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Sodium Chloride Affects *Helicobacter pylori* Growth and Gene Expression[▽]

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Epidemiological evidence links high-salt diets and *Helicobacter pylori* infection with increased risk of developing gastric maladies. The mechanism by which elevated sodium chloride content causes these manifestations is unclear. Here we characterize the response of *H. pylori* to temporal changes in sodium chloride concentration and show that growth, cell morphology, survival, and virulence factor expression are all altered by increased salt concentration.

Helicobacter pylori is a microaerophilic, gram-negative bacterium whose presence in the gastric environment is correlated with diseases such as gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma (7). Several epidemiological studies have shown that secondary risk factors affect the occurrence of certain gastric maladies associated with *H. pylori* infection (for a review, see reference 30). In these studies, dietary sodium chloride intake emerged as a key player in enhancing the likelihood of severe disease outcomes (25, 30). In support of this, experimental animal infections have shown that there is a synergistic effect between *H. pylori* and salt in terms of disease progression (2, 8, 21, 23). Thus, the bacterium likely senses fluctuations in salt concentration due to dietary intake and alters its growth and gene expression accordingly.

The ability to adapt to changing environments is critical for bacterial survival, and thus sodium chloride and osmotic stress have been shown to affect bacterial gene expression in a number of bacterial species (for reviews, see references 12 and 32). In several of these bacteria, salt resistance involves changes in expression of outer membrane proteins, such as the transporters ProP, ProU, BetT, and BetU in *Escherichia coli* (32). For pathogenic bacteria, osmotic stress can also serve as a signal that controls expression of virulence factors. Specifically, it has been suggested that sodium chloride concentration serves as a cue for the regulation of virulence in *Listeria monocytogenes* (10, 20) and *Leptospira interrogans* (17). In keeping with this, a recent study showed that elevated salt concentrations result in alterations in expression of the virulence factor CagA in *H. pylori* strain 26695 (16). Loh et al. showed that an increased salt concentration slows *H. pylori* growth and affects expression of a large number of genes (16). Concomitant studies by our group that explored this effect in further detail are described here.

Given the fact that *H. pylori* is adept at survival in the tumultuous environment of the stomach and the fact that there is a strong epidemiological link between salt intake and *H. pylori*-induced disease, we first sought to determine the effect of increasing the salt concentration on bacterial growth and survival of *H. pylori*. Strain G27 (Table 1) liquid cultures were grown at 37°C with shaking at 100 rpm in brucella broth (BB) containing various NaCl concentrations. The salt concentrations ranged from 5 g/liter (BB5) (which is the normal salt concentration found in BB) to 25 g/liter (BB25). All liquid growth media were supplemented with fetal bovine serum (FBS) (10%) and 10 μg/ml vancomycin, and cultures were grown in gas evacuation jars under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) generated with an Anoxomat gas evacuation and replacement system (Spiral Biotech) as previously described (3). Since in our hands we often find that optical density readings do not provide an adequate assessment of bacterial numbers due to cell clumping and transition to the coccoid form at later time points, we measured growth and survival by plating on blood agar plates as previously described (9). We found that as the concentration of NaCl in the liquid growth media increased, *H. pylori* cells stopped multiplying and eventually died (Fig. 1). This effect was also evident when the bacteria were exposed to increasing levels of salt both in F-12 defined media (27) and in F-12 media supplemented with 1% FBS (data not shown).

Our data differ from the data presented by Loh et al., who found that similar salt concentrations slowed the growth of strain 26695 but did not have a bactericidal effect on the *H. pylori* cells (16). While the difference could be attributed to the use of slightly different medium formulations and growth conditions, we wondered if strain-specific differences in salt resistance between G27 and 26695 were responsible for this phenotype. To assess strain-to-strain variability in the ability to grow in the presence of elevated concentrations of NaCl, we decided to directly compare G27 and 26695 (Table 1). The strains were first grown in liquid BB5 overnight and then serially diluted and plated side by side on clear agar-based medium mimicking the composition of the liquid medium (BB supplemented with 1.2% Agar-agar, 10% FBS, and a standard *H. pylori* antibiotic mixture [9]). Plates contained the normal concentration of NaCl (5 g/liter) or 10, 13, 15, or 17 g/liter of NaCl. Salt sensitivity was assessed by enumerating single colonies after growth for 7 to 10 days in the conditions described above. The data are expressed as percentages of survival relative to the growth on medium containing the normal salt

