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Effect of Growth Phase and Acid Shock on *Helicobacter pylori cagA* Expression

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***Helicobacter pylori* strains possessing *cagA* are associated with peptic ulceration. To understand the regulation of expression of *cagA*, *picB*, associated with interleukin-8 induction, and *ureA*, encoding the small urease subunit, we created gene fusions of *cagA*, *ureA*, and *picB* of strain 3401, using a promoterless reporter (*xylE*). Expression of XylE after growth in broth culture revealed that basal levels of expression of *cagA* and *ureA* in *H. pylori* were substantially greater than for *picB*. For *cagA* expression in stationary-phase cells, brief exposure to acid pH caused a significant increase in *xylE* expression compared with neutral pH. In contrast, expression of *xylE* in *ureA* or *picB* decreased after parallel exposure to acid pH (pH 7 > 6 > 5 > 4), regardless of the growth phase. Expression of the CagA protein varied with growth phase and pH exposure in parallel with the observed transcriptional variation. The concentration of CagA in a cell membrane-enriched fraction after growth at pH 6 was significantly higher than after growth at pH 5 or 7. We conclude that the promoterless reporter *xylE* is useful for studying the regulation of gene expression in *H. pylori* and that regulation of CagA production occurs mainly at the transcriptional level.**

Helicobacter pylori is a gram-negative microaerophilic bacterium that colonizes the human stomach (43). Although infection with *H. pylori* persists despite seemingly hostile conditions in the stomach, the basis for this survival is not well understood. There is essentially no microbial competition, and the immune system is ineffective in eliminating *H. pylori* (5). Therefore, the host may rely chiefly on gastric acidity and peristalsis to eliminate organisms (5). Thus, it may be favorable for *H. pylori* to be able to adapt to low pH, for example, by secreting products that can inhibit acid secretion (6, 29).

H. pylori may possess other constituents involved in acid response, and their production may be pH regulated. Not surprisingly, there is evidence that *H. pylori* is an acidophile, with optimal in vitro growth at pH 5.5 (21). Recent data indicate that those *H. pylori* strains that produce the high-molecular-weight CagA proteins (2, 8, 9, 13) (encoded by *cagA*) are strongly associated with duodenal ulceration, which often is accompanied by hyperacidity. Despite the potential importance of *cagA*, its function is unknown and its regulation, particularly in relation to pH, has not been studied.

xylE from *Pseudomonas putida* has been used as a reporter for gene fusions in several bacterial species (14, 23, 27, 30). Activity of the *xylE* gene product, catechol 2,3-dioxygenase, can easily be determined in whole cells or crude extracts and provides quantitation of gene expression (32, 33). The goals of this study were to develop genetic tools for analysis of expression of *H. pylori* genes and to understand the role of pH variation in transcriptional regulation of *cagA*. The current work indicates that *cagA* is up-regulated by acid pH.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *H. pylori* CPY3401 (hereafter referred to as 3401), isolated from a patient with duodenal ulcer and able to colonize the mouse stomach (22), was the host strain used to create chromosomal gene fusions. Stock cultures were maintained at −70°C in brucella broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 15% glycerol. *Escherichia coli* DH5α was used for transformation. Plasmids used in this study are listed in Table 1. *E. coli* strains were cultured overnight in LB medium at 37°C with shaking. *H. pylori* strains were cultured for 24, 36, or 48 h in brucella broth (pH 6.95) supplemented with 5% fetal bovine serum and on Trypticase soy agar plates (BBL) containing 5% sheep blood in a microaerobic atmosphere as described previously (11). Growth phase was assessed by viable counts.

Genetic techniques. Restriction enzymes and Klenow fragment of *E. coli* DNA polymerase I were purchased from either New England BioLabs (Beverly, Mass.) or Promega (Madison, Wis.). *H. pylori* chromosomal DNA was isolated by lysing the bacteria with GES (60% guanidium thiocyanate, 0.1 M EDTA, 0.5% Sarkosyl), and the DNA was purified by chloroform extraction and isopropyl alcohol precipitation (37). Oligonucleotide primers listed in Table 2 were synthesized with an ABI 392 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). PCR conditions used in this study were 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, with 35 cycles of amplification (Perkin-Elmer Thermal Cycler; Perkin-Elmer, Norwalk, Conn.). Colony hybridization was performed as described previously (41).

Construction of *xylE* transcriptional fusions. A 4.5-kb *H. pylori* insert from strain 84-183 containing *cagA* and *picB* had been previously cloned into pMT1 (42). A 1.8-kb *Kpn*I-*Xho*I fragment from pTs117 that contains *xylE* with its promoter and a 1.3-kb *Sal*I fragment that carries a kanamycin resistance (Kan^r) cassette from pUC4K were ligated into unique *Kpn*I and *Xho*I sites in pMT5054 to create pMKQ (Table 1; Fig. 1). A 3.2-kb *Sca*I fragment that contains promoterless *xylE* and the Kan^r cassette was excised from pMKQ (Table 1) and blunt-end ligated into Klenow enzyme-filled *Hin*dIII sites in the open reading frame (ORF) of *cagA* or *picB* (pMT1) to create pMK25 (*cagA*::[*xylE*-Kan^r]) or pMK13 (*picB*::[*xylE*-Kan^r]), respectively (Table 1; Fig. 1). pHPT178 contains an 8.25-kb insert from *H. pylori* HPK5 including *ureC*, *-D*, *-A*, *-B*, *-I*, *-E*, *-F*, *-G*, and *-H* in vector pMT267. The *xylE* reporter was ligated into the unique *Eco*RV site in the ORF of *ureA* to create pSK2 (Table 1; Fig. 2). In each case, the promoterless *xylE* was inserted in the direction of transcription. The *Sca*I fragment with Kan^r and promoterless *xylE* also was ligated into *cagA*, *picB*, or *ureA* ORFs in the opposite orientation to create pMK2, pMK27, and pSK3, respectively.

