**Helicobacter pylori** virulence factors affecting gastric proton pump expression and acid secretion

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**HUMAN GASTRIC COLONIZATION** by the Gram-negative bacterium *Helicobacter pylori* causes chronic active gastritis that may progress to peptic ulcer disease, gastric mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma (40). A consistent feature of acute *H. pylori* infection in humans is transient hypochlorhydria that arises within days of infection and lasts for several weeks (18, 19, 28, 34, 41, 49). Antral and corpus mucosal inflammation follows the same time course and lasts for several weeks (18, 19, 28, 34, 41, 49). Antral and transient hypochlorhydria that arises within days of infection.

**Acute** *Helicobacter pylori* infection of gastric epithelial cells and human gastric biopsies represses H,K-ATPase α subunit (HKα) gene expression and inhibits acid secretion, causing transient hypochlorhydria and supporting gastric *H. pylori* colonization. Infection by *H. pylori* strains deficient in the cag pathogenicity island (cag PAI) genes *cagL*, *cagE*, or *cagM*, which do not transfer CagA into host cells or induce interleukin-8 secretion, does not inhibit HKα expression, nor does a cagA-deficient strain that induces IL-8. To test the hypothesis that virulence factors other than those mediating CagA translocation or IL-8 induction participate in HKα repression by activating NF-κB, AGS cells transfected with HKα promoter-Luc reporter constructs containing an intact or mutated NF-κB binding site were infected with wild-type *H. pylori* strain 7.13, isogenic mutants lacking cag PAI genes responsible for CagA translocation and/or IL-8 induction (cagA, cagG, cagE, cagZ, and cagB), or deficient in genes encoding two peptidoglycan hydrolases (*sIt* and *cagY*). *H. pylori*-induced AGS cell HKα promoter activities, translocated CagA, and IL-8 secretion were measured by luminometry, immunoblotting, and ELISA, respectively. Human gastric biopsy acid secretion was measured by microphysiometry. Taken together, the data showed that HKα repression is independent of IL-8 expression, and that CagA translocation together with *H. pylori* transglycosylases encoded by *sIt* and *cagY* participate in NF-κB-dependent HKα repression and acid inhibition. The findings are significant because *H. pylori* factors other than CagA and IL-8 repression are now implicated in transient hypochlorhydria which facilitates gastric colonization and potential triggering of epithelial progression to neoplasm.

acid secretion; *Helicobacter pylori*; proton pump; type 4 secretory system; virulence factors

facilitates gastric colonization (31) and corpus gastritis, initiate a pathological progression that may culminate in gastric cancer, understanding their mechanistic basis clarifies disease etiology and illustrates a novel and important bacterial adaptation to a hostile ecological niche.

A major virulence determinant of *H. pylori* is the cytotoxin-associated gene (cag) pathogenicity island (PAI), a 40-kb genetic locus encoding ~28 protein components of type IV secretory system (T4SS) forming pili that project from the bacterial outer membrane. *H. pylori* strains expressing T4SS genes induce secretion of the proinflammatory cytokine IL-8 in vitro (3, 24), and in vivo lead to increased mucosal IL-8 expression, mucosal inflammation, ulceration, and increased risk of gastric cancer (1, 5, 12, 37, 38). However, the relevance of cag PAI or other *H. pylori* genes and induction of IL-8 secretion to acid inhibition is still not fully understood. T4SS pili mediate *H. pylori* adherence to cell membranes, enabling delivery of virulence factor(s) into the host cell. CagE subunits with ATPase activity in the cytoplasmic part of the T4SS energize such transfer. The extracellular shaft of the T4SS incorporates the structural protein CagM and also expresses surface-bound CagL, which interacts specifically with α5β1 and other integrins on gastric epithelial cells, contributing to bacterial adherence and facilitating T4SS function (26, 55). CagA, a secreted protein encoded by cagA, the 3′-terminal gene of the cag PAI, has been extensively studied as a mediator of *H. pylori* pathogenesis and is the first reported bacterial oncprotein that acts in mammals (2, 36). CagA transferred into gastric epithelial cells through the T4SS mimics adapter proteins by interacting with host cell proteins through tyrosine phosphorylation-dependent and -independent mechanisms (20).

