Regulation of *Helicobacter pylori cagA* Expression in Response to Salt

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Abstract

***Helicobacter pylori* infection and a high dietary salt intake are risk factors for the development of gastric adenocarcinoma. In this study, we tested the hypothesis that high salt concentrations might alter gene expression in *H. pylori*. Transcriptional profiling experiments indicated that the expression of multiple *H. pylori* genes, including *cagA*, was regulated in response to the concentrations of sodium chloride present in the bacterial culture medium. Increased expression of *cagA* in response to high salt conditions was confirmed by the use of transcriptional reporter strains and by immunoblotting. *H. pylori* CagA is translocated into gastric epithelial cells via a type IV secretion pathway, and on entry into target cells, CagA undergoes tyrosine phosphorylation and causes multiple cellular alterations. Coculture of gastric epithelial cells with *H. pylori* grown under high salt conditions resulted in increased tyrosine-phosphorylated CagA and increased secretion of interleukin-8 by the epithelial cells compared with coculture of the cells with *H. pylori* grown under low salt conditions. Up-regulation of *H. pylori cagA* expression in response to high salt concentrations may be a factor that contributes to the development of gastric adenocarcinoma.** [Cancer Res 2007;67(10):4709–15]

Introduction

Helicobacter pylori is a Gram-negative bacterium that persistently colonizes the human stomach (1, 2). *H. pylori* elicits a gastric mucosal inflammatory response, and infection with this organism is a risk factor for gastric cancer and peptic ulcer disease (1, 2). *H. pylori* has been classified as a class I carcinogen by the WHO (3).

H. pylori exhibits a high level of intraspecies genetic diversity (4). Whether symptomatic disease develops in *H. pylori*–infected persons is determined in part by characteristics of the *H. pylori* strains with which individuals are infected. Some *H. pylori* strains contain a 40-kb region of chromosomal DNA known as the *cag* pathogenicity island (PAI; refs. 5–7). The risk of gastric cancer or peptic ulcer disease is higher among persons infected with *cag*-positive *H. pylori* strains than among persons infected with *cag*-negative strains (5–7). The *cag* PAI encodes a protein, CagA, that enters gastric epithelial cells, as well as a type IV secretion system that delivers CagA into host cells (8, 9). Within gastric epithelial cells, CagA can interact with multiple target molecules and can cause a wide array of cellular alterations, including changes in the morphology of gastric epithelial cells, cell scattering, cell proliferation, alterations of tight junctions, dephosphorylation of cellular proteins, activation of β-catenin, and activation of the Ras/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase/ERK pathway (6). Based on these observations, it has been proposed that CagA functions as a bacterial oncoprotein (6, 10).

The risk of gastric carcinoma is influenced not only by *H. pylori* strain characteristics but also by host genetic determinants and environmental factors. One of the environmental factors associated with an increased risk of gastric cancer is high dietary salt intake (11–13). A link between high salt intake and gastric cancer has been shown in human epidemiologic studies and also in animal models (14, 15). The mechanisms by which high salt intake contributes to the development of gastric carcinoma remain poorly understood.

Gene expression in several bacterial pathogens, including *Vibrio cholerae, Salmonella enterica*, and *Listeria monocytogenes* (16, 17), is regulated in response to salt concentration. In the present study, we tested whether *H. pylori* gene expression is altered in response to changes in salt concentration in the bacterial culture medium. We report that the expression of numerous *H. pylori* genes is regulated in response to sodium chloride concentration. We describe in detail the effect of salt concentration on the expression of CagA, a bacterial factor that may contribute to the development of gastric cancer.

Materials and Methods

Bacterial strains and culture conditions. Mutant strains were derived from *H. pylori* 26695 SC#7, which contains an intact *cag* PAI (18). *H. pylori* strains were grown on trypticase soy agar plates containing 5% sheep blood, or in sulfite-free Brucella broth medium supplemented with 5% fetal bovine serum (FBS; i.e., BB-FBS). The previously described sulfite-free Brucella broth medium (19) was modified by the use of Bacto Proteose peptone (Becton Dickinson) in place of peptamin. The BB-FBS medium routinely used for growing *H. pylori* was prepared by addition of 0.5% NaCl. In the current study, BB-FBS was supplemented with NaCl concentrations ranging from 0.25% (43 mmol/L) to 2% (342 mmol/L) NaCl. When appropriate, agar-based culture media were supplemented with kanamycin (10 μg/mL) or chloramphenicol (5 μg/mL). All *H. pylori* strains were grown at 37°C in room air supplemented with 5% CO_2. *Escherichia coli* strains were grown in Luria Bertani (LB) medium or in LB liquid medium supplemented with ampicillin (50 μg/mL), kanamycin (25 μg/mL), or chloramphenicol (30 μg/mL), as appropriate.