Introduction of *xylE* gene fusion constructs into the *H. pylori* chromosome. Each of the recombinant plasmids was used to introduce the *xylE* reporter into the chromosome of wild-type *H. pylori* 3401 by natural transformation, selecting for kanamycin resistance, as described previously (12). Allelic exchange by marker rescue created the following strains: CPY3401 *cagA*::[*xylE*-Kan^r] (3401A$^-$), CPY3401 *picB*::[*xylE*-Kan^r] (3401B$^-$), and CPY3401 *ureA*::[*xylE*-Kan^r] (3401U$^-$), (Table 1; Fig. 1 and 2). Parallel procedures also were performed to create mutants carrying promoterless *xylE* in the opposite orientation. In each case, the authenticity of the construct was verified using PCR with reverse primers to *xylE* or targeted gene, and forward primers for Kan^r or the targeted gene (Table 2), and by Southern hybridization.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacAYA-argF*)U169 *deoR* [F′ 80d*lac*Δ(*lacZ*)M15]	18
H. pylori		
Tx30a	*cagA* wild type	41
60190	*cagA*$^+$ wild type	41
84-183	*cagA*$^+$ wild type	41
3401	*cagA*$^+$ wild type	22
3401A$^-$	3401 *cagA*::[*xylE*-Kan^r]	This study
3401B$^-$	3401 *picB*::[*xylE*-Kan^r]	This study
3401U$^-$	3401 *ureA*::[*xylE*-Kan^r]	This study
Plasmids		
pUC4K	pUC4::Kan^r, source of Tn*903*	40
pTs117	pACYC177::*xylE*	32
pMT5054	Derivative of pBend (Ap^r)	M. Tsuda
pMT267	Derivative of pBR322 (Ap^r)	M. Tsuda
pMKQ	pMT5054 with *xylE* from pTs117 inserted upstream of Kan^r from pUC4K	This study
pMT1	pBS(Cm^r) with 4.5-kb insert containing 5′ *cagA*, *picA*, and 5′ *picB*	42
pMT25	2.6-kb fragment with promoterless *xylE* and Kan^r from pMKQ inserted into *cagA* of pMT1	This study
pMT13	2.6-kb fragment with promoterless *xylE* and Kan^r from pMKQ inserted into *picB* of pMT1	This study
pHPT178	pMT267 with 8.25-kb fragment containing *ureC*, *-D*, *-A*, *-B*, *-I*, *-E*, *-F*, *-G*, and *-H*	M. Tsuda
pSK2	2.6-kb fragment with promoterless *xylE* and Kan^r from pMKQ inserted into *ureA* of pHPT178	This study

Assay conditions for XylE activity. The wild-type and mutant strains were grown in broth culture for 24, 36, or 48 h representing log and stationary phases, respectively, for *H. pylori* cells in culture (Fig. 3). Viable counts were determined by serial dilution and plate culture. Cells from 24-, 36-, or 48-h cultures were aliquoted, centrifuged at 2,500 × *g* for 10 min at 4°C, and resuspended in McIlvain's buffer, at pH 4, 5, 6, or 7, as described previously (28). After brief exposure (5 or 50 min) to the solution of defined pH, cells were centrifuged, resuspended in brucella broth, and incubated at 37°C in an atmosphere containing 5% CO_2 for the remainder of an hour to allow pH to equilibrate. Then viable counts and XylE activity in each mutant and in the wild-type strain were assessed. The *xylE* product, catechol 2,3-dioxygenase, converts catechol (Sigma, St. Louis, Mo.) to a product with bright yellow color, α-hydroxymuconic ε-semialdehyde. Bacterial colonies expressing XylE turn yellow after being sprayed with a 10 mM solution of catechol. The bacterial cells were washed with 0.9% saline, resuspended in 50 mM potassium phosphate buffer (pH 7.5) with 10% acetone, and sonicated (Braun SonicU; B. Braun, Melsungen, Germany) on ice two times for 15 s each with a 15-s rest, and XylE activity was measured spectrophotometrically by assessing the change in A_{375}. Protein concentrations were determined by the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.). One unit of XylE activity oxidizes 1 μmol of catechol min^{-1} at 24°C (32, 33). All assays were done in quadruplicate.

ELISA for detection of UreA protein. UreA protein expression in sonicated cells was determined in an enzyme-linked immunosorbent assay (ELISA) using rabbit antisera specific for UreA as described previously (34). Wild-type *H. pylori* 84-183 and *ureA* isogenic knock-out mutant HPT47 were used as positive and negative controls for UreA production, respectively. Bacterial cell suspensions were diluted in 0.5% carbonate buffer (pH 9.6) to yield a protein concentration of 3 μg per well for coating microtiter plates. The rabbit serum monospecific for UreA protein was used at a 1:1,000 dilution, and a peroxidase conjugate of anti-rabbit immunoglobulin G (IgG) was used at a 1:2,000 dilution.