Gastric acid secretion is mediated by the parietal cell H,K-ATPase proton pump (48). Our studies of infected gastric epithelial AGS cells have shown that the cag PAI-encoded T4SS proteins CagL, CagM, and CagE, which encode structural T4SS components, and secreted CagA participate in repression of transfected H,K-ATPase α subunit (HKα) promoter constructs (15–17, 43–46) and that such repression is mediated by NF-κB p50 homodimer interaction with an HKα promoter NF-κB binding site (46). Notably, HKα promoter activity was also repressed, but to a lesser extent, by an *H. pylori* cagA deletion mutant (43), suggesting that bacterial factors other than Cag A may play a role in HKα repression. Recently, we reported that in vitro *H. pylori* infection of AGS cells and human gastric biopsies upregulates a microRNA (miR-1289) that represses translation of HKα mRNA in a CagA-dependent manner (57). miR-1289 upregulation was

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also dependent on another *H. pylori* virulence factor, a product of bacterial peptidoglycan (PGN) cleavage by soluble lytic transglycosylase (SLT) (7) encoded by the non-cag PAI gene *slt* (HP0645 homolog). *H. pylori* also expresses putative PGN hydrolase activity (*CagY*) encoded by the *cag* PAI gene *cagY* (HP0523 homolog) (58). The present study sought to determine the role of *H. pylori*-induced IL-8 secretion and the *H. pylori* virulence genes *slt* and *cagY* on acid inhibition. Infection of AGS cells with a series of isogenic *H. pylori* knockout mutants and measurement of transfected HKα promoter activity indicated that bacterial factors other than those mediating CagA transfer or IL-8 secretion participate in HKα gene repression.

**MATERIALS AND METHODS**

*Cells, bacteria, and reagents.* Human gastric epithelial AGS cells (ATCC, Manassas, VA) were maintained in Ham’s F12 medium containing 10% FBS as described (46). The carcinogenic *H. pylori* wild-type strain 7.13 and isogenic mutants (Table 1) were cultured on *Brucella*-agar plates at 37°C under microaerobic conditions (5% O2, 7% CO2, 88% N2). Bacterial multiplicity of infection (MOI) were calculated as described (46). All reagents were of molecular biology grade.

HKα promoter-luc reporter plasmid transfection. An HKα promoter-Luc reporter construct (HKα206) was formed by integrating 206 bp of proximal human gastric HKα 5’-flanking sequence into the luciferase reporter plasmid pGL2-Basic Vector as described (16). Point mutations at −159 bp (A>C, forward strand; T>G, reverse strand) and at −161 bp (G>A, forward strand; C>T, reverse strand) were introduced into the NF-kB1 site of the HKα construct using QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene) (46), and mutagenesis was verified by dideoxy sequencing. AGS cells were cotransfected with wild-type or mutated promoter-Luc reporter construct (HKαΔ206) and pMaxGFP for transfection efficiency control and normalization, and promoter-reporter activities were measured and normalized as described (44).

**Generation of isogenic *H. pylori* strain 7.13 mutants.** Five wild-type *H. pylori* strain 26695 DNA plasmids (gift of Dr. Rainer Haas) containing a chloramphenicol acetyltransferase (*cat*) gene cassette in place of the *cag* PAI genes *cagA, cagC, cagB, cat*, and *cagY*, respectively, were used to naturally transform wild-type *H. pylori* strain 7.13 (11). The replacement of specific *cag* PAI genes with *cat* cassettes utilized TnMax5 or TnMax5fp transposon insertion mutagenesis, yielding isogenic mutant plasmids with no polar effects on expression of downstream genes (10). Briefly, *H. pylori* strain 7.13 were grown for 2–3 days on *Brucella*-agar with 10% serum and diluted to an A550 ~0.1 in 1 ml BHI broth with 10% serum in a 24-well culture plate. After 2–3 days culture at 37°C with 5% CO2, when the culture density increased to A550 ~0.2, supercoiled DNA plasmid (1–5 μg) was added and culture continued for 12 h (37°C, 5% CO2). Culture aliquots (100 μl) were spread on *Brucella*-agar plates with 10% serum and chloramphenicol (8 μg/ml). Chloramphenicol-resistant transformants were isolated after 3–6 days incubation under microaerobic conditions (5% O2, 7% CO2, 88% N2), at 37°C and were checked for correct insertion of the cassette into the *cag* PAI by PCR. Insertion of a kanamycin resistance cassette into the BamHI site of a cloned PCR product of the full-length *slt* gene (HP0645 homolog), followed by transformation into *H. pylori* and kanamycin selection, yielded a Δ*slt* mutant, confirmed to be nonpolar by equivalent expression of the downstream gene HP0646 in both wild-type 7.13 *H. pylori* and the Δ*slt* mutant. The resulting isogenic mutants are shown in Table 1.