RNA isolation and array analyses. *H. pylori* strain 26695 (SC#7) was grown for 15 h in BB-FBS medium containing 0.5% sodium chloride (i.e., BB-FBS-0.5%). The bacterial cells (A_{600}, 0.4–0.5) were then centrifuged and resuspended in fresh BB-FBS-0.25% or BB-FBS-1.5% medium at an A_{600} of 0.1. The bacteria were grown for 10 h, harvested by centrifugation, and frozen at −80°C. Extraction of RNA was done using the Trizol method (Invitrogen), and the extracted RNA was further purified with RNeasy spin columns (Qiagen). Labeling of RNA with [^{33}P]dATP was carried out with SuperScript II Reverse Transcriptase (Invitrogen) as described previously

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(20). Each labeled RNA preparation was then hybridized to *H. pylori* Panorama arrays (Sigma Genosys) according to the manufacturer's instructions. The *H. pylori* Panorama arrays contain 1,681 PCR-amplified open reading frames (ORF), including 1,590 ORFs from *H. pylori* strain 26695, as well as 91 strain-specific ORFs found in *H. pylori* strain J99. Scanning of the hybridized arrays was done with a PhosphorImager (Fuji, Inc.), and the hybridization signals were quantified with ImageQuant (Fuji) and Array Vision (Imaging Research, Inc.) software. For each array feature, a normalized signal intensity was calculated as a ratio of the signal intensity for the individual array feature divided by the total signal intensity of the entire array. The results of multiple array experiments were then used to calculate mean normalized signal intensities for each array feature. Array data have been deposited in the Gene Expression Omnibus database, accession number GSE7183.

Plasmid construction and generation of *H. pylori* transcriptional reporter strains. PCR was carried out using Vent DNA Polymerase (NEB). To construct *H. pylori* transcriptional reporter strains, target genes of interest were PCR amplified from *H. pylori* strain 26695 using the following forward and reverse primers sets: 5′-AATCGTTCTAGCCTTTAGACGCC-3′ and 5′-CAGGTTCTATGCCATTATGACTCCC-3′ for *cagA* (HP0547); 5′-GCCCTCATCGCCCACGCTTTCCCC-3′ and 5′-TGCGGGTATGGGACTAAACAAC-3′ for HP1322; and 5′-CATGGAAAATAAGCTCACGCCAGC-3′ and 5′-TGAGATCGCTTTTATCCATGCC-3′ for HP0209. Each amplified product was then cloned into pGEMT-Easy (Promega). The resulting plasmids (pHP0547a, pHP0209a, and pHP1322a) were then digested with *Hin*dIII (*cagA*) or *Stu*I (HP0209 and HP1322), blunt ended with Klenow (NEB), and a blunt-ended promoterless chloramphenicol acetyl transferase (*cat*) reporter cassette from plasmid pCM7 (ATCC 37173; ref. 21) was inserted into the respective blunt-ended restriction sites. Plasmids containing the *cat* cassette in the desired orientation were identified by PCR. The plasmid used for construction of the *ureA-cat* transcriptional fusion has been described previously (20). *H. pylori* reporter strains were generated by transformation of *H. pylori* strain 26695 SC#7 with the plasmids described above, and transformants were selected on BB-FBS-0.5% agar plates containing 5 μg/mL chloramphenicol. The plasmids used in these experiments are unable to replicate in *H. pylori*; therefore, chloramphenicol-resistant strains result from the integration of the *cat* reporter cassettes into the *H. pylori* chromosome. PCR was used to confirm the insertion of the *cat* reporter cassettes into the appropriate *H. pylori* genes and to verify that double crossover events had occurred. *H. pylori* strains harboring insertions of a kanamycin cassette into either *cagA* or *cagE* (*picB*) were generated by transformation of *H. pylori* strain 26695 SC#7 with either pMC3-Kan (22) or picB-Kan (23), respectively.

Transcriptional reporter analyses. The level of CAT expression in each transcriptional reporter strain was determined using a CAT ELISA kit (Roche Diagnostics). For analyses of CAT expression, bacterial cultures were grown overnight in BB-FBS-0.5% medium. The bacteria were centrifuged at 1,000 g, resuspended in BB-FBS-0.25% medium, and then transferred to medium containing varying salt concentrations and cultured for the indicated times. Aliquots of *H. pylori* cells were lysed using the ELISA kit lysis buffer, and protein concentrations of lysates were determined using the bicinchoninic acid (BCA) reagent kit (Pierce). The amounts of lysate protein added to each ELISA plate well were 2 μg (for HP0209-CAT and HP1322-CAT reporter strains), 0.25 μg (for *ureA*-CAT), and 0.05 μg (for *cagA*-CAT). The levels of CAT bound per well were quantified using protocols provided by the manufacturer. Absorbance data were obtained using an ELISA plate reader (Dynatech).

Immunoblot analysis of CagA expression. In experiments designed to analyze CagA protein expression during bacterial growth, *H. pylori* was grown in broth cultures containing varying concentrations of NaCl. Bacteria were lysed using NP40 lysis buffer [150 mmol/L NaCl, 1% NP40, 50 mmol/L Tris-HCl (pH 8.0)] supplemented with protease inhibitors (Complete Mini; Roche Applied Science). Protein concentrations were measured using the BCA reagent kit and samples were standardized based on protein concentration. Proteins in the cell lysates were then separated by SDS-PAGE (8% SDS-acrylamide gels) and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were incubated in blocking buffer (TBS containing 0.1% Tween, 3% nonfat powdered milk) and immunoblotted for 1 h with rabbit polyclonal antibodies generated against CagA (1:6,000 dilution in blocking buffer; Austral Biologicals) followed by rabbit antiserum to soluble *H. pylori* proteins (1:10,000 dilution; ref. 24). The membranes were then washed with TBS-Tween and incubated for 1 h with horseradish peroxidase (HRP)–conjugated goat anti-rabbit immunoglobulin G (IgG; Bio-Rad), diluted 1:6,000 in blocking buffer. Signals were detected on X-ray film using chemiluminescent methods (Amersham Bioscience). Antibodies were removed from membranes with a stripping buffer (Pierce) before being reprobed.

