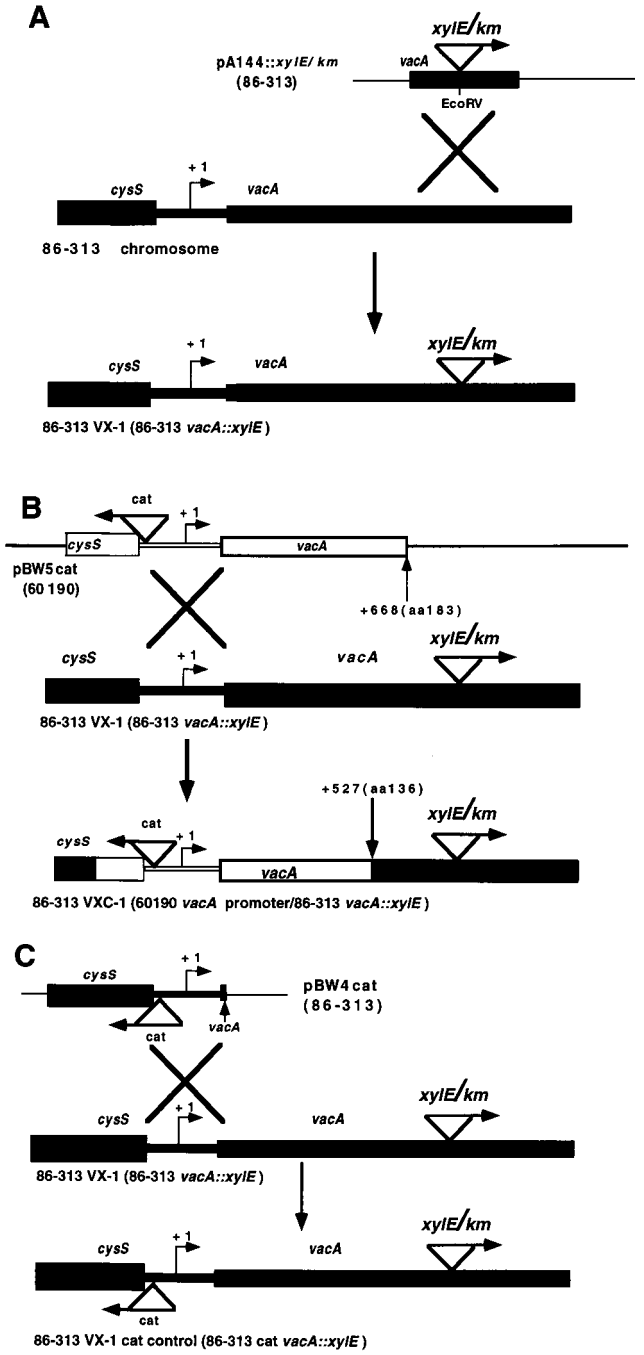

FIG. 3. Construction of *vacA*::*xylE* transcriptional fusions in Tox$^-$ *H. pylori* 86-313. Sequences derived from Tox$^+$ strain 60190 are represented by open boxes. Sequences derived from Tox$^-$ strain 86-313 are indicated by black boxes. Vector sequences are shown as thin, single lines. The *vacA* TSP (+1) is represented by a bent arrow. The directional arrow in the *xylE*/*km* cassette denotes the orientation of *xylE*. The kanamycin resistance gene (*km*) is transcribed under the control of its native promoter and in the same direction as *xylE*. (A) Construction of a *vacA* transcriptional reporter strain. The *xylE*/*km* cassette was cloned into the *Eco*RV site of pA144, which contains a 1.3-kb *vacA* fragment from Tox$^-$ strain 86-313 (yielding pA144::*xylE*/*km*). This construct was introduced into the chromosome of strain 86-313 by natural transformation and allelic exchange, and the resultant strain (86-313 *vacA*::*xylE*) was designated 86-313 VX-1. (B) Introduction of Tox$^+$ *vacA* promoter region sequences into Tox$^-$ strain 86-313. To place the *vacA*::*xylE* fusion in 86-313 VX-1 under the control of a heterologous *vacA* promoter from Tox$^+$ strain 60190, the *cat* gene was cloned into a fragment from strain 60190 containing the entire *cysS-vacA* intergenic region (to yield pBW5*cat*). Natural transformation and allelic exchange were used to introduce this sequence into the 86-313 VX-1 chromosome. The extent of *vacA* sequence exchange in the chimera was experimentally determined to be up to +527, relative to the TSP. The resultant strain, which now contains the *vacA* promoter region from 60190, was designated 86-313 VXC-1. (C) Construction of a chloramphenicol-resistant control strain. The construction of the chimeric reporter outlined in panel B required the use of a marker for chloramphenicol resistance. To determine the effect of the *cat* gene alone on *vacA* transcription, an isogenic control strain was constructed by transforming Tox$^-$ *vacA* reporter strain 86-313 VX-1 with plasmid pBW4*cat*. The resultant Cm^r Km^r strain bears the *cat* gene at the same location and in the same orientation as in the chimeric reporter strain, 86-313 VXC-1, described above. This isogenic control strain was designated 86-313 VX-1 *cat* control.