**Human gastric biopsies.** Gastric endoscopic biopsies were acquired from patients who provided written informed consent (21–60 years old) to undergo esophagogastroduodenoscopy or endoscopic ultrasound at the MUSC Digestive Disease Center (IRB-approved protocol HR16941). Exclusion criteria included patients with positive urea breath and CLO tests. Four endoscopic biopsies (6–42 mg each) per patient were obtained from normal-appearing corpus mucosa on the greater curvature of the stomach. Corpus mucosa, unlike antral mucosa, is responsible for physiological acid secretion by virtue of parietal cell expression of H,K-ATPase, the focus of this study. Single biopsies were incubated in individual wells of 96 well culture plates with F12 culture medium (100 μl, 1 h, 37°C), infected for varying periods of time with 24 h cultures of *H. pylori* (1–2 × 105 bacteria/ml wet weight biopsy), and then rinsed 3 × with F12 medium. Same-patient biopsies incubated with F12 medium alone served as mock-infection controls.

**Biopsy acid secretion.** Gastric biopsies were infected in vitro with *H. pylori* (1–2 × 105 bacteria/ml wet weight biopsy, 24 h), and then treated for 30 min with 5-(N-ethyl-N-isopropyl) amiloride (EIPA; 150 μM) to block Na+/H+ exchanger activity. Biopsies were divided into four parts along the mucosal-serosal axis, distributed into wells of a 24-well XF24 culture plate thermostatted to 37°C, and overlaid with pyruvate- and bicarbonate-free DMEM (600 μl; Meditech, Manassas VA). The wells had been pretreated overnight with Cell-Tak (1 μl; BD Biosciences, Bedford, MA) to facilitate adhesion of biopsies. After 15 min acclimatization, measurement of gastric biopsy medium acidification was initiated using an XF24 Extracellular Flux Analyzer (Seahorse Biosciences, Boston, MA). Automated placement of fiber-optic pH-sensitive fluorescent hydrogel probes within ~300 μm of the biopsies created a “virtual chamber” enclosing ~28 μl of medium immediately overlaying a biopsy. Measurements of [H+] in this limited diffusion region were carried out in six 10-min cycles, each cycle involving probe retraction and vibration for 4 min to mix and reequilibrate formerly enclosed medium with bulk medium, a 3 min pause, and [H+]−dependent fluorescence measurement for 3 min. The rate of medium acidification was calculated from the slope of change in [H+], pH measurements were made without significant depression of oxygen tension or medium acidification, achieving microphysiometer-like sensitivity. Constitutive H+ secretion was measured for ~30 min before programmed remote injection of histamine (1 mM final concentration).

**IL-8 secretion.** IL-8 protein levels in AGS cell/H. pylori-conditioned media were measured by ELISA (R&D Systems, Minneapolis-St. Paul, MN) according to the manufacturer’s protocol.

**Immunoblotting.** AGS cells (80% confluent, serum-deprived for 15–20 h) were infected (MOI = 50, 1 h) with WT and isogenic mutant *H. pylori* strain 7.13, washed and harvested in ice-cold PBS, and centrifuged (520 g, 5 min). Cell pellets were dissolved in SDS-PAGE sample buffer and analyzed by immunoblotting as described (14) using pan-phosphotyrosine specific antibody PY99 (Santa Cruz Bio-technology, CA) (5 μg antibody/ml lysate protein). Uniformity of sample loading onto gels was confirmed by sequential probing of PVDF gel replicas with PY99 and GAPDH or β-actin specific anti-

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**Table 1. Genetic characterization of *H. pylori* strains used in this study**

<table>
<thead>
<tr>
<th><em>H. pylori</em> Strains</th>
<th>Inactivated Gene</th>
<th>Proposed Gene Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type 7.13</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Δ<em>cagA</em></td>
<td>HP0547</td>
<td>CagA oncprotein</td>
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<tr>
<td>Δ<em>cagC</em></td>
<td>HP0520</td>
<td>Unknown</td>
<td>20</td>
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<tr>
<td>Δ<em>cagB</em></td>
<td>HP0521</td>
<td>Unknown</td>
<td>10</td>
</tr>
<tr>
<td>Δ<em>cagβ</em></td>
<td>HP0524</td>
<td>Nucleoside triphosphatase coupling protein</td>
<td>10</td>
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<tr>
<td>Δ<em>cagZ</em></td>
<td>HP0526</td>
<td><em>cag</em>-specific translocation factor</td>
<td>23</td>
</tr>
<tr>
<td>Δ<em>cagY</em></td>
<td>HP0523</td>
<td>Peptidoglycan hydrolase</td>
<td>56, 58</td>
</tr>
<tr>
<td>Δ<em>slt</em></td>
<td>HP0645</td>
<td>Soluble lytic transglycosylase</td>
<td>7</td>
</tr>
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CagA translocation. A previous seminal study utilizing systematic mutagenesis of 27 individual genes in the cag PAI genes but still competent in terms of CagA phosphorylation and IL-8 secretion, respectively. These data suggest that neither CagA-nor IL-8-mediated pathways, alone or in concert, fully account for the observed impact of H. pylori on gastric acid secretion.