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description[a]	Reference
Plasmids		
pTM117	Modified pHP666 including *E. coli* origin of replication, *aphA-3* cassette (Kan^r), multiple cloning site, and promoterless *gfpmut3*	3
pDSM196	pTM117 *cagA* promoter::*gfpmut3* fusion	This study
pDSM463	pTM117 *ureA* promoter::*gfpmut3* fusion	This study
pDSM223	pTM117 *vacA* promoter::*gfpmut3* fusion	This study
pDSM134	pGEM-T Easy::internal fragment of *ureA* for riboprobe generation	This study
pDSM393	pGEM-T Easy::internal fragment of *cagA* for riboprobe generation	This study
pDSM394	pGEM-T Easy::internal fragment of *vacA* for riboprobe generation	This study
H. pylori strains		
G27	Wild-type *H. pylori*	5
26695	Wild-type *H. pylori*	29
J99	Wild-type *H. pylori*	1
HPAG1	Wild-type *H. pylori*	22
SS1	Wild-type *H. pylori*	14
J166	Monkey-colonizing strain, isolate 316-3.4	J. Solnick lab collection
K6	Clinical isolate from a 45-year-old female gastric cancer patient	J. Cha and I. Chung lab collection
K112	Clinical isolate from a 57-year-old male gastritis patient	J. Cha and I. Chung lab collection
DSM195	G27(pDSM196), Kan^r (25 μg/ml)	This study
DSM215	G27(pTM117), Kan^r (25 μg/ml)	3
DSM241	G27(pDSM223), Kan^r (25 μg/ml)	This study
DSM464	G27(pDSM463), Kan^r (25 μg/ml)	This study

[a] Relevant antibiotic concentrations are indicated in parentheses.

concentration. As shown in Fig. 2A and B, strain 26695 was able to grow in the presence of 15 g/liter NaCl, while strain G27 could not. This shows that 26695 is more resistant to an increased salt concentration than G27. This fact may partially explain why no bactericidal effect of salt was observed in the study of Loh et al. (16). We next wondered whether there was variation in the level of salt resistance in other *H. pylori* strains. To study this, we examined strains J99, HPAG1, SS1, J166, and B128, as well as K6 and K112, which are two recent Korean clinical isolates (Table 1). We found a wide range of sensitivity to increased salt concentrations (Fig. 2C). Some strains (HPAG1, J99, B128, and K112) exhibited virtually no decrease in viable counts at salt concentrations that completely inhibited the growth of or killed other *H. pylori* strains (J166, SS1, and K6). This variation may imply that different *H. pylori* strains have evolved to withstand different NaCl pressures that could arise from specific host dietary habits. The strain difference in sensitivity to salt is particularly interesting when it is considered in the context of the epidemiological findings concerning salt and *H. pylori* infection; the salt resistance phenotype of the