ELISA for detection of CagA protein. CagA protein expression in either whole cells or sonicated cells was determined in an ELISA using rabbit antisera specific for CagA as described previously (10). Wild-type *H. pylori* 84-183 and 93-6 were used as positive and negative controls for CagA production, respectively. Bacterial cell suspensions were diluted in 0.5% carbonate buffer (pH 9.6) to yield a protein concentration of 3 μg per well for coating microtiter plates. To improve specificity, the antiserum was absorbed with a lysate of *cagA*-negative *H. pylori* Tx30a cells conjugated to silica as described previously (19, 41). Specificity of the absorbed antiserum for CagA was demonstrated by immunoblot analysis and ELISA. For immunoblotting, *cagA*$^+$ (60190 and 3401) and *cagA*-negative (Tx30a) wild-type strains and 3401A$^-$, the isogenic *cagA* knockout mutant, were used (Fig. 4). For ELISA, multititer plates were coated with preparations of strain 84-183, 3401, or 3401A$^-$ (0 to 3.0 μg of protein per well) (Fig. 5). The absorbed rabbit serum monospecific for CagA protein was used at a 1:1,000 dilution, and a peroxidase conjugate of anti-rabbit IgG was used at a 1:2,000 dilution.

Detection of CagA protein in cells grown at different pHs. Wild-type strain 3401 was grown for 24 h in broth culture adjusted to pH 7, 6, or 5. Growth at 24 h was selected because 48-h growth at these different pHs showed large differences in cell number and viability. Cells from each growth condition were centrifuged at 2,500 × *g* for 10 min at 4°C, resuspended in 1 ml of 0.01 M Tris (pH 7.4), and fractionated as previously described (4). In brief, pellets of frozen cells were thawed and then sonicated (Braun SonicU; B. Braun) on ice two times for 15 s with a 15-s rest, and an aliquot was saved for analysis of CagA protein. The preparation then was centrifuged at 5,000 × *g* for 10 min to remove whole cells, and an aliquot of this supernatant saved for analysis. After centrifugation at 100,000 × *g*, the supernatant representing cytoplasmic fraction was aspirated, and the pellet representing the total membrane fraction was resuspended in 500 μl of distilled water. The concentration of CagA protein in each fraction was determined by ELISA as described above. The efficiency of the fractionation procedure was assessed by determination of Lewis^x antigen (Le^x) in the fractions by ELISA (44). Le^x has been shown to be present in the lipopolysaccharide (LPS) of strain 3401 (44). Multititer plates were coated at pH 9.6 with 1.0 μg of protein per well of cellular or subcellular fractions of strain 3401. After washing, Le^x was detected with a mouse monoclonal IgM antibody to Le^x (Signet Laboratories Inc., Dedham, Mass.) at a 1:1,000 dilution. Bound antibodies were recognized by a goat anti-mouse IgM antibody conjugated to alkaline phosphatase (Sigma) exactly as described previously (45).

Statistical analyses. Results are expressed as the mean ± standard deviation. Distributions of XylE activities and optical densities were examined by using Student's *t* test for comparison of means of independent samples. All *P* values

TABLE 2. Oligonucleotides used in this study

Gene	Name	Position	Strand	Length (bp)	Sequence (5′-3′)	Reference
cagA	B2136	1170–1191	+	22	GATAACGCTGTCGCTTCATACG	41
cagA	A2308	1557–1578	−	22	CTGCAAAAGATTGTTTGGCAGA	41
picB	A1603	1327–1341	+	15	TGTTTGGTTTCCCTG	42
picB	A1704	2649–2663	−	15	ACGCATTCCTTAACG	42
ureA	B2339	2659–2678	+	20	ATGAAACTCACCCCAAAAGA	24
ureA	A2801	3261–3281	−	21	TTGTCTGCTTGTCTATCAACC	24
xylE	941114A	4733–4753	−	21	GTTCATGACGTCACCTCTTCA	32
Kan^r	941114B	1601–1622	+	22	CTTGTGCAATGTAACATCAGAG	40