**NF-κB dependency of H. pylori-induced HKα repression.** To determine whether intracellular CagA alone and its phosphorylation are sufficient to repress HKα and whether NF-κB signaling plays a role in repression, AGS cells expressing wild-type HKα (HKα206) or mutated HKα promoter (HKαΔ206) were transfected with wild-type or phosphorylation-resistant (PR) CagA expression plasmids. HKαΔ206 is a Luc reporter construct in which the NF-κB binding site on the HKα promoter is rendered nonfunctional by mutation of two nucleotides (46), and the PR CagA expression plasmid contains a tyrosine > alanine mutation in the COOH-terminal EPIYA motif. Intracellular expression of CagA was confirmed by immunoblotting (Fig. 2, inset), and was associated with significant repression of wild-type HKα promoter activity (Fig. 2). Mutation of the HKα promoter NF-κB binding site partially reduced CagA-mediated HKα promoter repression, indicating that CagA activates mobilization and nuclear targeting of NF-κB. Interestingly, wild-type CagA expression repressed HKα promoter activity significantly more than PR CagA expression in infected cells. Wild-type infection inhibited transfected HKα promoter activity by ~80%, but ΔcagZ and Δcage infections inhibited HKα by only 50%, comparable to HKα inhibition exerted by ΔcagA. Δcage infection induced ~50% less IL-8 secretion than wild-type H. pylori or ΔcagZ, HKα promoter activity and IL-8 secretion were unaffected by ΔcagZ mutant infection. Phospho-CagA was detected only in cells infected with wild-type H. pylori and ΔcagZ and Δcage. Two mutants that did not induce phospho-CagA (ΔcagA and ΔcagZ) nevertheless inhibited HKα promoter activity by ~50%, and induced 14-fold and 5-fold induction of IL-8 secretion, respectively. These data suggest that neither CagA-nor IL-8-mediated pathways, alone or in concert, fully account for the observed impact of H. pylori on gastric acid secretion.

**RESULTS**

cag PAI gene dependency of HKα repression.** Having previously shown that H. pylori-induced repression of HKα transcription was only partially relieved when cells were infected with a cagA-deficient isogenic mutant (43), we reasoned that other bacterial factors may also exert HKα-repressive effects. Thus we examined the effects on HKα transcription of gastric epithelial cell infection with H. pylori isogenic mutants deficient in selected cag PAI genes but still competent in terms of CagA translocation. A previous seminal study utilizing systematic mutagenesis of 27 individual genes in the cag PAI of strain 26695 H. pylori and subsequent infection of gastric epithelial cells in vitro had revealed that 12 cag PAI genes were required for induction of IL-8 secretion, and 9 of these genes were also required for CagA transfer into host cells (10). We selected four strain 26695 cag PAI gene knockout strains that retained both CagA phosphorylation and IL-8 secretion competence to generate corresponding H. pylori mutants in the 7.13 strain background (ΔcagG, ΔcagE, ΔcagB, and ΔcagZ; Table 1). Figure 1 shows constitutive HKα promoter activity (corresponding to ~5500 RLU) and IL-8 secretion (~200 pg/ml) in mock-infected cells and corresponding data for H. pylori-infected cells. Wild-type infection inhibited transfected HKα promoter activity by ~80%, but ΔcagZ and Δcage infections inhibited HKα by only 50%, comparable to HKα inhibition exerted by ΔcagA. Δcage infection induced ~50% less IL-8 secretion than wild-type H. pylori or ΔcagZ. HKα promoter activity and IL-8 secretion were unaffected by ΔcagZ mutant infection. Phospho-CagA was detected only in cells infected with wild-type H. pylori and ΔcagZ and Δcage.
pression; both repressive effects were sensitive to NF-κB binding site mutation. These data suggest that although phosphorylated and nonphosphorylated CagA both signal to NF-κB, the latter CagA does so more efficiently than the former.