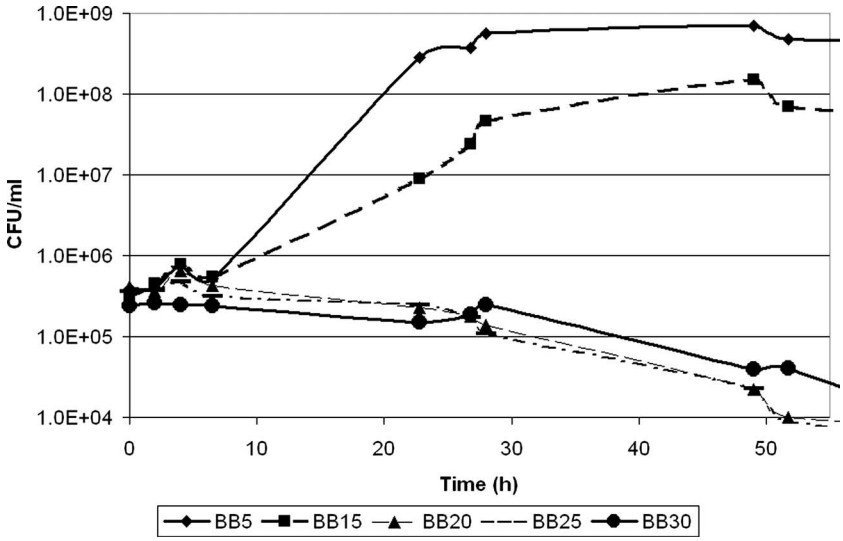

FIG. 1. Growth and survival of *H. pylori* G27 in liquid cultures containing different concentrations of NaCl. A broth-grown, overnight culture of *H. pylori* G27 was diluted into fresh growth media containing different concentrations of NaCl (BB5, BB15, BB20, BB25, and BB30). Growth and survival were monitored by plating.

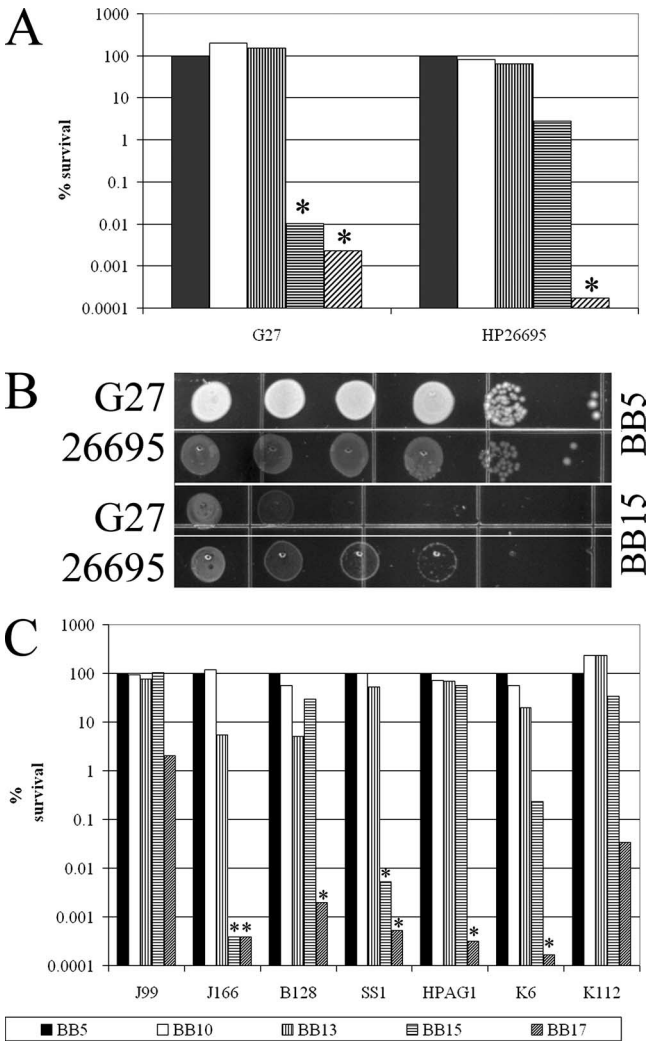

FIG. 2. Relative NaCl sensitivities of various *H. pylori* strains. (A) *H. pylori* cultures of strains G27 and 26695 were grown in liquid BB5. The numbers of starting cells were normalized by using optical density at 600 nm. The relative salt sensitivity was assessed by serial dilution and plating on BB-based agar plates containing increasing concentrations of salt. CFU were enumerated, and the percentages of survival relative to the number of CFU on the BB5 plates are presented. (B) Relative growth of *H. pylori* strains G27 and 26695 on BB5 and BB15 plates. Serial dilutions of cultures normalized using optical density at 600 nm were plated side by side on BB5 and BB15. (C) *H. pylori* strains J99, J166, B128, SS1, HPAG1, K6, and K112 were examined as described above. An asterisk indicates that no single colonies were detected, and the bars below the asterisks indicate the limits of detection at the concentrations used. The results are representative of multiple biological repeats.

infecting strain may be important for determining the disease outcome.