of *vacA* transcription (Fig. 1), and because both strains were known to be naturally competent for transformation (unpublished data). The introduction of *vacA*::*xylE* transcriptional fusions into strains 60190 and 86-313 yielded strains 60190 VX-1 and 86-313 VX-1, respectively (Fig. 3A and 4A). The XylE activity was more than 30-fold higher in Tox$^+$ reporter strain 60190 VX-1 than in Tox$^-$ reporter strain 86-313 VX-1 (76,500 ± 500 versus 2,136 ± 500 mU/OD$_{600}$, $P < 0.001$). Thus, both primer extension analysis and *vacA*::*xylE* transcriptional fusion data indicated that these two strains differ in the level of *vacA* transcription.

Comparison of *vacA* promoter strengths in *H. pylori* 60190 and 86-313. One possible explanation for differential *vacA* transcription among strains is the occurrence of variations in *vacA* promoters. Such a phenomenon accounts for the active transcription of the pertussis toxin operon in *Bordetella pertussis* and the presence of a silent toxin operon in *B. parapertussis* and *B. bronchiseptica* (1). Although the precise locations of *vacA* promoter sequences in *H. pylori* have not been determined, putative −10 and −35 hexamers can be inferred based on spacing relative to the *vacA* transcriptional start point and comparison with *E. coli* consensus sequences. A comparison of the putative *vacA* −10 and −35 sequences in *H. pylori* 60190 and 86-313 reveals no obvious differences that would account for the different levels of *vacA* transcription in these two strains (Fig. 2). A second potential explanation for the demonstrated difference in *vacA* transcription might be varying numbers of binding sites for a *trans*-acting factor. This possibility is relevant because unlike that of Tox$^+$ strain 60190, the *cysS-vacA* intergenic region of Tox$^-$ strain 86-313 contains a 63-bp insertion (14). This 63-bp insertion contains a 36-bp segment that is duplicated a few base pairs farther downstream (Fig. 2).

To determine experimentally whether sequence differences in the *cysS-vacA* intergenic region might account for different levels of *vacA* transcription, the *vacA*::*xylE* fusion in Tox$^-$ reporter strain 86-313 VX-1 was placed under the control of the *vacA* promoter region from Tox$^+$ strain 60190. Sequence analysis confirmed that in this chimeric reporter strain (86-313 VXC-1), an exchange of promoter and signal sequences had taken place and that the 63-bp insertion had been eliminated (Fig. 3B). Nevertheless, there was no increase in XylE activity in response to the heterologous promoter sequences (Fig. 5). These data indicate that the constraint on transcription in strain 86-313 is not the consequence of either a weak promoter or *cis*-acting sequences in the promoter region.