To determine whether \textit{H. pylori}-induced HKα repression was mediated by NF-κB, whether or not cagA was delivered into host cells, AGS cells expressing wild-type HKα206 or HKαΔ206 Luc reporter constructs were infected (6 h, MOI = 50) with wild-type or Δcagγ, Δcage, or Δcagβ \textit{H. pylori} mutants, and HKα promoter activity was measured as before. As shown in Fig. 3, HKα repression induced by infection of transfected cells with 7.13 wild-type or Δcagγ, Δcage, or Δcagβ isogenic mutants was significantly or completely abrogated by the presence of a nonfunctional NF-κB binding site mutation. These data suggest that although phosphorylation; both repressive effects were sensitive to NF-κB binding site mutation. These data suggest that although phosphorylated and nonphosphorylated CagA both signal to NF-κB, the latter CagA does so more efficiently than the former.

Role of \textit{slt} and \textit{cag} genes in HKα repression. Bacterial soluble lytic transglycosylase (SLT) activity was previously shown to signal to host intracellular nucleotide-oligomerization domain (NOD)1 receptor with subsequent NF-κB activation (54). Additionally, the \textit{H. pylori} cag PAI gene cagγ (HP0523) has been reported to express PGN hydrolase activity, generating GM-tripeptide (58). To determine whether \textit{slt} and/or cagγ gene products repress HKα gene transcription, AGS cells expressing HKα206 or HKαΔ206 were infected (6 h, MOI = 50) with wild-type \textit{H. pylori}, Δslt, Δcagγ, or Δslt/Δcagγ \textit{H. pylori} isogenic mutants. As shown in Fig. 4A, Δcagγ and Δslt isogenic mutants repressed HKα promoter activity by ∼75%, the same degree of repression exerted by wild-type \textit{H. pylori}. In contrast, in AGS cells infected with the double mutant Δslt/Δcagγ, HKα promoter activity was repressed by only 35%, suggesting some degree of participation in HKα repression by cooperative SLT and Cagγ secretory activity. IL-8 secretion into culture medium was markedly stimulated by wild-type \textit{H. pylori} infection of AGS cells, and was equally stimulated by Δcagγ, Δslt, and Δslt/Δcagγ isogenic mutants (Fig. 4A), indicating that \textit{H. pylori}-induced IL-8 secretion is not dependent on bacterial Cagγ or SLT expression. As shown by phospho-CagA immunoblotting of infected AGS cells (Fig. 4B), Δslt, Δcagγ, and Δslt/Δcagγ isogenic
mutants remained capable of CagA transfer into the cells. When transfected AGS cells were separated from bacteria by Transwell filters, HKα promoter activity was unaffected by infection with Δslt, Δcagγ, or Δslt/Δcagγ isogenic mutants (data not shown). As shown in Fig. 4C, AGS cells expressing HKαΔ206 infected with Δslt or Δcagγ isogenic mutants showed significantly less repression of HKα promoter activity than observed with cells expressing wild-type HKα promoter.

Since both SLT and Cagγ give rise to N-acetyl-glucosamine-N-acetyl-muramic acid-l-alanine-n-glutamate-mesodiaminopimelic acid (GM-3), we investigated the potential role of this PGN in modulating HKα transcription. HKα206 or HKαΔ206-transfected AGS cells were treated with the GM-3 mimetic Tri-DAP (MurNac-l-Ala-γ-p-Glu-mDAP), a diaminopimelic acid-containing muramyl tripeptide NOD1 receptor agonist. Tri-Lys, a peptide found in the PGN of Gram-positive bacteria that is not recognized by NOD1 receptor, was used as a negative control for Tri-DAP. Tri-DAP dose-dependently repressed HKα206 promoter-Luc reporter activity (Fig. 5A), and HKα promoter activity in Tri-DAP-treated cells (10 μg/ml, 6 h) was repressed by ~50% compared with control; Tri-Lys had no effect on HKα promoter activity (Fig. 5B). The repressive effect of Tri-DAP was significantly reduced (P = 0.02) by mutation of the HKα NF-κB site (Fig. 5B). Taken together, these data indicate that cooperative secretory activity of both SLT and Cagγ is involved in cell contact-dependent, NOD1-mediated, and NF-κB-dependent repression of HKα promoter activity.