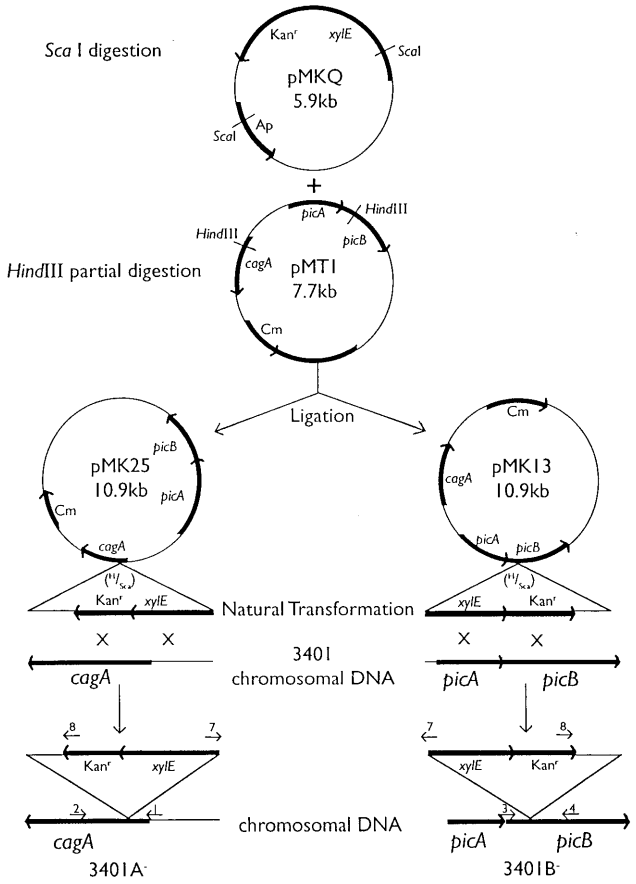

FIG. 1. Steps in the construction of *H. pylori* 3401A$^-$ and 3401B$^-$. A 3.2-kb *Sca*I fragment from pMKQ which contains *xylE* (Table 1) was blunt-end ligated into the filled *Hin*dIII (H) site in *cagA* or *picB* to introduce the promoterless *xylE* and Kan^r cassette into *cagA* (to create pMK25) or *picB* (to create pMK13). *H. pylori* 3401 was transformed with pMK25 or pMK13, and transformants were selected on kanamycin-containing plates to create 3401A$^-$ or 3401B$^-$, respectively. Gene fusions were analyzed by restriction enzyme mapping and PCR analysis. Small arrows with numbers indicate the locations and directions of the primers used in the PCR amplifications. Cm and Ap, chloramphenicol and ampicillin resistance genes, respectively.

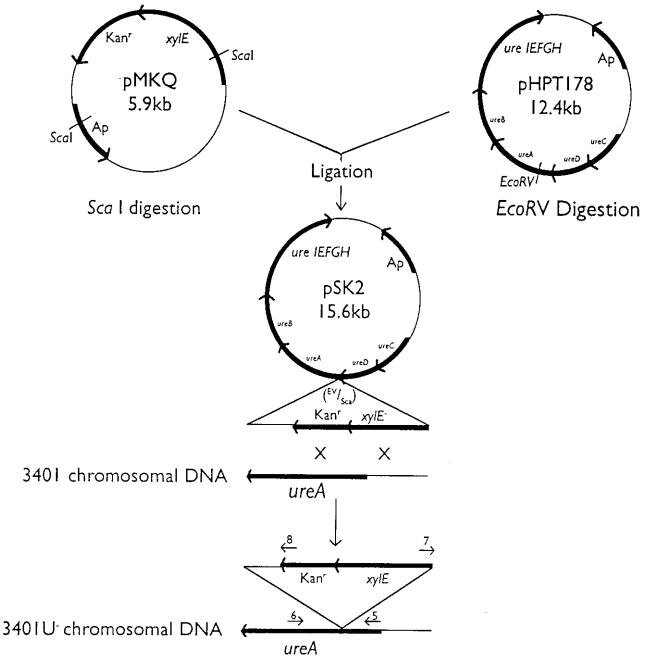

FIG. 2. Steps in the construction of *H. pylori* 3401U$^-$. A 3.2-kb *Sca*I fragment from pMKQ (Table 1) was blunt-end ligated into the *Eco*RV (EV) site of *ureA* in pHPT178 (Table 1) to create pSK2 (Table 1). *H. pylori* 3401 was transformed with pSK2, and transformants were selected on kanamycin-containing plates to identify 3401U$^-$. Gene fusions were analyzed by restriction enzyme mapping and PCR analysis. Small arrows with numbers indicate the locations and directions of the primers used in the PCR amplifications. Ap, ampicillin resistance gene.

were calculated for two-tailed significance levels, and values of <0.05 were considered significant.

RESULTS

Construction and characterization of *xylE* fusions in *H. pylori* mutant strains 3401A$^-$, 3401B$^-$, and 3401U$^-$. To study the regulation of *cagA*, we constructed a *cagA*::*xylE* fusion to mutate *H. pylori* 3401 and to create strain 3401A$^-$ (Fig. 1). To verify that the *xylE* construct in 3401A$^-$ was in the proper site and orientation, we performed Southern hybridizations using *km*, *xylE*, and *cagA* probes (data not shown) and showed that a double crossover event with insertion of *km* and *xylE* had occurred within *cagA* to create *cagA*::[*xylE*-Kan^r]. We also performed PCR analyses (Table 2; Fig. 1) that indicated that the position and orientation of *xylE* in 3401A$^-$ were exactly as expected. Western blot (immunoblot) analysis of 3401 and 3401A$^-$ whole cells with rabbit antiserum specific for CagA showed a reactive band of approximately 120 kDa in the wild-type strain but not the mutant strain (Fig. 4). Similarly, the identities of *H. pylori* 3401B$^-$ and 3401U$^-$ and of mutants carrying promoterless *xylE* in the opposite orientation were verified by Southern hybridization and PCR analyses (Table 2; Fig. 1 and 2). Cells of *H. pylori* 3401A$^-$, 3401U$^-$, 3401B$^-$ or 3401 grown for 24 and 48 h represented log and stationary phases, respectively (Fig. 3). The growth rates of the wild-type and mutant strains were similar.

