**Helicobacter pylori** Induces Gastric Epithelial Cell Invasion in a c-Met and Type IV Secretion System-dependent Manner*  

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**Helicobacter pylori** interacts with gastric epithelial cells, activating signaling pathways important for carcinogenesis. In this study we examined the role of **H. pylori** on cell invasion and the molecular mechanisms underlying this process. The relevance of **H. pylori** cag pathogenicity island-encoded type IV secretion system (T4SS), CagA, and VacA for cell invasion was also investigated. We found that **H. pylori** induces AGS cell invasion in collagen type I and in Matrigel invasion assays. **H. pylori**-induced cell invasion requires the direct contact between bacteria and cancer cells. **H. pylori**-mediated cell invasion was dependent on the activation of the c-Met receptor and on increased MMP-2 and MMP-9 activity. The abrogation of the c-Met receptor using the specific NK4 inhibitor or the silencing increased MMP-2 and MMP-9 activity. The abrogation of the c-Met receptor using the specific NK4 inhibitor or the silencing increased MMP-2 and MMP-9 activity. The abrogation of the c-Met receptor using the specific NK4 inhibitor or the silencing increased MMP-2 and MMP-9 activity. 

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4 Numerous studies have shown that **H. pylori** is able to interact with gastric epithelial cells, activating signaling pathways, modifying host cellular functions, and inducing cell phenotypes important for carcinogenesis (4–7). One of the less explored cell phenotypes induced by **H. pylori** is cellular invasion. Although little is known about the mechanisms involved in this process, **H. pylori** was shown to activate tyrosine kinase receptors frequently involved in invasion-related pathways, such as the epidermal growth factor receptor (EGFR),4 Her2/Neu (ErbB-2), and c-Met (8–10). Another group of molecules associated with cancer cell invasion and influenced by **H. pylori** are matrix metalloproteinases (MMPs) (11). MMP expression and activity are frequently enhanced in tumors as compared with normal tissue (11, 12). It has been shown that **H. pylori** up-regulates the expression and activity of several MMPs, both in gastric epithelial cell lines and in the gastric mucosa (13–15).

**H. pylori** virulence factors differentially interfere with signaling pathways in gastric epithelial cells (16). One well-established **H. pylori** virulence factor is the presence of a cluster of about 30 genes, known as the **cag** pathogenicity island (**cag** PAI). The **cag** PAI encodes a type IV secretion system (T4SS), a multimolecular complex that mediates the translocation of bacterial factors into the host cell (17, 18). The T4SS translocates the CagA protein into the host cell cytoplasm, where it can stimulate cell signaling through interaction with several host proteins (6, 18, 19), such as the tyrosine kinase c-Met receptor. The intracellular interaction between CagA and c-Met induces a motogenic response in gastric epithelial cells (10).

VacA is another **H. pylori** virulence factor. This bacterial toxin with multiple activities is inserted in the host cell membrane, inducing cytoplasmic vacuolation (20, 21). In Western populations, the presence of a functional T4SS, of CagA, and VacA cytotoxicity are frequently associated with severe gastric inflammation, ulceration, and increased risk of gastric carcinoma (22–25), although the precise molecular mechanisms underlying these associations are poorly understood.

In this study our first goal was to examine the influence of **H. pylori** on epithelial gastric cancer cell invasion. After demonstrating that **H. pylori** is able to induce cell invasion in this model, we examined the molecular mechanisms underlying this process. The relevance of **H. pylori** cag pathogenicity island-encoded type IV secretion system (T4SS), CagA, and VacA for cell invasion was also investigated. We found that **H. pylori** induces AGS cell invasion in collagen type I and in Matrigel invasion assays. **H. pylori**-induced cell invasion requires the direct contact between bacteria and cancer cells. **H. pylori**-mediated cell invasion was dependent on the activation of the c-Met receptor and on increased MMP-2 and MMP-9 activity. The abrogation of the c-Met receptor using the specific NK4 inhibitor or the silencing increased MMP-2 and MMP-9 activity. The abrogation of the c-Met receptor using the specific NK4 inhibitor or the silencing increased MMP-2 and MMP-9 activity. 

**Helicobacter pylori** is a bacterial pathogen that colonizes the gastric mucosa of more than half of the human population (1). In most individuals the infection induces chronic superficial gastritis, a condition that will remain throughout life. However, in some individuals, more severe outcomes of the infection may develop, such as peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma (2). This diversity of clinical outcomes associated with **H. pylori** infection is probably a result of the interactions among host, environmental, and bacterial virulence factors (2, 3).