Coculture of *H. pylori* with AGS gastric cells. Human gastric carcinoma-derived AGS epithelial cells (CRL-1739; American Type Culture Collection) were grown in RPMI 1640 (Life Technologies) containing 2 mmol/L L-glutamine and 10% FBS at 37°C in 5% CO_2. AGS cells were routinely passaged every 3 days. AGS cells were seeded into 24-well plates (2.5×10^5 per well) and grown for 24 h before addition of *H. pylori*. *H. pylori* cells were cultured overnight in BB-FBS-0.5% and then subcultured for 12 h in fresh BB-FBS containing different concentrations of added NaCl. Bacteria were then directly added to AGS cells (50:1 ratio of *H. pylori* to AGS cells) for the indicated times. Following infection, AGS cells were analyzed to detect a morphologic change known as the hummingbird phenotype (25).

Quantification of *H. pylori* binding to AGS cells was done as described by Franco et al. (26). Briefly, *H. pylori*-AGS cocultures were washed five times with PBS (pH 7.5) to remove unbound bacteria, and AGS cells were then detached by scraping with a rubber policeman. Serial 10-fold dilutions of the cell suspensions were plated on blood agar plates, and the numbers of colony-forming units (CFU) were determined 5 days after plating.

To analyze tyrosine phosphorylation of CagA, *H. pylori*-AGS cocultures were lysed with NP40 lysis buffer (described above) containing protease inhibitors and 2 mmol/L sodium orthovanadate. Protein concentrations were measured using the BCA reagent kit and samples were standardized based on protein concentration. Proteins in the cell lysates were then analyzed by immunoblotting as described above, using mouse anti-phosphotyrosine antibody PY99 (1:3,000 dilution; Santa Cruz Biosciences) or rabbit antiserum to soluble *H. pylori* proteins (1:10,000 dilution; ref. 24). The membranes were then washed with TBS-Tween and incubated for 1 h with either HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences) or HRP-conjugated goat anti-rabbit IgG, diluted 1:6,000 in blocking buffer. Signals were detected on X-ray film using chemiluminescent methods. Antibodies were removed from membranes with a stripping buffer before being reprobed.

Analysis of interleukin-8 secretion. Following infection of AGS cells for 5 h with *H. pylori* 26695 wild-type (WT) or isogenic mutant strains as described above, supernatants were removed and the concentrations of interleukin-8 (IL-8) in the supernatants were determined using an IL-8 cytometric bead assay (BD Biosciences), according to the manufacturer's recommendation. After 2 h of incubation, samples were analyzed using a FACSCalibur four-color flow cytometer (Becton Dickinson), and the data were analyzed using FlowJo software (Treestar, Inc.). IL-8 concentrations were determined using purified human IL-8 standards provided with the IL-8 kit.

Results

Effects of salt concentration on *H. pylori* growth. Because both high salt intake and *H. pylori* infection are associated with an increased risk for gastric cancer, we investigated the possibility that high salt concentrations might alter gene expression in *H. pylori*. As a first step, we analyzed the growth of *H. pylori* strain 26695 (SC#7) in BB-FBS medium supplemented with different NaCl concentrations (0.25–2.0%). This range includes salt concentrations that are both lower and higher than the 0.5% NaCl concentration typically used for preparation of BB-FBS. In agreement with the results of a previous study (27), growth of *H. pylori* strain 26695 was observed at each of the NaCl concentrations tested. The rate of bacterial growth was relatively similar for cultures grown in NaCl

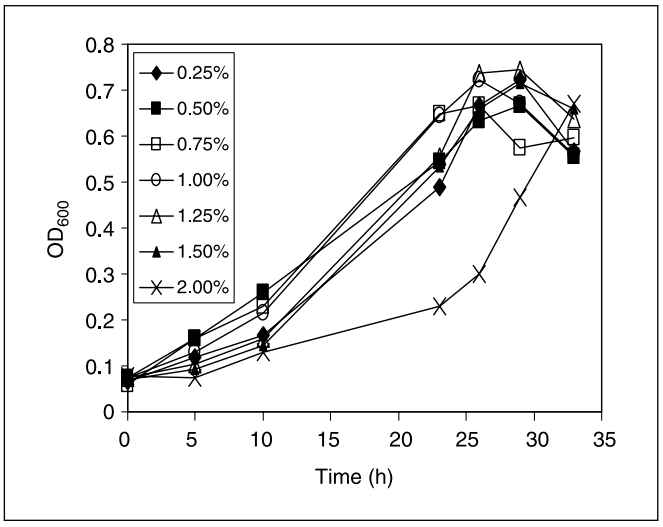

Figure 1. Growth of *H. pylori* in medium containing different salt concentrations. *H. pylori* strain 26695 was grown overnight in BB-FBS-0.5% medium. Bacteria from this culture were harvested by centrifugation, resuspended in BB-FBS-0.25% medium, and inoculated into fresh Brucella-FBS broth containing different concentrations of added NaCl (0.25–2.0% NaCl). At the indicated time points, A_{600} values of the cultures were measured. Mean results based on analysis of triplicate cultures from a single experiment are reported; all SDs were <12% of the reported mean values and are not depicted. At time points >32 h, clumping of the bacteria resulted in an artifactual reduction in absorbances; therefore, data for time points >32 h are not reported. The experiment was repeated five times with similar results.

concentrations ranging from 0.25% to 1.5% (Fig. 1). However, the rate of bacterial growth was slower in cultures containing 2.0% NaCl (Fig. 1). To test the effects of NaCl on viability of the bacteria, cultures containing different concentrations of NaCl were harvested before the onset of stationary phase, normalized to an A_{600} of 0.1, serial dilutions were plated onto blood agar plates, and the numbers of CFU (CFU/mL) were determined. The numbers of CFUs recovered from the normalized cultures were similar, suggesting that high salt concentrations (2.0% NaCl) retard *H. pylori* growth but do not have a bacteriocidal effect (data not shown).