In a converse experiment, the *vacA*::*xylE* transcriptional fusion in Tox$^+$ reporter strain 60190 VX-1 was placed under the control of the *vacA* promoter from Tox$^-$ strain 86-313. Sequence analysis of the DNA from the resulting chimeric reporter, 60190 VXC-1, confirmed that all 73 bp upstream from the promoter, the putative −35 and −10 sequences, and the 5′ untranslated region through +87 in this chimeric strain had been replaced with sequences from strain 86-313 (Fig. 4B). The level of XylE activity in this chimera was about 65% less

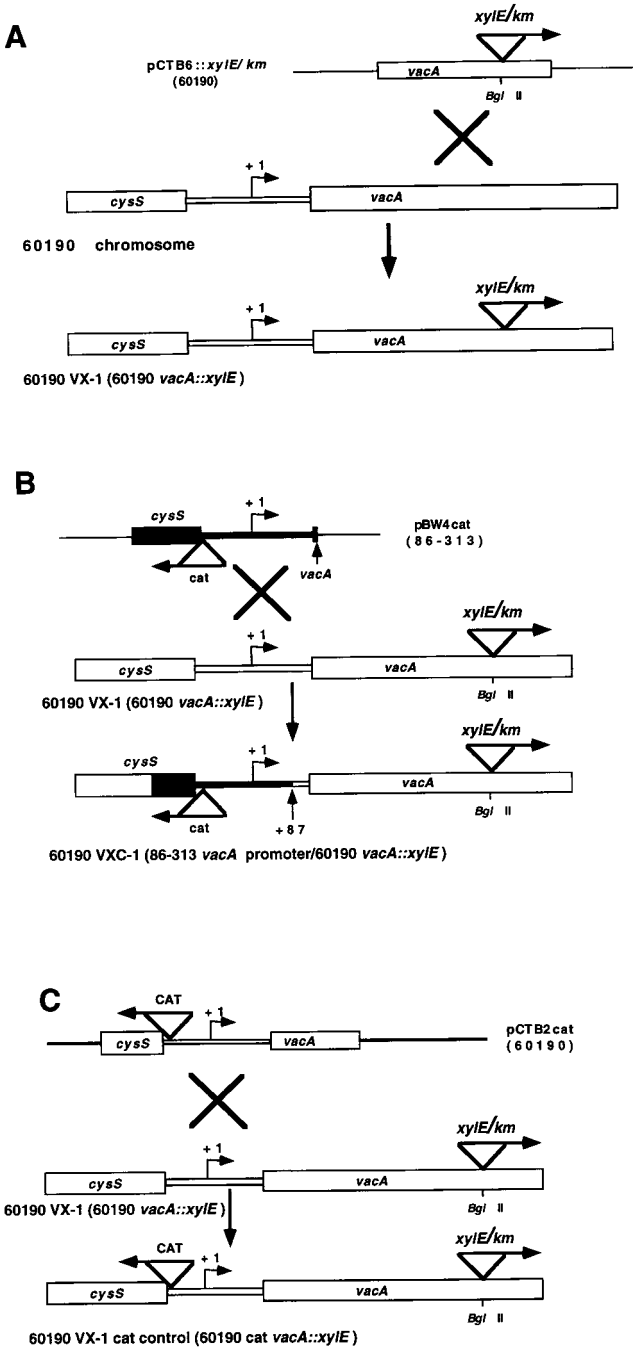

FIG. 4. Construction of *vacA*::*xylE* transcriptional fusions in Tox$^+$ *H. pylori* 60190. Sequences derived from Tox$^+$ strain 60190 are represented by open boxes. Sequences derived from Tox$^-$ strain 86-313 are indicated by black boxes. Vector sequences are shown as thin, single lines. The *vacA* transcriptional start point (+1) is represented by a bent arrow. The directional arrow in the *xylE*/*km* cassette denotes the orientation of *xylE*. The kanamycin resistance gene (*km*) is transcribed under the control of its native promoter and in the same direction as *xylE*. (A) Construction of a *vacA* transcriptional reporter strain. The *xylE*/*km* cassette was cloned into the *Bgl*II site of pCTB6, which contains a 3.2-kb *vacA* fragment from Tox$^+$ strain 60190 (yielding pCTB6::*xylE*/*km*). This construct was introduced into the chromosome of strain 60190 by natural transformation and allelic exchange, and the resultant strain (60190 *vacA*::*xylE*) was designated 60190 VX-1. (B) Introduction of Tox$^-$ *vacA* promoter region sequences into Tox$^+$ strain 60190. To place the *vacA*::*xylE* fusion in 60190 VX-1 under the control of a heterologous *vacA* promoter from Tox$^-$ strain 86-313, the *cat* gene was cloned into a fragment from strain 86-313 containing the entire *cysS-vacA* intergenic region (to yield pBW4*cat*). Natural transformation and allelic exchange were used to introduce this sequence into the 60190 VX-1 chromosome. The extent of *vacA* sequence exchange in the chimera was experimentally determined to be up to +87 relative to the *vacA* TSP. The resultant strain, which now contains the *vacA* promoter region from strain 86-313, was designated 60190 VXC-1. (C) Construction of a chloramphenicol-resistant control strain. The construction of the chimeric reporter strain outlined in panel B required the use of a marker for chloramphenicol resistance. To determine the effect of the *cat* gene alone on *vacA* transcription, an isogenic control strain was constructed by transforming Tox$^+$ *vacA* reporter strain 60190 VX-1 with plasmid pCTB2*cat*. The resultant Cm^r Km^r strain bears the *cat* gene at the same location and in the same orientation as in the chimeric reporter strain, 60190 VXC-1, described above. This isogenic control strain was designated 60190 VX-1 *cat* control.