Colonies of the 3401A$^-$, 3401B$^-$, and 3401U$^-$ mutants turned bright yellow within minutes after being sprayed by the catechol solution, indicating their expression of XylE activity, whereas the wild-type strain and the mutants with promoterless *xylE* in the reverse orientation remained white. These findings indicated that only when *xylE* was properly introduced downstream of an active promoter of *cagA*, *picB*, or *ureA* in *H. pylori* could its expression be readily detected. To assess quantitative *xylE* activity in each mutant, cells from 48-h broth cultures were sonicated and XylE activity in these extracts was measured. The magnitude (mean ± standard deviation) of XylE activity in *cagA* (829.6 ± 7.0 mU/mg of protein) or *ureA* (988.8 ± 10.1 U/mg of protein) cells was more than 30-fold greater than that in *picB* cells (24.4 ± 1.0 U/mg of protein), which indicates that under these in vitro growth conditions, levels of expression of both *cagA* and *ureA* are significantly higher than that of *picB*.

pH regulation of *cagA* expression. To determine whether *cagA* is regulated by pH, we examined *xylE* expression in *cagA* after exposure of 3401A$^-$ cells to several pH conditions. As expected (28), the brief exposure (5 or 50 min) of the *H. pylori* cells from the wild-type or mutant strains to pH 4 to 7 did not affect viability (data not shown). For 3401A$^-$ cells from 24-h (log-phase) cultures, exposure to pH 4 led to markedly lower *xylE* expression compared with exposure to pH 5, 6, or 7 (Fig. 6). For 3401A$^-$ cells at 48 h (stationary phase), exposure to pH 5 or 6 led to increased *xylE* expression, but exposure to pH 4 or

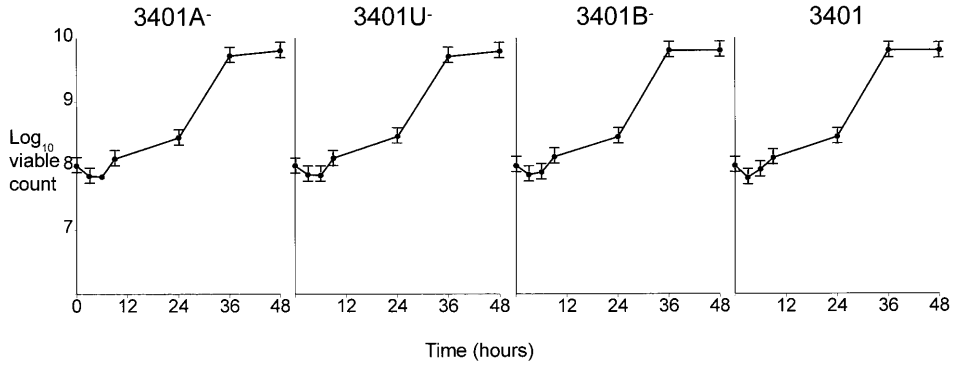

FIG. 3. Growth of mutant strains 3401A$^-$, 3401U$^-$, and 3401B$^-$ and of wild-type strain 3401 in brucella broth with 5% fetal bovine serum for 48 h. Each point represents the mean values of four determinations of $\log_{10}$ viable counts.

7 induced little change compared with the values observed for the log-phase cells. For the stationary-phase cells, the brief (5-min) exposure to pH 6 yielded activity 1.7-fold (± 0.1-fold) higher than that at pH 7. Varying the time of exposure to pH 4, 5, 6, or 7 from 5 to 50 min had essentially no impact on *xylE* expression.

To determine whether the expression characteristics observed for *cagA* through the *xylE* reporter are gene specific, we examined *xylE* expression in *ureA* or *picB* cells as a control. For stationary-phase 3401U$^-$ cells, *xylE* expression was higher after exposure to pH 6 or 7 than in log-phase cells, but no such variation was seen after exposure to pH 4 or 5 (Fig. 6). Highest expression occurred after exposure to pH 7. For 3401B$^-$ cells, there was little difference in *xylE* expression throughout the growth cycle for cells exposed to pH 4 or 5. In contrast, stationary-phase cells showed decreased expression compared with log-phase cells after exposure to pH 6 or 7 (Fig. 6). For cells at each growth phase, optimal expression occurred after exposure to pH 7.

Thus, for each of the genes studied, growth phase of the cells and pH exposure affected *xylE* expression in its own unique pattern. In contrast to strain 3401A$^-$, 3401U$^-$ or 3401B$^-$ cells exposed to pH 7 showed the highest *xylE* expression, with progressively less at lower pHs (7 > 6 > 5 > 4), regardless of growth phase. Consistent with our earlier observations, the magnitude of *xylE* expression in *ureA* and *cagA* was 20- to 30-fold greater than in *picB* cells under each of the induction conditions. These findings indicated that *cagA* expression in response to acid pH is gene specific.

pH regulation of *cagA* expression is gene specific. Next, we sought to determine whether *cagA* up-regulation at acid pH is gene specific by assessing the level of another translated product in strain 3401A$^-$ and whether the studies with transcriptional fusions are indicative of the amount of product actually translated. To accomplish these aims, using an ELISA with monospecific antisera, we examined production of UreA, the product of *ureA*, in the wild-type strains and each of the mutant strains. Aliquots of the cells exposed to different pH conditions from the above-described studies of *xylE* expression had been preserved for studies of protein translation. As expected, 3401U$^-$ cells had no UreA protein detectable since *ureA* was interrupted (Fig. 7). UreA levels in 3401A$^-$, 3401B$^-$, and the wild-type cells were nearly identical and paralleled the findings for *xylE* expression in *ureA* (Fig. 7). These results indicated that under the conditions studied, mutation of *cagA* or *picB* had no effect on UreA expression. Moreover, that the *ureA* product in strain 3401A$^-$ was maximally expressed at pH 7, whereas the *cagA*::*xylE* fusion product was maximally expressed after expo-