Effect of salt concentration on *H. pylori* gene expression. We next investigated the effect of salt concentration on *H. pylori* gene expression. RNA was isolated from cultures of *H. pylori* strain 26695 that were grown for 10 h under two different conditions (either BB-FBS-0.25% or BB-FBS-1.5% medium). RNA preparations from eight independent cultures (four from each condition) were each hybridized independently with *H. pylori* DNA macroarrays. The mean normalized signal intensity for each array feature was calculated as described in Materials and Methods. To compare gene expression in bacteria grown under the two different conditions, ratios of normalized signal intensities were generated (signal intensity when grown in BB-FBS-1.5% divided by the signal intensity when grown in BB-FBS-0.25%). The mean ± SD of intensity ratios was 1.05 ± 0.27. Genes that had expression ratios >2 SD above or below the mean ratio were considered to be potentially regulated in response to salt concentration. Based on these criteria, 63 genes were classified as potentially up-regulated and 61 genes as potentially down-regulated in response to high salt concentration (Table 1). The list of candidate regulated genes (Table 1) includes genes that encode heat shock proteins (e.g., HP0010, HP0011, and HP1024), genes encoding outer membrane proteins (e.g., HP0229, HP0671, HP1177, and HP0722), and several genes associated with *H. pylori* virulence [(e.g., *cagA* (HP0547), *napA* (HP0243), and *iceA* (HP1209)]. Several genes that encode heat shock proteins (e.g., HP0010 and HP1024) have been reported previously to be up-regulated in response to osmotic shock (28). These data indicate that gene expression in *H. pylori* is altered when the bacteria are grown in environments containing different salt concentrations.

Effect of salt on *cagA* transcription. One of the genes regulated in response to salt was *cagA*. Array analyses indicated that there was increased *cagA* transcription (a 2-fold increase in normalized signal intensity) in bacteria grown in BB-FBS-1.5% medium compared with bacteria grown in BB-FBS-0.25% medium. Based on the criteria for identifying salt-regulated genes described above, the transcription of other genes in the *cag* PAI was not regulated in response to salt. To validate the observation that *cagA* transcription was regulated in response to salt, we inserted a promoterless CAT reporter into *cagA*, thereby generating a *H. pylori cagA*-CAT transcriptional reporter strain. In addition, we constructed three control reporter strains, in which the CAT cassette was inserted into genes that were not regulated in response to salt, based on array experiments [*ureA*, HP0209 (encoding an outer membrane protein designated HofA) and HP1322 (an ORF of unknown function)]. The *H. pylori* reporter strains were cultured in medium containing different NaCl concentrations for varying lengths of time. After growth for >6 h in BB-FBS-1.5% medium, the *cagA-cat* reporter strain expressed higher levels of CAT activity than when it was grown for the same length of time in BB-FBS-0.25% or BB-FBS-0.5% medium (Fig. 2*A*). The expression of CAT activity by the *cagA-cat* reporter strain was also stimulated by overnight growth in medium containing 1.0% NaCl (Fig. 2*B*). In contrast, the expression of CAT activity in *ureA*-CAT, HP0209-CAT, or HP1322-CAT reporter strains was not affected by salt concentration (Fig. 2*B*; data not shown). When the bacteria were cultured in medium containing low salt concentrations (0.25% or 0.5% NaCl), *cagA* expression was not affected by the bacterial growth phase (Fig. 2*A*). Specifically, under these conditions (0.25% or 0.5% NaCl), there was no increase in *cagA* expression when the bacteria were in stationary phase (36 h) compared with earlier time points.

Analysis of CagA protein expression. We next investigated the effects of salt concentration on CagA protein expression. As shown in Fig. 3, Western blot analyses using a polyclonal anti-CagA antibody revealed the presence of high molecular mass immunoreactive bands in *H. pylori* 26695 (SC#7) but not in an isogenic *cagA* mutant strain. The presence of multiple immunoreactive bands is consistent with results of several previous studies that provided evidence for post-translational modification of CagA (18). Consistent with the transcriptional analysis, the levels of CagA protein expression were higher in *H. pylori* cells grown in high-salt medium than in *H. pylori* cells grown in medium containing 0.25%, 0.5%, or 0.75% NaCl (Fig. 3). Increases in CagA expression in response to NaCl were also detected in immunoblotting experiments with *H. pylori* strains 60190 and SS1 and several *H. pylori* strains isolated from Japanese patients. However, salt-dependent changes in CagA expression were not detected for several other strains, including *H. pylori* strain J99 (data not shown). Western blot analysis of *H. pylori* whole cells did not reveal any consistent effect of salt concentration on VacA expression (data not shown), and similarly, no effect of salt concentration on *vacA* transcription was detected in array experiments (Table 1). Supplementation of the bacterial culture medium with potassium chloride or potassium acetate (osmolarity of 0.43 Osm, osmoequivalent to 1.25% NaCl) resulted in

Table 1. *H. pylori* genes differentially expressed in response to NaCl concentration