Since *H. pylori* has been shown to modulate virulence factor expression in response to low pH, iron limitation, and other stressful environments (18, 19), we wondered if an increased salt concentration would affect expression of the virulence factors *cagA* (HP0527), *vacA* (HP0887), and *ureA* (HP0073) (4). To determine if there was any effect on expression of any of these three genes, transcriptional fusions of each gene to the promoterless *gfpmut3* carried on pTM117 (3) were made. The transcriptional fusions were constructed by amplification of the predicted promoters of *ureA* and *vacA* using primers ureA_promoter_F and ureA_promoter_R and primers VAF1 and VAR1, respectively (Table 2). To facilitate directional ligation into pTM117, primers ureA_promoter_F and VAF1 have KpnI restriction sites at the 5′ end, and primers ureA_promoter_R and VAR1 have XbaI restriction sites at the 3′ end. In a similar manner, the *cagA* promoter was amplified using primers cagA_promoter_F and cagA_promoter_R, which have SacII and BamHI restriction sites. The reporter plasmids constructed were moved into wild-type strain G27 by natural transformation, and transformants were selected on plates containing 25 μg/ml kanamycin.

Strains DSM195, DSM464, and DSM241 (Table 1) were grown as described above in BB5 and BB15 for 16 to 20 h. At this point, cells were harvested by centrifugation and resuspended in phosphate-buffered saline (pH 7.0), and 100,000 events were analyzed using a Beckman Coulter Epics XL-MCL flow cytometer with a laser setting of 750 V. The resulting data were analyzed using WinList 3D, version 6.0 (Verity Software House). We found that the level of green fluorescent protein (GFP) expression was significantly elevated for both the *ureA* and *vacA* reporter strains but only moderately increased for the *cagA* reporter strain (Fig. 3A and data not shown). Interestingly, we observed a marked change in the forward scatter of each of the *H. pylori* strains that were grown in BB15 (Fig. 3B and data not shown). Since forward scatter is a relative indication of cell size and shape (6), we concluded that salt might have affected the morphology of the *H. pylori* cells. Thus, observed changes in the level of GFP expression may be influenced by changes in cell size or shape and not solely attributed to true changes in transcription. We therefore decided to monitor expression of the genes directly using RNase protection assays (RPAs). We also investigated alterations in cell morphology arising from exposure to NaCl.

RPAs were conducted with RNA from overnight *H. pylori* G27 cultures grown in BB5, which had been harvested by centrifugation and then exposed to a salt shock in BB25, whose salt concentration completely halts *H. pylori* growth but does not result in immediate cell death (Fig. 1). Cells were harvested for RNA isolation at 0, 15, 30, and 60 min after resuspension. The RNA was extracted as previously described (28), and 1 or 2 μg of RNA was used in each RPA reaction using riboprobes for *cagA*, *ureA*, and *vacA*. For each gene, the riboprobe template was generated by PCR using the primer pairs listed in Table 2. Radiolabeled riboprobes were produced, and the RPAs were performed as previously described (9). *ureA* and *cagA* showed no change in expression in response to salt shock (Fig. 4C and data not shown). However, as shown in Fig. 4A and B, expression of the *vacA* transcript was significantly increased upon exposure to salt. These results differ from the results of Loh et al., who found that the salt concentration affected expression of *cagA* but not expression of *vacA* in *H. pylori* strain 26695 (16). To determine whether salt shock resulted in strain-specific differences in gene expression in G27 and 26695, we repeated our experiment using our media as well as the sulfite-free media used by Loh et al. As shown in Fig. 4A and B, we observed strain-specific differences in response to salt shock; expression of the *vacA* transcript in-