4 The abbreviations used are: EGFR, epidermal growth factor receptor; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; siRNA, short interfering RNA; MOI, multiplicity of infection.
this process. Finally, we also evaluated whether there were differences between strains in their ability to stimulate cell invasion, and assessed the role of the cag PAI-encoded T4SS, CagA, and VacA in this process.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—AGS cells, derived from a human gastric carcinoma, were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum, 2.5 μg/ml fungizone (Bristol-Myers Squibb, Brussels, Belgium), 200 μg/ml streptomycin, and 200 international units/ml penicillin (Invitrogen) at 37 °C, under a 5% CO₂ humidified atmosphere.

Pharmacological inhibitors PD168393, ZD1839, and LY294002, were obtained from Calbiochem (Darmstadt, Germany), AstraZeneca (Macclesfield, UK), and Sigma, respectively. NK4 was kindly provided by W. Jiang (Dept. of Surgery, University of Wales College of Medicine, Cardiff, UK). The concentrations of inhibitors used were 2 μM, 1 μM, 100 μM, and 100 ng/ml, for PD168393, ZD1839, LY294002, and NK4, respectively. These concentrations were previously shown to inhibit cell invasion induced by stimulators, such as heregulin-β1, EGF, lipochoelic acid, and HGF in AGS cells and in other cell lines (26, 27). Drug toxicity was controlled by measuring cell viability with the trypan blue dye exclusion test.

**Bacterial Strains and Growth Conditions**—Bacteria were grown in tryptic soy agar (TSA) supplemented with 5% sheep blood (BioMérieux, Marcy l’Etoile, France) and incubated for 48 h at 37 °C under a microaerophilic atmosphere. Bacterial density was estimated by the absorbance measurement at 600 nm. Heat-killed *H. pylori* was obtained by boiling during 30 min at 56 °C followed by an incubation for 10 min at 80 °C. Unless otherwise stated, experiments were performed with *H. pylori* strain 26695 (ATCC 700392, cag PAI⁺, vacA s1/m1), obtained from the American Type Culture Collection (ATCC). *H. pylori* insertion mutants with inactivation of the *cagA* (60190ΔcagA), *cagE* (60190ΔcagE) or *vacA* (60190ΔvacA) genes (14, 28) were studied together with their parental wild type strain 60190 (ATCC 49503, cag PAI⁺, vacA s1/m1). In parallel experiments, *H. pylori* strain Tx30a (ATCC 51932, cag PAI⁺, vacA s2/m2) was also used.

**Preparation of Conditioned Medium**—Conditioned medium from *H. pylori* (CM 26695) was prepared by washing three times in serum-free medium, 1 × 10⁸ bacteria growing in TSA. Bacteria were added to 1.5 ml of RPMI 1640 serum and antibiotic-free medium, and incubated on Matrigel-coated filters in the absence of cells at 37 °C, under a microaerophilic atmosphere. Control-conditioned medium (CM control) was prepared similarly, in the absence of bacteria. Conditioned media were collected after 48 h, centrifuged at 3220 × g for 2 min and passed through 0.2-μm pore-size filters (Schleicher & Schuell, Dassel, Germany) prior to test on invasion assays.

**Infection of Gastric Cells**—Prior to infection, 80% confluent AGS monolayers were washed twice in PBS and incubated overnight in serum and antibiotic free medium (Invitrogen). For infection, 48 h colonies of *H. pylori* were collected and added to cells at, unless otherwise stated, a multiplicity of infection (MOI) of 100. Cultures were maintained at 37 °C under a 5% CO₂ humidified atmosphere. Control monolayers were processed similarly in the absence of bacteria.

**Collagen Invasion Assay**—Collagen invasion assays were performed as previously described (29). Briefly, collagen gels were prepared in 6-well plates (Becton and Dickinson, Bedford, MA), using a collagen type I solution (Upstate Biotechnology, Lake Placid, NY), and polymerized overnight at 37 °C. AGS cells (1 × 10⁵) were incubated on top of the gels for 24 h at 37 °C, in the presence or absence (control) of *H. pylori*, and in some experiments with pharmacological inhibitors. Invasion was scored as the ratio between the number of invasive cells inside the gel and the total number of cells, counted in at least 12 microscopic fields with a computer-assisted inverted microscope. Cell viability was evaluated by trypan blue dye exclusion test at the end of each assay.

**Matrigel Invasion Assay**—Prior to each experiment, 24-well Matrigel-coated invasion inserts of 8-μm pore size filters (Becton and Dickinson) were introduced into 24-well plates. For re-hydration, the inner and outer compartments of the system were filled with RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotic-free, and incubated for 60 min at 37 °C. After rehydration, 5 × 10⁵ cells were incubated for 24 h at 37 °C, in the presence or absence (control) of *H. pylori* or of *H. pylori*-conditioned medium, and pharmacological inhibitors. Filters were washed in PBS, fixed in 4% paraformaldehyde, removed from the insert, and mounted in Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Invasive cells were scored in at least 25 microscopic fields (×20 objective), when DAPI-counterstained nuclei passed through the pores of the filter.