Up-regulated genes	Fold induction	Down-regulated genes	Fold repression
HP0010	1.6	HP0022	1.7
HP0011	1.8	HP0071	2.8
HP0023	1.7	HP0083	1.7
HP0041	1.8	HP0085	1.9
HP0103	1.9	HP0091	2.1
HP0130	1.6	HP0093	1.7
HP0144	2.0	HP0126	1.6
HP0149	1.7	HP0136	1.7
HP0218	2.0	HP0137	1.6
HP0229	1.8	HP0139	1.6
HP0237	1.6	HP0164	1.7
HP0243	1.8	HP0166	2.0
HP0292	1.6	HP0168	1.8
HP0304	2.2	HP0225	1.6
HP0337	2.1	HP0247	1.8
HP0344	1.8	HP0248	1.7
HP0414	1.9	HP0377	1.9
HP0448	1.8	HP0424	1.6
HP0490	1.7	HP0429	1.7
HP0513	1.6	HP0446	2.2
HP0547	2.0	HP0565	1.6
HP0663	1.6	HP0630	1.7
HP0671	1.8	HP0705	1.7
HP0685	1.6	HP0717	1.7
HP0718	1.6	HP0719	1.7
HP0722	1.8	HP0720	2.1
HP0723	1.7	HP0721	2.2
HP0822	2.3	HP0772	1.8
HP0893	1.7	HP0873	2.1
HP0894	1.6	HP0874	2.2
HP0948	1.7	HP0967	1.9
HP0983	1.6	HP1033	2.3
HP0999	1.8	HP1040	1.7
HP1005	2.2	HP1084	2.0
HP1008	1.9	HP1109	1.8
HP1023	1.7	HP1172	2.4
HP1024	1.6	HP1182	1.6
HP1081	1.6	HP1192	2.1
HP1083	2.0	HP1200	1.7
HP1112	1.9	HP1204	1.7
HP1125	1.8	HP1209	1.8
HP1150	1.7	HP1245	2.4
HP1154	1.8	HP1286	1.8
HP1177	2.3	HP1292	1.7
HP1180	3.3	HP1299	1.6
HP1232	1.8	HP1305	1.8
HP1258	1.6	HP1308	2.1
HP1264	1.6	HP1310	1.6
HP1288	1.8	HP1311	1.6
HP1329	2.5	HP1314	1.6
HP1338	1.7	HP1316	2.0
HP1351	1.8	HP1355	1.7
HP1432	1.7	HP1372	2.2
HP1459	1.6	HP1433	1.8
HP1588	2.7	HP1444	1.8
JHP0045	1.8	HP1446	1.7
JHP0540	2.0	HP1554	1.7
JHP0814	1.8	HP1564	1.8
JHP0827	1.7	HP1586	2.8
JHP0922	2.0	HP1590	1.7
JHP0936	1.7	JHP0826	2.3
JHP0946	1.6		
JHP0961	1.7		

NOTE: *H. pylori* strain 26695 was grown in medium containing either 1.5% or 0.25% NaCl. Transcriptional profiling and identification of differentially expressed genes were accomplished as described in the text. The indicated genes were up-regulated or down-regulated during growth in BB-FBS-1.5% medium compared with growth in BB-FBS-0.25% medium.

increased CagA expression, albeit to a lesser extent than observed with NaCl. Supplementation of the bacterial culture with osmoequivalent concentrations of several other solutes (magnesium sulfate, magnesium chloride, magnesium acetate, sorbitol, or sucrose) did not result in increased CagA expression.

Effect of salt on *H. pylori*-AGS cell interactions. When *H. pylori* is cocultured with AGS gastric epithelial cells, CagA is translocated into the epithelial cells via a type IV secretion pathway, and it subsequently undergoes tyrosine phosphorylation within host cells (8, 25). To analyze translocation and tyrosine phosphorylation of CagA, AGS cells were infected with *H. pylori* 26695 for 5 h and then washed to remove unbound *H. pylori*. The AGS cells were then lysed and tyrosine-phosphorylated CagA was detected by immunoblotting. As shown in Fig. 4*A*, the levels of phosphorylated CagA and total CagA were each markedly increased in AGS cells infected with *H. pylori* cells grown in BB-FBS-1.0% medium compared with AGS cells infected with *H. pylori* grown in low-salt medium.

Translocation of CagA into AGS gastric epithelial cells can result in a morphologic change known as the hummingbird phenotype, which is characterized by the appearance of elongated, needle-shaped cells (25). To determine if varying salt concentrations altered the ability of *H. pylori* to cause the hummingbird phenotype, *H. pylori* strains were cultured for 12 h in BB-FBS containing 0.25%, 0.5%, or 1.0% NaCl, and the bacteria were then incubated with AGS cells. Cells were inspected microscopically after incubation for 5 h. The proportion of AGS cells showing epithelial cell elongation was higher when infected with *H. pylori* 26695 grown in BB-FBS-1.0% medium than when infected with *H. pylori* 26695 grown in medium containing either 0.25% or 0.5% NaCl (55 $\pm$ 12% versus 10 $\pm$ 5% and 15 $\pm$ 5% elongated cells, respectively). Representative micrographs are shown in Fig. 4*B*. Parallel infections of AGS cells with either an isogenic *H. pylori cagA* mutant strain or a *cagE* mutant strain did not result in the hummingbird phenotype, regardless of the salt concentrations in which these *H. pylori* mutant strains were grown (data not shown). Similarly, the addition to AGS cells of uninoculated (bacteria-free) BB-FBS medium containing 0.25%, 0.5%, or 1.0% NaCl did not produce the hummingbird phenotype. Growth of *H. pylori* in medium containing different concentrations of NaCl did not influence the number of viable *H. pylori* associated with gastric epithelial cells (data not shown). Moreover, immunoblot analyses