TABLE 2. Primers used in this study

Primer (restriction endonuclease)	Sequence (5′–3′)[a]	Reference
Promoter::*gfp* fusion primers		
cagA-promoter_F (SacII)	**CCGCGG**CAAATTGAAATCAATCG	This study
cagA_Promoter_R (BamHI)	**GGATCC**TACTATACCTAGTTTCATAC	This study
ureA_Promoter_F (KpnI)	**GGTACC**CAAAAACAAAACAAAATTAAGGCATA	This study
ureA_Promoter_R (XbaI)	**TCTAGA**TGGGGTGAGTTTCATCTCATT	This study
VAF1 (KpnI)	**GGTACC**GCGCCTATTTCCAATTAGGCG	This study
VAR1 (XbaI)	**TCTAGA**GGGTGCGACTTTAGACTAG	This study
RPA primers		
cagA-RPA-F	AATGTTGTCGCTTCATTTGATCCT	This study
cagA-RPA-R	GATAGGGGGTTGTATGATATTTTC	This study
vacA-RPA-F	CACACCGCAAAATCAATCGCC	This study
vacA-RPA-R	CCATTGCCCGCCTTTGACCC	This study
ureA-RPA-F	CGGATGATGTGATGGATGG	This study
ureA-RPA-R	TTCTACGGATTTTTCTTCG	This study
Sequencing primer		
apha3-2	CGGTGATATTCTCATTTTAGCC	3

[a] Restriction endonuclease sites are indicated by bold type.

creased only in G27, and expression of the *cagA* transcript changed in neither strain. While the reason for the difference between our results and the results of Loh et al. is not completely clear, the findings likely reflect the fact that we used short-term salt shock to assess changes in gene expression, whereas Loh et al. assessed extended growth in the presence of salt (16). Moreover, it should be noted that Loh et al. reported that they found strain-specific differences in the effect of salt on *cagA* expression (16); this fact, along with the strain-specific differences in survival in response to salt stress that we observed (Fig. 2C), may suggest that the specific response to salt stress of the infecting strain may be important for determining the *H. pylori*-induced disease outcome.

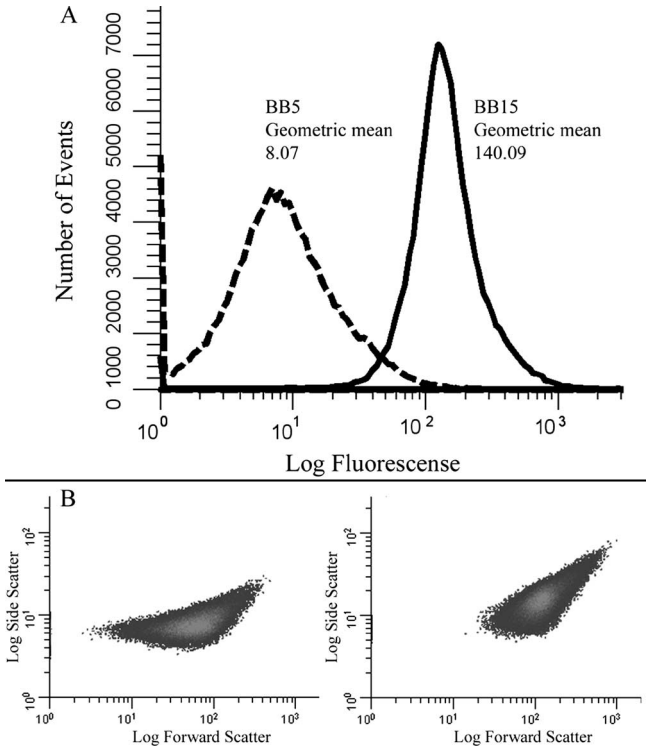

FIG. 3. Flow cytometry analysis of the *vacA* GFP transcriptional fusion. Strain DSM241 bearing a *vacA* promoter fusion was grown in either BB5 or BB15 for 20 h and analyzed by flow cytometry. (A) Fluorescence histograms for cells grown in BB5 (dashed line) and for cells grown in BB15 (solid line). Fluorescence is expressed in relative units, and the geometric mean is indicated. The data are representative of multiple independent flow analyses. (B) Density plots of side scatter versus forward scatter for DSM241. The density plot for cells grown in BB5 is on the left, and the density plot for cells grown in BB15 is on the right.