Role of slt and cagγ genes in physiological acid secretion. To assess the contribution of H. pylori slt and cagγ genes to functional regulation of H,K-ATPase in terms of physiological acid secretion, human gastric corpus biopsies were infected (MOI ~ 50, 24 h) with wild-type 7.13 H. pylori, Δslt, or Δcagγ isogenic mutant strains. The biopsies were then treated with EIPA (150 μM, 30 min), and biopsy medium [H+] was recorded continuously for 3 min at intervals of 7 min. After 15 min, histamine (1 mM final) or vehicle alone was added to some biopsies. The slopes of the initial rates of change of extracellular pH were transformed for buffer capacity to yield the proton production rate (PPR: pmol H+/min) (43). Urease-induced ammonia production factors into the measured PPR, as does CO2 production during substrate oxidation (33). Both processes contribute to measured PPR in wild-type and mutant H. pylori infections alike, and so changes in PPR following infection can be ascribed to changes in parietal cell proton secretion (43). As shown in Fig. 6A, mock-infected biopsies (●) maintained a stable constitutive PPR of ~180 pmol H+/min for 15 min. Histamine addition to mock-infected biopsies (●) increased PPR to ~220 pmol H+/min within 10 min, and this rate of acid secretion was maintained over the next 30 min. Pretreatment of biopsies with 50 μM SCH28080 for 30 min abrogated histamine-stimulated medium acidification (not shown), confirming mechanistic involvement of biopsy H,K-ATPase activity in this acidification. In contrast, wild-type H. pylori-infected biopsies (▲) showed significantly attenuated PPR of ~75 pmol H+/min, and histamine addition caused minimal change in PPR. Biopsies infected with the Δslt isogenic mutant (■) exhibited an intermediate relatively stable PPR of ~140 pmol H+/min throughout the experimental time-course with no sensitivity to added histamine. Since wild-type H. pylori and Δslt isogenic mutant both express CagA, these data are consistent with SLT acting synergistically with CagA in parietal cells to suppress acid secretion. In contrast, as shown in Fig. 6B, the acid secretory response in biopsies infected with the Δcagγ isogenic mutant was more attenuated than that observed in biopsies infected with wild-type H. pylori, although both wild type-infected and Δcagγ-infected biopsies had reduced constitutive PPR compared with mock-infected controls, and histamine addition failed to stimulate extracellular acidification. Since wild-type H. pylori and Δcagγ isogenic mutant both express CagA, the data are consistent with products of the putative PGN hydrolase activity expressed by the cagPAI gene cagγ countering to some extent CagA-driven inhibition of acid secretion. Overall, microphysiometric data confirm our previous report of inhibition of parietal cell H,K-ATPase-mediated acid secretion as a physiological consequence of H. pylori infection (43), and additionally implicate H. pylori soluble lytic transglycosylase activity expressed by the slt gene in the form of GM-3 tripeptide as a mechanistic intermediary in the inhibitory pathway.
cagA and slt genes work in concert to repress HKα transcription. Given our findings that CagA and GM-3 independently promote repression of HKα transcription, we reasoned that concurrent deletion of both the cagA and the slt genes would deprive *H. pylori* of its acid-suppressive capabilities. Accordingly, AGS cells transfected with the HKα206 promoter-Luc reporter construct were infected (6 h, MOI = 50) with an *H. pylori* double isogenic mutant deficient in both genes. As shown in Fig. 7, HKα promoter activity was not significantly attenuated by infection of transfected AGS cells with the double mutant, although infection with the single mutants markedly repressed HKα promoter activity. These data indicate that expression of both the cagA and the slt genes contributes to repression of HKα transcription, and together with our foregoing findings of NF-κB participation in this repression, strongly suggest that CagA and GM-3 tripeptide

![Graph](image_url)

Table 2. Experimental end points of *H. pylori* infection of AGS cells

<table>
<thead>
<tr>
<th>Outcome of Infection*</th>
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<tbody>
<tr>
<td><strong>CagA</strong></td>
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<tr>
<td>translocation</td>
</tr>
<tr>
<td>wild-type 7.13</td>
</tr>
<tr>
<td>ΔcagA</td>
</tr>
<tr>
<td>ΔcagZ</td>
</tr>
<tr>
<td>ΔcagB</td>
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<tr>
<td>ΔcagZ</td>
</tr>
<tr>
<td>ΔcagY</td>
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<td>Δslt</td>
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*Numbers denote low (1), intermediate (2), or maximal (3) IL-8 secretion or HKα promoter repression.