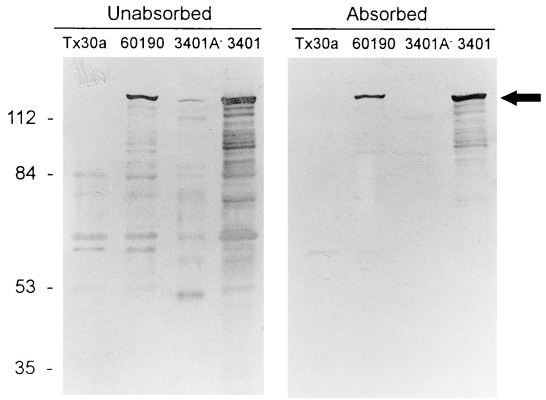

FIG. 4. Immunoblotting of *H. pylori* Tx30a, 60190, 3401A$^-$, and 3401 with unabsorbed or absorbed immune rabbit antiserum for CagA. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lysates and electrotransfer, the nitrocellulose membrane was reacted with unabsorbed or absorbed antiserum for CagA and alkaline phosphatase-conjugated anti-rabbit IgG antibody. The absorbed antiserum specifically recognized an approximately 120-kDa native CagA band of strains 60190 and 3401 as well as a series of bands of lower molecular mass. Complete absence of these bands in 3401A$^-$ indicates that these bands represent degradation products of CagA. Sizes on the left are in kilodaltons.

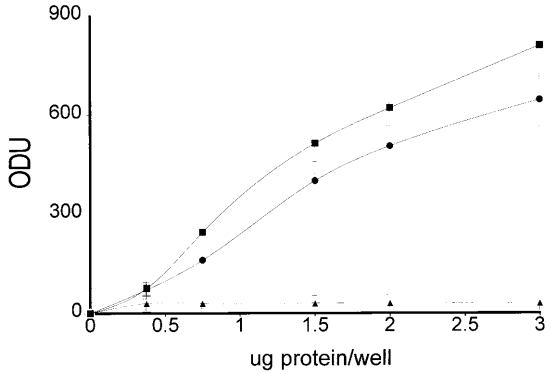

FIG. 5. Determination of CagA protein in *H. pylori* whole cells by ELISA using absorbed antiserum. Wells were coated with 0 to 3.0 μg of protein of *H. pylori* whole cells of mutant strain 3401A$^-$ (▲) or wild type strain 3401 (●) or 84-183 (■). Results represent the mean values of four determinants. ODU, optical density units multiplied by 10^3.

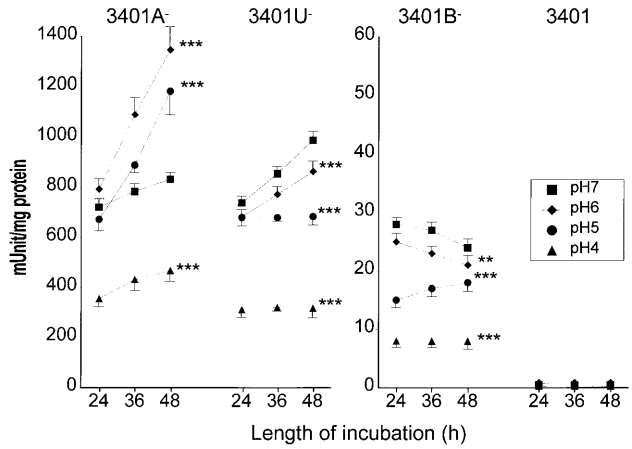

FIG. 6. Expression of *xylE* gene fusions in wild-type and mutant *H. pylori* strains in response to different pH conditions. The mutant (3401A$^-$, 3401U$^-$, and 3401B$^-$) and wild-type (3401) strains were grown in culture for 24 h (log phase), 36 h, or 48 h (stationary phase); then cells were exposed to buffers at pH 4 to 7 for 5 min, pelleted, and resuspended in brucella broth for 55 min; then cells were sonicated, an aliquot was removed, and *xylE* expression was assessed. The results shown are the mean values of four determinations. **, $P < 0.01$ compared with pH 7; ***, $P < 0.001$ compared with pH 7.

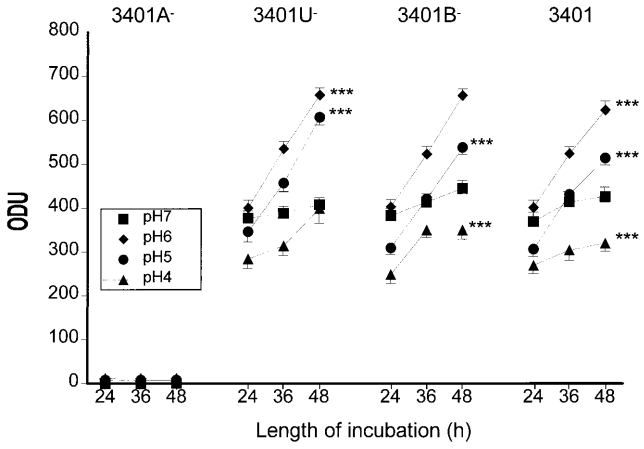

FIG. 8. Determination by ELISA of CagA protein levels in sonicated *H. pylori* cells of strains 3401A$^-$, 3401U$^-$, and 3401B$^-$ and wild-type strain 3401. An aliquot parallel to that described in the legend to Fig. 6 was used for these experiments. Results represent the mean values of four determinants. ODU, optical density units multiplied by 10^3. ***, $P < 0.001$ compared with pH 7.