Finally, given the pronounced effect on growth of G27 that we observed (Fig. 1) and the fact that we observed large differences in the flow cytometry profiles of cells exposed to salt (Fig. 3B), we sought to determine the specific effect that salt had on the bacterial cells. To this end, electron microscopy was performed with samples of cells grown overnight in BB5 or BB15. The cells were harvested by centrifugation and then resuspended in 2% electron microscopy grade glutaraldehyde in phosphate-buffered saline for ~1 h. Subsequently, samples were washed three times with double-distilled H_2O, dried, and examined with a Philips CM100 electron microscope. As show in Fig. 5, *H. pylori* grown in BB5 exhibited a characteristic spiral shape and possessed multiple unipolar flagella. Conversely, the bacterial cells grown in BB15 showed marked morphological changes; they lost the spiral shape, became elongated, and formed chains. This suggests that salt stress results in a delay in septum formation. Interestingly, despite this significant effect on cell morphology, the cells still had unipolar flagella (Fig. 5). However, perhaps not surprisingly, there was a clear reduction in cell motility when the cells were observed by bright-field microscopy (data not shown). Similar salt-induced changes in cell morphology were also observed when we examined bacteria grown in F-12 and F-12 containing 1% FBS (data not shown). Additionally, we observed the same morphological changes for *H. pylori* strains HPAG1, J99, SS1, and J166, and a similar effect has previously been shown for strain HPK5 (26). This suggests that despite strain-specific differences in gene expression and survival of *H. pylori*, high salt concentrations have a physiological effect that results in changes in cell morphology. Similar results have also been