than that of the control strain, 60190 VX-1 *cat* control (Fig. 5; $P < 0.001$). However, the level of XylE activity in the chimeric strain was still about 10-fold higher than that in Tox$^-$ reporter strain 86-313 VX-1 (Fig. 5).

The results of these promoter exchange experiments suggest that strains 60190 and 86-313 differ in *vacA* promoter strength. However, any such difference must be dictated by sequences outside the putative −10 and −35 hexamers, since these sequences are identical in the two strains. An important finding is that the Tox$^-$ (strain 86-313) *vacA* promoter is capable of initiating higher levels of *vacA* mRNA synthesis in the strain 60190 background than in the strain 86-313 background. Therefore, it seems likely that the *vacA* transcription level difference between these two strains is not due solely to a difference in *vacA* promoter strength. One possibility is the expression of a *trans*-acting repressor factor in strain 86-313, or alternatively, that strain 60190 produces an activator factor which is absent or reduced in quantity or function in strain 86-313.

Another possible explanation for these data is that strains 60190 and 86-313 differ in *vacA* transcript stability. To investigate this possibility, we attempted to determine the half-lives of *vacA* transcripts in these two strains by using serial quantitative primer extension analyses of bacterial cells that had been treated with rifampin to inhibit RNA polymerase activity. These experiments repeatedly yielded nonlinear patterns of primer extension signal decay, and therefore, it remains unclear whether strains 60190 and 86-313 differ in *vacA* transcript stability. Important determinants of mRNA stability in prokaryotic organisms include stem-loop structures located at either the 5′ or the 3′ ends of transcripts (4, 12, 13). In the promoter switching experiments described in this report, we replaced the entire 5′ untranslated region of *vacA* from strain 86-313 with that from strain 60190 and failed to demonstrate any significant increase in *vacA* transcription in the chimeric strain (86-313 VXC-1, Fig. 3). This suggests that sequences at the 5′ end of *vacA* mRNA do not significantly alter *vacA* mRNA stability. Both Tox$^+$ and Tox$^-$ strains that have been analyzed thus far contain prominent stem-loop structures at the 3′ ends of *vacA* transcripts (2, 10), and thus, there is also no evidence that sequence differences in this region would contribute to different *vacA* mRNA stability.

Determinants of the vacuolating cytotoxin phenotype. The two groups of *H. pylori* strains analyzed in this study (Tox$^+$ and Tox$^-$) clearly differ in the capacity to induce vacuolation of HeLa cells. One explanation for this difference, supported by data in this study, as well as previous studies (6, 8), is that there are higher concentrations of VacA in broth culture supernatant from Tox$^+$ strains than in supernatant from Tox$^-$ strains. Heterogeneity among strains in the level of *vacA* transcription would undoubtedly be a factor that contributes to this phenomenon. In addition, there also may be heterogeneity among strains in the efficiency of *vacA* secretion, possibly related to differences in *vacA* signal sequences (2). In support of this