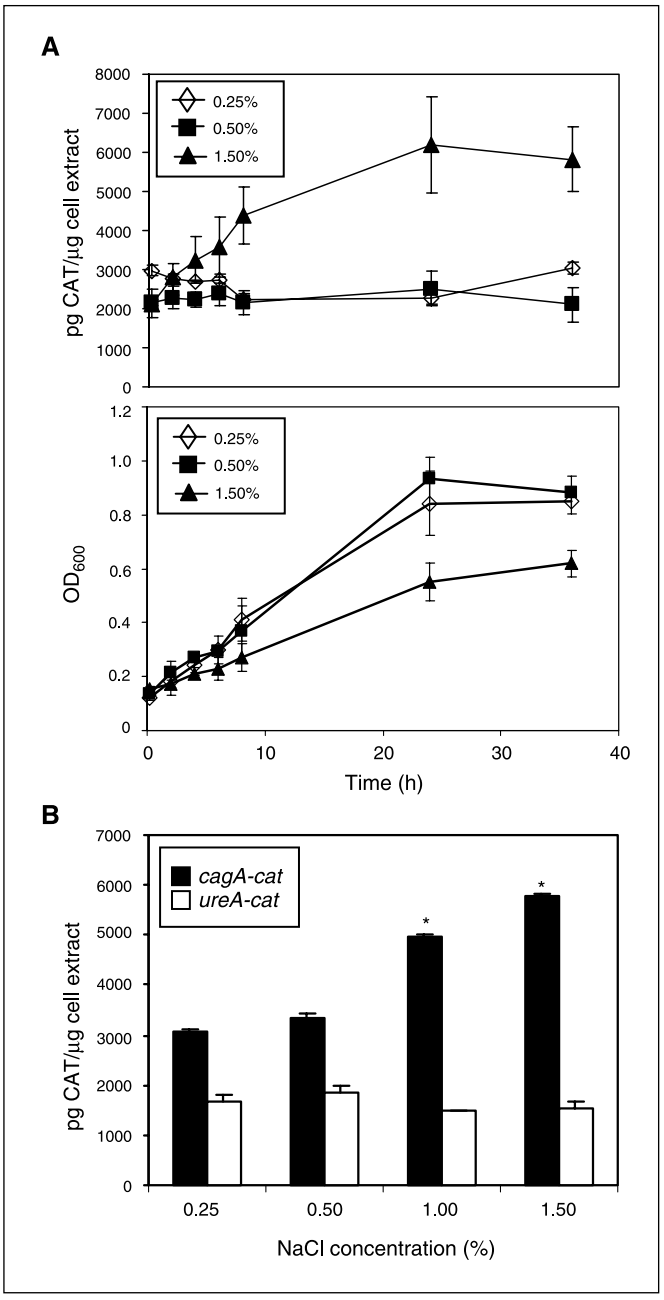

Figure 2. Analysis of *cagA* expression in *H. pylori* strain 26695. An overnight culture of *H. pylori* strain 26695 harboring a *cagA*-cat transcriptional fusion was inoculated (1:100) into BB-FBS broth containing different concentrations of NaCl (0.25%, 0.5%, or 1.5% NaCl) and grown for varying lengths of time. *A,* aliquots of cultures were removed at the indicated time points, A_{600} of the cultures was measured (*bottom*), and the cultures were analyzed for CAT expression (*top*). *B,* reporter strains containing *cagA-cat* or *ureA-cat* transcriptional fusions were grown for 15 h in medium containing 0.25%, 0.5%, 1.0%, or 1.5% NaCl, and CAT expression was then analyzed. *Points* and *columns,* mean based on analysis of triplicate cultures from a single experiment; *bars,* SD. *, $P < 0.05$, levels of CAT expression significantly different from the CAT levels of cultures grown in BB-FBS-0.25% medium. The experiments were conducted thrice with similar results.

of the lysates with an anti–*H. pylori* antiserum did not reveal any obvious effect of salt on *H. pylori* interactions with the AGS cells (Fig. 4*A*). These results suggest that exposure of *H. pylori* to high salt concentrations increases the level of CagA expression, which in turn leads to an increased amount of CagA translocated into AGS cells and an enhanced ability of *H. pylori* to cause alterations in cell morphology.

Effect of salt on *H. pylori*–induced IL-8 production by AGS cells. Two studies (29, 30) showed recently that CagA can stimulate increased expression of IL-8 in AGS cells. We thus sought to determine if increased levels of CagA in *H. pylori* strains grown in high salt concentrations could affect the ability of *H. pylori* to stimulate IL-8 production by AGS cells. *H. pylori* strains grown in medium containing varying salt concentrations were added to AGS cells, and supernatants from the *H. pylori*-AGS cocultures were removed after 5 h. The levels of secreted IL-8 were analyzed by flow cytometry as described in Materials and Methods. As shown in Fig. 5, WT *H. pylori* grown in BB-FBS-1.0% medium induced higher levels of IL-8 secretion than did *H. pylori* grown in medium containing lower salt concentrations. The addition of bacteria-free BB-FBS-0.25% or BB-FBS-1.0% medium to the AGS cells did not alter IL-8 production (data not shown). As expected, based on previously reported results (31), *cagE* (*picB*) mutant strains were defective in the ability to induce IL-8 secretion, regardless of the levels of salt in the growth medium. The *cagA* mutant strain induced higher levels of IL-8 production than did the *cagE* mutant strain (Fig. 5), but growth of the *cagA* mutant in high-salt medium did not alter its ability to elicit IL-8 production in the AGS cells (Fig. 5). These results indicate that exposure of *H. pylori* to high salt conditions enhances the ability of the bacteria to stimulate AGS cell expression of IL-8 in a CagA-dependent manner.