Fig. 7. Repression of HKα promoter activity by wild-type *H. pylori*, ΔcagA, or Δslt *H. pylori* isogenic mutants is NF-κB-dependent. AGS cells were transfected with HKα206 or HKαΔ206 promoter-Luc reporter constructs, and independently mock-infected or infected 24 h later (MOI = 50, 6 h) with Δslt or ΔcagY *H. pylori* isogenic mutants. Data are shown as means ± SD, n = 3. *P < 0.05; **P < 0.01.
pylori, and is unaffected by ΔcagE, ΔcagM, and ΔcagL. H. pylori mutants (16, 43). These data demonstrate a role for CagA and a requirement for T4SS integrity in H. pylori-induced HKα repression. We have also shown that H. pylori stimulates AGS cell ADAM 17 activity and represses transected HKα promoter activity. Mechanistic data in that study established that acute H. pylori infection causes CagL to dissociate ADAM 17 from α5β3 integrin, activating ADAM 17-dependent, NF-κB-mediated repression of HKα promoter (42). The mechanisms underlying H. pylori-induced hyphochlorhydria include inhibition of H.K-ATPase α-subunit (HKα) gene expression by ERK 1/2-mediated NF-κB p50 subunit homodimer binding to HKα promoter (46). We recently reviewed the mechanistic evidence for H. pylori-induced acid secretary inhibition, including evidence that acute H. pylori-induced acid inhibition is not caused by parietal cell ablation, neutrophil IL-1β secretion, or H. pylori vacuolating toxin (VacA) (48). More recently, we reported that H. pylori infection upregulates gastric epithelial cell microRNA (miR-1289), that CagA and bacterial soluble lytic transglycosylase (SLT) (54) are implicated in HKα-specific miR-1289 upregulation, and that miR-1289 with a highly-conserved HKα distal UTR binding site plays a role in repressing HKα mRNA translation (57).

The present study investigated the role of selected cag and non-cag PAI genes in H. pylori-induced IL-8 secretion and transected HKα promoter activity, with a view to establishing their participation in H. pylori-induced hyphochlorhydria. Cag PAI1 H. pylori strains have been characterized as high (>2,000 pg/ml) or low (<500 pg/ml) IL-8 inducers, depending on specific COOH-terminal sequence variations in CagA (3). Our finding that isogenic ΔcagA mutant induced significantly less IL-8 than wild-type infection suggests that H. pylori strain 7.13 is a high IL-8 inducer strain and confirms CagA participation in IL-8 secretion. High IL-8 inducer H. pylori strains induce IL-8 secretion by activation of a Ras > Raf > MEK > ERK > NF-κB signaling pathway (3), and neither Shp-2 tyrosine phosphatase, a binding partner of CagA (22), nor c-Met, nor MAP kinases p38 and c-Jun, nor PKC are required for signaling to IL-8 secretion. We have shown that NF-κB-mediated transcriptional regulation of HKα promoter activity is dependent on a functional ERK1/2 signaling pathway, and that neither p39 nor the JNK signaling pathways are involved in NF-κB-mediated inhibition of HKα promoter activity (46). Our findings here that the highest levels of H. pylori-induced IL-8 secretion coexist with maximal, intermediate or minimal stimulation of HKα promoter activity, depending on specific cag PAI or non-cag PAI gene knockouts, and that both high (1,500 pg/ml) and intermediate (700 pg/ml) levels of IL-8 secretion induced by Δcagζ and Δcag mutants, respectively, were accompanied by comparable HKα promoter activity, not significantly different from that induced by ΔcagA mutant infection, indicate that the IL-8 pathway per se plays no role in HKα transcription. The data confirm a previous report that cagζ and cagε gene products are unnecessary for IL-8 induction and that the cag PAI does not encode a secreted IL-8 inducing effector protein (10).

Notably, AGS cell infection with ΔcagB mutant, which did not translocate CagA, nonetheless repressed constitutive HKα promoter activity by ~60%, comparable to the repression exerted by the ΔcagA mutant. Yeast two-hybrid and immuno-precipitation pull-down assays have shown that Cagβ interacts with the Cag-specific translocation factor CagZ (4) and with CagA, forming a putative T4SS substrate-translocation factor complex (23). Thus partial repression of HKα promoter activity by a ΔcagB mutant deficient in T4SS function suggests involvement of H. pylori virulence factor(s) that by-pass the T4SS to infiltrate host cells. Partial abrogation of wild-type H. pylori or isogenic mutant-induced HKα repression by mutation of the HKα promoter NF-κB binding site indicated that nuclear localized NF-κB was a primary effector of acid inhibition, whether CagA or other T4SS-dependent or -independent H. pylori factors are responsible for triggering NF-κB nuclear translocation. The potential involvement in HKα repression of another H. pylori-induced signaling pathway, e.g., AKT targeting by phosphatidylinositol 3-phosphate kinase (35), remains to be investigated.