**Preparation of Cell Lysates and Immunoprecipitation**—At the end of the infection period, cells were lysed in cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 3 mM sodium vanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). To immunoprecipitate tyrosine-phosphorylated molecules, 750 μg of proteins were incubated for 2 h at 4 °C with a mouse monoclonal anti-phosphotyrosine antibody (PY-20, BD Biosciences-Transduction Laboratories, San Jose, CA). Immunocomplexes were incubated for 60 min with protein G-Sepharose beads (Amersham Biosciences, Buckinghamshire, UK), washed and eluted in sample buffer. Proteins were separated by SDS-PAGE and visualized by immunoblot analysis, using antibodies directed to the phosphorylated molecules of interest.

**Immunoblot Analysis**—After electrophoresis, proteins were transferred onto Hybond nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 4% bovine serum albumin (Sigma) in PBS + 0.5% Tween-20 (for detection of phosphorylated proteins) or with 5% nonfat milk in PBS + 0.5% Tween-20 (for overall protein detection) and incubated for 60 min with a rabbit polyclonal anti-c-Met antibody (Santa Cruz Biotechnology), a mouse monoclonal anti-α-tubulin antibody (Sigma), a rabbit polyclonal anti-MMP2 antibody (Labvision Neomarkers, Fremont, CA) or a mouse monoclonal anti-MMP-9 antibody (Calbiochem). A goat anti-rabbit (Santa Cruz Biotechnology) or a rabbit anti-mouse (Amersham Biosciences) horseradish peroxidase-conjugated secondary anti-
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bodies were used, followed by ECL detection (Amersham Biosciences). Immunoblots were quantified with the Quantity One Software (Bio-Rad).

Small Interference RNA (siRNA) Transfection—siRNAs targeting c-Met, MMP-2, or MMP-9 mRNA, previously tested for knockdown efficiency by quantitative RT-PCR, were obtained from Qiagen (Valencia, CA), and prepared according to manufacturer’s instructions. In parallel, non-silencing siRNA duplexes (Sense: 5’-UUCUGGAACGGUGUCACGU-3’ and antisense: 5’-ACGUGACACGGUUCCGAAGAA-3’) were used as negative control. Prior to transfection, 50% confluent AGS monolayers plated onto 6-well plates were washed with PBS and incubated in serum and antibiotic-free medium. For transfection with siRNAs targeting MMP-2 and MMP-9, cells were grown, also until 50% of confluency, on 6-well plates previously coated with a collagen type I solution. Cells were transiently transfected with 80 nM (for c-Met or non-silencing siRNA) or with 50 nM (for MMP-2 and MMP-9) of siRNA, using the Lipofectamine 2000 transfection reagent (Invitrogen). At the end of each transfection, putative cytotoxic effects were evaluated, analyzing cell viability by trypan blue dye exclusion test.

Zymography—To detect MMP enzymatic activity, transfected, or non-transfected AGS cells, were cultured for 48 h on top of collagen type I gels, in the presence or absence of H. pylori. 12 μg of protein from conditioned medium of such cultures were loaded on 10% SDS-PAGE containing 1 mg/ml gelatin (MMP-2 and MMP-9) or 1 mg/ml β-casein (MMP-3 and MMP-9) as substrates. Zymograms were run in Tris/glycine SDS running buffer under non-denaturing conditions. After electrophoresis, gels were washed twice in 2% Triton X-100, to remove SDS. Zymograms were subsequently incubated for 20 h at 37 °C in the appropriated MMP substrate buffer (10 mM CaCl$_2$ in 50 mM Tris-HCl, pH 7.5 for MMP-2 and MMP-9; 0.2 M NaCl, 5 mM CaCl$_2$, 1% Triton X-100 in 50 mM Tris-HCl, pH 7.4 for MMP-3 and MMP-9). Proteolytic activity was visualized as the presence of clear bands against a blue background of Coomassie Blue-stained gelatin or β-casein substrates.

Statistical Analysis—Data were analyzed with Student’s t test and were expressed as mean values of at least three independent experiments ± S.D. Differences in data values were considered significant at a p value of less than 0.05.

RESULTS

H. pylori Stimulates AGS Cell Invasion—To investigate whether H. pylori was capable of inducing invasion of gastric epithelial cells, non-invasive AGS cells were infected with H. pylori and evaluated using two well established invasion assays. H. pylori significantly stimulated the invasion of these cells into both collagen type I gels (Fig. 1A) and Matrigel-coated filters (Fig. 1B).

To test whether the proportion of bacteria:cell used in the experiments would influence the cellular phenotype, invasion was assessed using different MOIs (Fig. 1A). Because the highest MOI used (100) did not affect cellular viability, as assessed by the trypan blue dye exclusion test (data not shown), this proportion bacteria:cell was used in all further experiments.