Discussion

Many epidemiologic studies have reported a correlation between high levels of dietary salt intake and an increased risk of gastric cancer (11–13). This association has been detected in prospective studies (32), case-control studies (33), and in a study that compared urinary salt excretion with gastric morbidity rates (34). The mechanisms by which a high-salt diet increases the risk of gastric cancer in humans are incompletely understood. One possibility is that salt may have direct effects on the gastric mucosa that lower the threshold for malignant transformation (35). Another proposed explanation is that salt damages the gastric mucosa (36), thereby allowing increased entry of carcinogens into gastric tissue. Whether *H. pylori* infection and high salt intake are independent or interdependent risk factors is not entirely clear. A prospective study of a Japanese population (37) and a case control study in

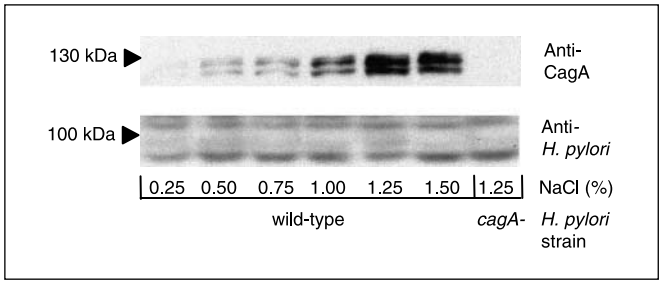

Figure 3. Analysis of CagA protein expression. Overnight cultures of *H. pylori* (either WT strain 26695 or a *cagA* null mutant derivative) were inoculated into BB-FBS broth containing the indicated concentrations of NaCl. After culture for 12 h, *H. pylori* cells were harvested, lysed, and standardized based on protein concentration. Cell extracts (2 μg) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, and the membrane was probed sequentially with anti-CagA antiserum (1:6,000) followed by anti–*H. pylori* serum (1:10,000).

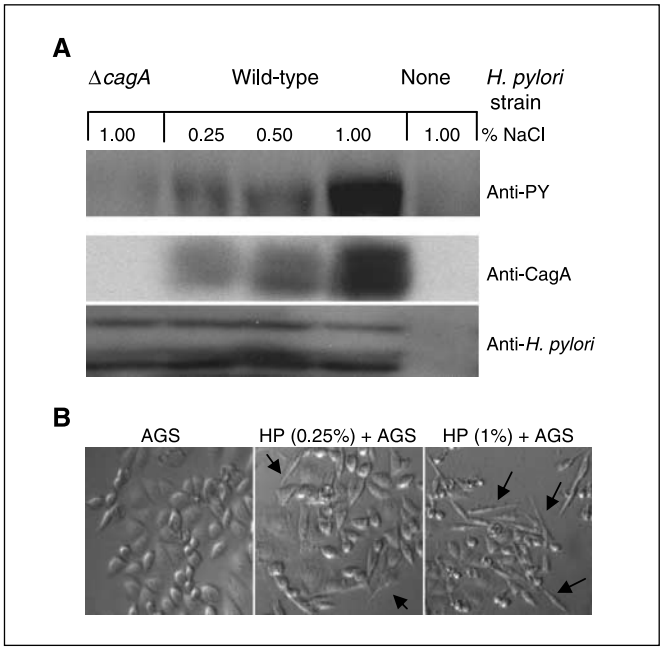

Figure 4. Effects of salt on *H. pylori* interactions with AGS cells. *H. pylori* cells (WT strain 26695 or an isogenic *cagA* mutant strain) were grown overnight in medium containing the indicated NaCl concentrations. AGS cells were infected with *H. pylori* at a multiplicity of infection of 50:1. *A,* AGS cells infected with *H. pylori* were lysed, and extracts were standardized by protein concentration and then analyzed by SDS-PAGE and immunoblotting. The membrane was probed sequentially with an anti-phosphotyrosine (*PY*) antibody, anti-CagA antiserum, and anti–*H. pylori* serum. *B,* AGS cells were cocultured with *H. pylori* (*HP*) that had been grown in BB-FBS-0.25% or BB-FBS-1.0% medium (*middle* and *right*). An equivalent amount of uninoculated BB-FBS-1.0% medium was added to control AGS cells (*left*). *Arrows,* cells exhibiting the hummingbird phenotype.

Korea (33) each reported that *H. pylori*–infected subjects consuming a high-salt diet had an increased risk of gastric cancer when compared with *H. pylori*–infected subjects who consumed lower levels of salt. Another study reported a correlation between the prevalence of *H. pylori* infection and levels of dietary salt intake (38). Studies using a Mongolian gerbil model have shown that the presence of *H. pylori* and a high-salt diet have a synergistic effect on gastric carcinogenesis (15, 39).