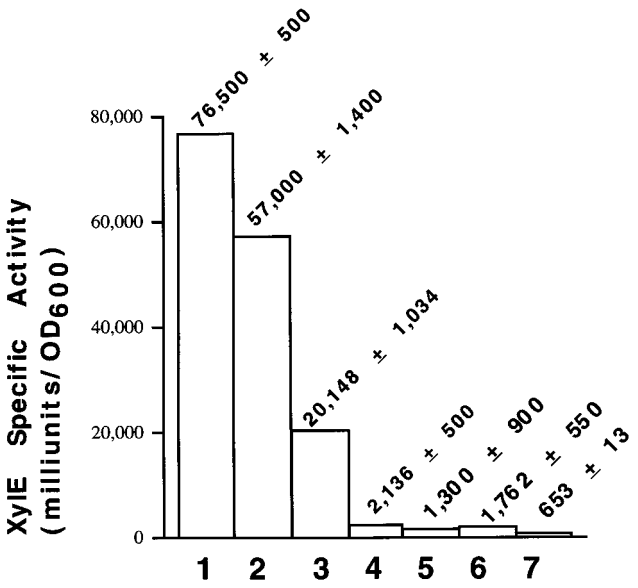

FIG. 5. XylE activity of *H. pylori vacA*::*xylE* transcriptional reporter strains. Specific XylE activities (milliunits per OD_{600}) were determined by using bacteria that had been grown in brucella broth–5% FBS for 18 h (late-log phase to early stationary phase). In all assays, the densities of bacterial suspensions were standardized by OD_{600}. XylE activity was quantified as described in Materials and Methods. Results represent the mean ± the standard deviation of three assays from a representative experiment. Absolute values varied slightly from trial to trial, but the overall pattern shown here is representative of three independent experiments. Results from *H. pylori* 86-313 (parental strain, no *xylE* fusion) are consistent with background levels of 2-hydroxymuconic semialdehyde production. Levels of XylE activity were significantly higher in strain 60190 VX-1 (lane 1) than in strain 86-313 VX-1 (lane 4), $P < 0.001$. Placement of the 86-313 *vacA* promoter upstream from *vacA* in strain 60190 VX-1 resulted in a significant decrease in XylE activity (compare lanes 2 and 3; $P < 0.001$) but did not reduce activity to the same level as in strain 86-313 VX-1 (lanes 4 and 5).

hypothesis, in the present study, we detected 10-fold higher concentrations of VacA in supernatant from Tox^+ *H. pylori* 60190 than in supernatant from Tox^- *H. pylori* 86-338 but found that the two strains did not differ substantially in the level of *vacA* transcription (Fig. 1 and Table 1).

A second explanation for different vacuolating phenotypes is that the Tox^+ and Tox^- strains analyzed in this study produce *vacA* products (types s1/m1 and s2/m2, respectively) that have markedly different amino acid sequences. Specifically, type s1/m1 and type s2/m2 VacA proteins are only about 58% identical within a 250-amino-acid midregion segment (2). These substantial differences would be expected to result in considerably different structural and functional properties. Nevertheless, in previous studies, we have demonstrated that a type s2/m2 VacA protein is capable of assembling into a complex oligomeric structure that is almost identical to that of type s1/m1 VacA proteins (9). To determine whether the different amino acid sequences of type s1/m1 and s2/m2 VacA proteins are important determinants of the vacuolating cytotoxin phenotype, we purified VacA oligomers from culture supernatants of strains 60190 (type s1/m1 VacA) and 86-338 (type s2/m2 VacA) and tested equal concentrations of the two acid-activated proteins in a HeLa cell vacuolation assay. This experiment indicated that the s1/m1 VacA protein produced prominent cell vacuolation, as expected, whereas the type s2/m2 VacA protein lacked any detectable activity in this assay. Thus, equal concentrations of VacA from Tox^+ and Tox^- strains are not equal in toxicity.

In summary, the vacuolating cytotoxin phenotype of an *H. pylori* strain is dependent on the amino acid sequence of its *vacA* product but may also be modulated by other strain-specific factors, such as the level of *vacA* transcription or the efficiency of VacA secretion. The considerable variation in these determinants among *H. pylori* strains is consistent with the high level of genetic diversity that exists in the *H. pylori* species (15) and may be relevant to the occurrence of different clinical outcomes in *H. pylori*-infected humans.

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