sure to pH 6, indicates that up-regulation at acid pH is *cagA* specific.

pH regulation of CagA expression. To determine whether translation of the *cagA* product also reflects the pH-regulated transcription observed, in *H. pylori* cells exposed to different pH conditions, we assessed levels of CagA by ELISA using a rabbit antiserum to CagA that had been absorbed with lysates from a *cagA*-negative *H. pylori* strain (Tx30a). This absorption of the CagA antiserum created a highly specific reagent, since by Western blotting, no bands were recognized in *cagA* strains Tx30a and 3401A$^-$ (Fig. 4). By ELISA using this antiserum, *cagA*$^+$ strains 3401 and 84-183 were well recognized (Fig. 5). As expected, 3401A$^-$ cells had no detectable CagA protein since *cagA* was interrupted (Fig. 8 and 5). In total, CagA levels in the wild-type, 3401U$^-$, and 3401B$^-$ cells were nearly identical. Log- and stationary-phase cells showed similar responses after exposure to pH 4 or 7. After exposure to pH 5 or 6, responses were significantly ($P < 0.001$) different, with approximately twofold increases in stationary-phase compared with log-phase cells. These increases after exposure to pH 5 or 6 parallel those observed for *xylE* expression in *cagA*. Detection of CagA protein in whole (nondisrupted) *H. pylori* cells yielded results nearly identical to those for the sonicated cells (data not shown). In total, these findings indicate that transcription rates of *cagA* are the major determinants for CagA protein expression in *H. pylori* cells.

Effect of growth pH on CagA expression. *H. pylori* may grow in broth culture within a broad pH range (pH 5 to 7) (21). The results of the foregoing experiments suggest that *H. pylori* cells that are grown at pH 6 also might have higher-level expression of CagA than when grown at other pHs. To test this hypothesis, *H. pylori* cells were cultured at pH 5, 6, or 7 for 24 h and levels of CagA expression in each fraction were assessed by ELISA. As predicted, cells grown at pH 6 showed maximal CagA expression ($P < 0.01$ compared with pH 7) (Fig. 9). The cell sonic extracts were separated by ultracentrifugation into a

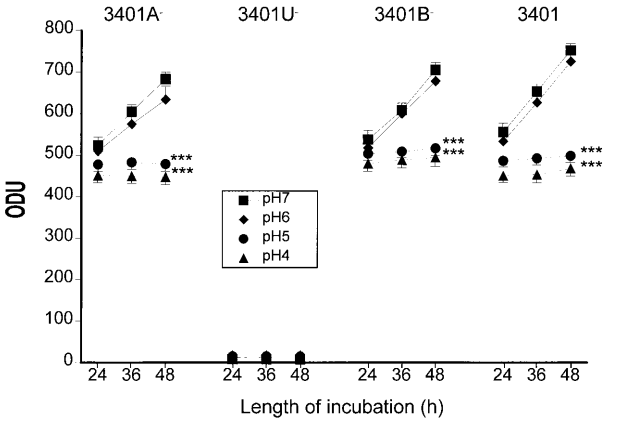

FIG. 7. Determination by ELISA of UreA protein levels in sonicated *H. pylori* cells of mutant strains 3401A$^-$, 3401U$^-$, and 3401B$^-$ and wild-type strain 3401. An aliquot parallel to that described in the legend to Fig. 6 was used for these experiments. Results represent the mean values of four determinants. ODU, optical density units multiplied by 10^3. **, $P < 0.01$ compared with pH 7; ***, $P < 0.001$ compared with pH 7.

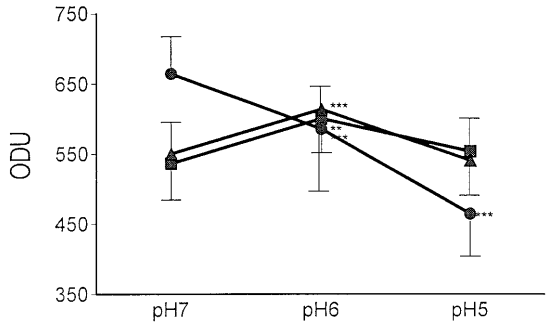

FIG. 9. Expression of CagA in *H. pylori* 3401 grown for 24 h at pH 5, 6, or 7. The strain was cultured at 37°C under microaerobic conditions, and 3.0-μg aliquots of sonic extracts of harvested cells were loaded into microtiter plate wells to assess total CagA production (■). Cell sonic extracts were fractionated into cytoplasmic (●) and membrane (▲) preparations as described in Materials and Methods. Results represent the mean values of four determinants. ODU, optical density units multiplied by 10^3. Total CagA and CagA concentrations in the membrane fraction were maximal at pH 6. **, $P < 0.01$ with pH 7; ***, $P < 0.001$ compared to pH 7.

pellet (membrane enriched) and a supernatant (cytoplasmic fraction). To confirm the identities of the fractions, an antibody to the Le^x LPS determinant of strain 3401 was used. The pellets were well recognized by the anti-Le^x antibody (mean optical density $\times 10^3 = 1{,}444$), whereas there was little recognition of the supernatant (mean = 64), confirming that the pellet was enriched in the bacterial membranes whereas the supernatant represented the cytoplasm. Maximal CagA expression in the membrane fraction was observed in cells grown at pH 6 ($P < 0.001$ compared with pH 7), whereas cytoplasmic levels significantly decreased for cells grown at progressively lower pH.