In this study, we investigated the changes in *H. pylori* gene expression that occur in response to a high-salt environment. Transcriptional profiling experiments revealed that the expression of multiple *H. pylori* genes was altered in response to changes in the salt concentration of the bacterial culture medium. The NaCl concentrations tested in the current study ranged from 0.25% (43 mmol/L) to 2.0% (342 mmol/L). Dietary salt intake varies widely among humans. Levels of dietary salt intake are reported to reach 41 g/d in Japan (40) and 55 g/d in China (41). Based on an average adult stomach volume of ~900 mL (42) and an intake of three meals a day, we estimate that gastric luminal salt concentrations of 300 mmol/L may commonly be attained or exceeded in some populations. Therefore, the NaCl concentrations tested in the current study are likely to be physiologically relevant. However, salt concentrations in the gastric mucus layer (the natural niche for *H. pylori*) are likely to be lower than in the gastric lumen.

A particularly interesting finding in the current study was the observation that *cagA* expression is regulated in response to salt concentration. Several previous studies provided evidence that expression of *cagA* is not constitutive. Specifically, modulation of *cagA* expression in response to iron concentration (43) and pH (44, 45) has been reported. The mechanisms by which *cagA* expression is regulated in response to environmental conditions are not yet understood. Potentially, regulation of *cagA* expression in response to multiple environmental stimuli is indicative of a generalized *H. pylori* stress response. Alternatively, there may be a variety of regulatory pathways that individually regulate *cagA* expression in response to specific environmental conditions. As shown in Fig. 1, *H. pylori* can survive and grow in NaCl concentrations up to 2.0% (342 mmol/L), a result that is consistent with previous reports (27). However, the growth rate of *H. pylori* was retarded when the bacteria were cultured in medium containing 2% NaCl. As gene expression in *H. pylori* is altered in response to growth phase, the observed effects of salt on gene expression in *H. pylori* could potentially be related to retardation of *H. pylori* growth. However, among the 138 genes listed in Table 1, only 8 (i.e., *cagA, napA*, HP0229, HP0630, HP0719, HP1172, HP1200, and HP1292) were reported previously to be differentially expressed based on growth phase (46). Moreover, in the current study, CagA expression was up-regulated in response to NaCl concentrations (1.0–1.5%) that did not have a substantial effect on *H. pylori* growth. Karita et al. (44) detected a growth phase-dependent change in *cagA* expression when the bacteria were grown at pH 5 but detected relatively little growth phase-dependent change in *cagA* expression when the bacteria were grown at pH 7. Similarly, under the experimental conditions tested in this study, we did not detect growth phase-dependent changes in expression of *cagA* when the bacteria were grown in BB-FBS-0.25% or BB-FBS-0.5% medium (Fig. 2*A*). These data suggest that the observed effects of NaCl on *cagA* expression are not attributable to salt-induced effects on the bacterial growth phase.

Epidemiologic studies indicate that the risk of gastric cancer is higher among humans infected with *cagA*-positive strains than among persons infected with *cagA*-negative strains, and the development of gastric cancer in the Mongolian gerbil model is

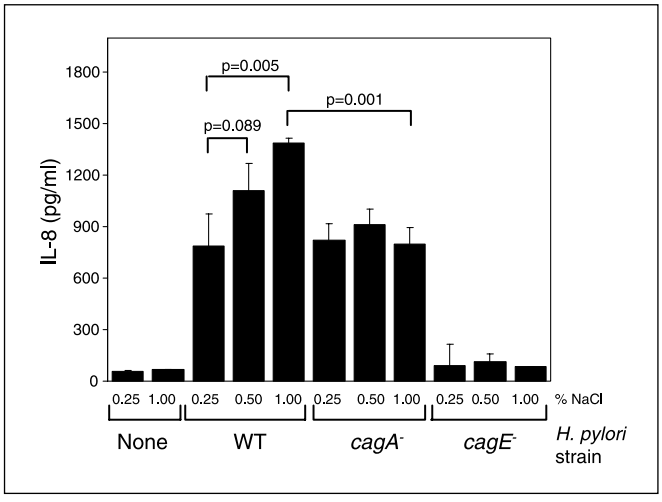

Figure 5. Effects of salt on *H. pylori*–induced IL-8 secretion. *H. pylori* 26695 WT or isogenic mutant strains were grown in broth containing the indicated NaCl concentrations for 12 h. Bacteria were then added to AGS cells and incubated for 5 h. The tissue culture media were collected, and IL-8 secreted by the AGS cells was assayed using an IL-8 cytometric bead assay as described in Materials and Methods. *Columns,* mean of triplicate samples; *bars,* SD. Statistical significance was analyzed using Student's *t* test, and *P* values for the indicated comparisons are shown.

also dependent on presence of the *cag* PAI (26, 47, 48). Our present findings provide further insight into mechanisms by which *cag*-positive *H. pylori* strains and high dietary salt intake may contribute to the development of gastric cancer. Specifically, the results of this study indicate that high salt concentrations stimulate increased expression of *H. pylori* CagA, which in turn leads to an increased amount of CagA translocated into gastric epithelial cells and an enhanced ability of *H. pylori* to alter gastric epithelial cell function. CagA may directly contribute to the pathogenesis of gastric cancer by altering signal transduction pathways in gastric epithelial cells (6). In addition, by causing enhanced secretion of the proinflammatory cytokine IL-8, CagA may indirectly contribute to the pathogenesis of gastric cancer by stimulating increased gastric mucosal inflammation (49). Thus, enhanced expression of *H. pylori* CagA in response to high salt concentrations may be an important mechanism by which a high-salt diet contributes to increased gastric cancer risk.

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