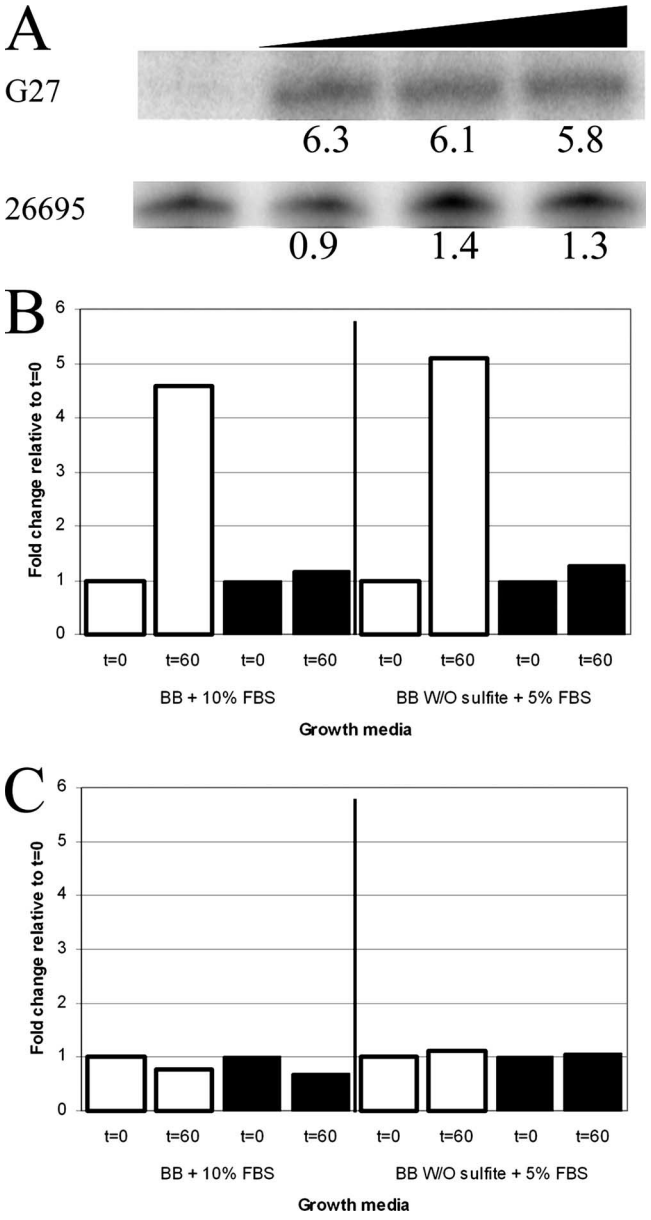

FIG. 4. Transcription of *vacA* and *cagA* following NaCl shock. (A) Logarithmically growing liquid cultures of G27 and 26695 were salt shocked by replacing BB5 with BB25. Samples were taken at 0, 15, 30, and 60 min after the change of medium. RNA was harvested, and RPAs were conducted as described in the text. Protected bands for the *vacA* transcript for G27 and 26695 and the changes (fold) relative to the zero-time results are shown. Increasing length of the salt shock is indicated by the triangle. (B) *vacA* transcript in G27 (open bars) and 26695 (filled bars). The changes in expression at 60 min are relative to the data for zero time. Cells were grown in BB5 and shocked in BB25 or in the comparable sulfite-free BB-based media containing 5% FBS used by Loh et al. (16). (C) *cagA* transcript in G27 (open bars) and 26695 (filled bars). The changes in expression at 60 min are relative to the data for zero time. Cells were grown in BB5 and shocked in BB25 or in the comparable sulfite-free BB-based media containing 5% FBS used by Loh et al. (16).

obtained for *Salmonella enterica* serovar Enteritidis and *L. monocytogenes* (11), which suggests that salt concentration may significantly impact a diverse number of bacteria. Interestingly, in recent studies to assess the ability of *H. pylori* to survive in seawater, Konishi et al. observed multiple elongated cells among the spiral and coccoid *H. pylori* forms (13). These authors suggested that these cells were bacterial contamination; however, the elongated forms closely resemble the salt stress phenotype that we observed here.

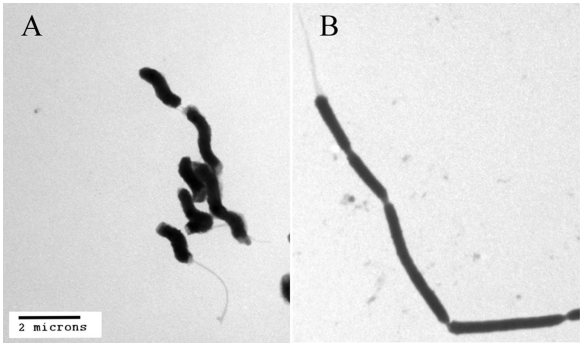

FIG. 5. Transmission electron microscopy of *H. pylori* cells grown under normal conditions or with an increased NaCl concentration. *H. pylori* cells from overnight liquid cultures in either BB5 (left panel) or BB15 (right panel) were examined using transmission electron microscopy. Bar = 2 μm.

H. pylori resides in an ever-changing environment, where pH, osmotic pressure, ionic composition, and other host-dependent factors are constantly changing. Given the fact that *H. pylori* is able to persistently colonize its human host, it must be particularly adept at responding to environmental stressors, such as osmotic or salt fluctuations. Thus, an understanding of stress adaptation is particularly important for the study of this bacterium. Mechanistically, other bacteria have been shown to utilize typical response regulatory proteins and a stress-related sigma factor (*rpoS* or σ^{38}) in adaptation to osmotic or salt stress (15, 24). However, relatively few response regulators and two-component systems have been described for *H. pylori* (31), and no *rpoS* homologue appears to be encoded in the *H. pylori* genome. Based on these limitations, it is possible that alternative effects on regulation may involve DNA supercoiling (33) or forms of posttranscriptional regulation. This mechanism has also been suggested by Wang et al. to be important for regulation of antioxidant proteins (31). Regardless, adaptation to the tumultuous environment of the stomach requires the ability to sense and respond to the myriad of environmental cues in a timely manner. Diet composition has been shown to be one factor that contributes to the outcome of *H. pylori* infection. Here we show that *H. pylori* responds to changes in the concentration of NaCl in its environment in such a way that growth, cell morphology, survival, and virulence factor expression are all altered by increased salt concentration. Moreover, the strain-specific differences that we observed provide increasing evidence suggesting that diet and the strain of *H. pylori* with which a person is infected may greatly affect the disease outcome.

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