DISCUSSION

Since wild-type *H. pylori* strains that possess *cagA* are associated with duodenal ulceration which often is related to enhanced gastric acidity, we hypothesized that *cagA*$^+$ strains may possess additional acid response mechanisms compared with *cagA*-negative strains. A corollary to this hypothesis is that expression of *cagA* may be regulated by pH. Other transcriptional fusion systems based on luciferase (1, 7, 20), β-galactosidase (3, 31, 39), and chloramphenicol acetyltransferase (17, 26) have been used to study regulation of gene expression in bacteria. Advantages of the *xylE* reporter used in this study include that catechol 2,3-dioxygenase activity produced by *xylE* is stable, can be rapidly detected in whole bacterial colonies, and can be measured spectrophotometrically, and that the substrate is inexpensive. Since both wild-type strains and mutants carrying *xylE* in the opposite orientation to gene transcription showed no background levels of XylE activity, the promoterless *xylE* cassette is well suited to study genetic regulation in *H. pylori*. We used brief exposures to low-pH conditions so that viability of the *H. pylori* cells would not be affected.

Although the pH in the lumen of the human stomach may be as low as 1, *H. pylori* does not colonize the lumen. It lives in the mucus layer, where the pH ranges from 4 to 7 in healthy humans (35). Furthermore, within the mucus layer, pH values approach 7 near the mucosa and are more acidic closer to the lumen (36). Because most *H. pylori* cells within the mucus layer live close to the mucosa, for this study we chose pH values ranging from 4 to 7 to examine the regulation of *cagA* expression. We chose a short exposure to low pH so as not to compromise viability of *H. pylori* cells, and because short exposure to low pH does not exactly reflect in vivo conditions, we broadened our study by examining CagA expression in cells grown at different pHs. Although not dramatic, the results of the growth and exposure experiments were internally consistent.

Two main findings emerged from our studies concerning exposure of *H. pylori* cells to acid pH. First, our studies of strain 3401A$^-$ indicated that pH 6 was the optimum for *cagA* expression in *H. pylori*, showing a 1.7-fold increase over pH 7. The *atrB* gene of *Salmonella typhimurium*, induced at pH 5.8 as part of an acid tolerance response in that organism, showed only a maximum 1.4-fold increase over pH 7 (16); the height of induction under in vitro conditions may be an imperfect indicator of in vivo events. That XylE activity in *picB* (in strain 3401B$^-$) or *ureA* (in strain 3401U$^-$) was maximal after induction at neutral pH indicates that the up-regulation of *cagA* at acid pH is specific. Similarly, that the levels of UreA activity in 3401A$^-$ cells were maximal after exposure to neutral pH further demonstrates that *cagA* up-regulation at acid pH is gene specific. The data obtained concerning expression of the translated CagA protein are consistent with the findings at the transcriptional level and provide independent confirmation of this phenomenon. The reason why the effect was maximal for *cagA* at pH 6 rather than at more acidic pH is not known; there is no a priori reason to assume otherwise, and results of several experiments (Fig. 6, 8, and 9) are consistent. The mechanisms whereby the low pH signal is transduced to result in heightened *cagA* expression are also not yet known; however, that transcription is higher at acid pH than at neutral pH indicates that *cagA* may be involved in an acid tolerance response of *H. pylori*, similar to that described for other organisms (15).

Brief exposure of the mutant and wild-type cells to pH 4 markedly reduced the level of the translated product (either the XylE or CagA protein) compared with exposure to the higher pHs. Since this phenomenon was observed for each of the three genes into which *xylE* was inserted, it likely represents a global mechanism due to cell damage caused by low pH. Although it is probable that the major effect was on transcription, pH shock also could affect posttranscriptional regulatory mechanisms (46).

Second, growth phase also affected *cagA* expression. From *xylE* reporter studies, it is clear that maximal *cagA* expression at stationary phase is almost twofold higher than that at log phase. This up-regulation of *cagA* in response to growth phase also is gene specific because studies using the *xylE* reporter inserted in *ureA* or *picB* showed no significant difference in the levels of their respective gene products between log and stationary phases. Differential gene expression during log and stationary phases is a common theme in bacterial physiology (38), but its particular role in *H. pylori* infection is not known.

Since brief exposure to low pH maximally induced *cagA*, we further examined whether growth of *H. pylori* cells at different pHs resulted in differences in expression of CagA or in the distribution of CagA within the bacterial cell. Total CagA expression and its concentration in the membrane fraction were maximal in cells grown at pH 6; although not dramatic, these differences were statistically significant and are consistent with the results of the brief-exposure studies. The reason for the decline in cytoplasmic levels of CagA by growth of cells in acidic pH is not known.

The studies reported here were performed in vitro; however, control of *H. pylori* gene expression in vivo may be substantially different, as occurs in other bacteria (25). Recent studies indicate that strain 3401 can colonize mice (22); thus, the *xylE* mutants created may be useful for assessing expression of the targeted genes in vivo as well.

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