Our data relating to the effects of Δslt and ΔcagY mutant infections on HKα promoter activity in AGS cells and on acid secretion by human gastric biopsies are consistent with SLT activity, and to a lesser extent CagY activity, playing a role in HKα gene repression and acid inhibition. SLT cleaves the unique diaminopimelidate-containing tripeptide GM-3 from the nonreducing ends of glycan strands constituting the cell wall PGN heteropolymer encapsulating H. pylori. GM-3 interacts with the intracellular NOD1 receptor, which in turn induces NF-κB activation and increased cell proliferation and migration (35), and inactivation of the H. pylori deacetylase PgdA decreases NOD1-dependent NF-κB activation and autophagy (52), conferring on GM-3 and NOD1 important roles in gastric carcinogenesis. The putative PGN hydrolysate activity (CagY) encoded by the cag PAI gene cagy (HP0523) has also been reported to digest H. pylori glycan strands resulting in the formation of a 1,6-anhydro bond in the MurNAc residue and degraded PGN fragments including GM-3 (56, 58). CagY expressed by H. pylori strain 26695 was reported to be essential for both CagA translocation and IL-8 secretion (10), consistent with the proposed role of PAI-encoded lytic transglycosylases in facilitating PGN penetration by the macromolecular complexes constituting the T4SS (25).

In the present study, however, human gastric mucosal biopsies infected in vitro with Δslt or ΔcagY H. pylori mutants failed to sustain the levels of histamine-induced acid secretion shown by mock-infected biopsies, indicating persistence of a functional T4SS. This finding recapitulates the wild-type H. pylori-induced acid inhibition that we reported previously (43), which was attributed to CagL and CagA signaling to NF-κB through ERK 1/2 pathways (42, 46). Infection with independent or joint slt and cagY preserved CagA phosphorylation and H. pylori-induced IL-8 secretion, consistent with H. pylori strain 7.13 being a CagA-dependent, high IL-8 inducer, and suggesting that structural and functional assembly of the T4SS in the 7.13 strain depends on lytic transglycosylases other than SLT or CagY. However, the partial abrogation of HKα promoter repression induced by the joint sltcagy deletion, or by mutation of the HKα promoter NF-κB binding site, argues for cumulative SLT and Cagγ-dependent GM-3 activation of intracellular NOD1 receptor as a significant contributor to NF-κB mobilization and nuclear localization, and thus HKα.
gene repression. This conclusion is further supported by our data demonstrating that a cell permeable, exogenous GM-3 NOD1 receptor analog (Tri-DAP), but not a Gram-positive-specific GM-3 analog (Tri-Lys), dose-dependently represses HKα promoter activity, but not in the presence of a mutated NF-κB site on the promoter. Complementary determination of wild-type or mutated HKα promoter activity following AGS cell infection with a ΔcagA/Δslt double mutant also pointed to synergistic activation of NF-κB by CagA and SLT activity; independent deletions of cagA orslt significantly repressed HKα promoter activity, while double mutant infections did not, and all repression was abolished by a dysfunctional HKα NF-κB binding site.

Involvement of the pro-carcinogenic cag PAI in acid secretory inhibition confers clinical relevance to mechanisms of H. pylori-induced hypochlorhydria. This study complements earlier findings that an intact T4SS is required for inhibition by presenting several lines of evidence that induction of host cell IL-8 secretion does not per se repress HKα gene expression, and that the translocated oncprotein CagA, together with secreted PGN, are the primary bacterial effectors of acid inhibition caused by acute H. pylori infection. The physiological relevance of such inhibition may be manifold. First, amelioration of an otherwise hostile acidic environment clearly facilitates gastric colonization by H. pylori. Second, while antral or antral-corpus transitional zone colonization is more typical, corpus and cardia may also be colonized (53). Infection of the acid-secreting corpus induces transient gastric hypochlorhydria which may predispose mucosal susceptibility to atrophic gastritis. The resulting parietal cell attrition reinforces hypochlorhydria, with consequent mucosal progression to intestinal metaplasia, dysplasia, and gastric cancer. Last, transient gastric H. pylori acid inhibition may contribute to gastrointestinal homeostasis by modulating gastrointestinal microbial composition. H. pylori-induced microbiota changes have been reported in gerbil large intestine (21), mouse stomach (27) and human stomach (30, 47). Since gut dysbiosis is associated with many diseases (39), dissection of the molecular mechanisms underlying transient H. pylori-induced hypochlorhydria may augment understanding of both the deleterious and potentially beneficial effects of gastric colonization by this microorganism.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