Stimulation of AGS Cell Invasion Requires Direct Contact with H. pylori—To investigate whether viable bacteria were necessary for AGS cell invasion, cells were cultured on collagen type I gels or Matrigel-coated filters, with intact or heat-killed H. pylori. On both substrates, heat-killed bacteria were no longer able to stimulate cancer cell invasion (Fig. 1, B and C). To determine whether stimulation of invasion occurred by direct contact between H. pylori and the cells or by release of soluble bacterial pro-invasive factors by H. pylori, conditioned medium of H. pylori incubated on Matrigel-coated filters without cells, was collected, filtered, and tested in the Matrigel assay. Conditioned medium from H. pylori induced significantly lower levels of cell invasion than those observed by bacterial direct stimulation (Fig. 1B), suggesting that the contact between H. pylori, and cells is necessary to induce an invasive phenotype.

H. pylori-mediated AGS Cell Invasion Is Blocked by NK4, a c-Met Inhibitor—To assess the involvement of EGFR, ErbB-2, c-Met, and of the downstream signaling molecule phosphoinositide 3-kinase (PI3K) in H. pylori-mediated cell invasion, AGS cells were infected with H. pylori in the presence of specific inhibitors for each of these molecules, and assessed for...
ZD1839, a specific EGFR inhibitor, the most striking effect of Matrigel-coated filters, although there was an inhibitory effect such as EGF, Heregulin concentrations known to block invasion by specific stimulators, the inhibitor, was able to block cell invasion stimulated by AGS cells infected for 24 h with H. pylori and with NK4, NK4 blocked not only H. pylori-mediated AGS cell invasion, but also c-Met tyrosine phosphorylation (Figs. 2, A and B), suggesting that H. pylori-mediated AGS cell invasion requires phosphorylation of the c-Met receptor. Neither H. pylori nor NK4 affected the expression levels of c-Met.

To further confirm that c-Met is directly involved in the stimulation of host cell invasion by H. pylori, we transiently transfected AGS cells with siRNA abrogating c-Met expression (Fig. 3C). c-Met silencing was maximal 48 h after transfection, as confirmed by immunoblot analysis (data not shown). Therefore, c-Met phosphorylation levels were investigated 48 h after transfection, and invasion assays started 24 h after transfection by incubating AGS cells with H. pylori strains 26695 and 60190 on Matrigel filters for an additional 24-h period. AGS cells transfected with siRNA to c-Met were resistant to both H. pylori-induced c-Met tyrosine phosphorylation and invasion (Fig. 3, A and B, C, and D). These findings demonstrate that invasion of AGS cells is stimulated by H. pylori through a c-Met-dependent mechanism.

H. pylori-mediated Cell Invasion Requires MMP-2 and MMP-9 Activity—To investigate the participation of MMPs in H. pylori-mediated cell invasion, cells were infected with H. pylori strain 60190 for 48 h on collagen type I gels. Supernatants of AGS cells cultured for 60 min with H. pylori and with NK4, showed increased MMP-2, MMP-9, and de novo MMP-3 activity (Fig. 4, A and B).

To determine whether the induction of MMP-2 and MMP-9 activity was essential for H. pylori-mediated invasion, AGS cells growing on collagen type I gels were transiently transfected with siRNA abrogating MMP-2 or MMP-9 expression (Fig. 4C). Silencing of both MMPs was maximal 48 h after transfection, as confirmed by immunoblot analysis (data not shown). At the end of each transfection, cell viability was evaluated by trypsin blue dye exclusion test. Because more than 90% of the cells remain viable, putative cytotoxic effects were excluded. Supernatants of transfected cells infected with H. pylori showed increased MMP-2, MMP-9, and de novo MMP-3 activity (Fig. 4, A and B).

involution on collagen and Matrigel assays. On collagen type I gels, only NK4, an HGF antagonist that inhibits c-Met tyrosine phosphorylation and activity (30), was able to inhibit AGS cell invasion (Fig. 2A). The other inhibitors, although used at concentrations known to block invasion by specific stimuliators, such as EGF, Heregulin β1 (Hrgβ1) (Fig. 2A), or lithocholic acid (27) had no effect on H. pylori-mediated cell invasion. On Matrigel-coated filters, although there was an inhibitory effect associated with PD168393, a dual EGFR/ErbB2 inhibitor, and ZD1839, a specific EGFR inhibitor, the most striking effect of inhibition of cell invasion was observed with NK4 (Fig. 2B). Identical results to those obtained with H. pylori strain 26695 were also observed with H. pylori strain 60190 (data not shown). In none of the assays LY294002, a specific PI3K inhibitor, was able to block cell invasion stimulated by H. pylori (Fig. 2, A and B). These results suggest that the c-Met receptor has an important role in H. pylori-mediated cell invasion.

Stimulation of Cell Invasion by H. pylori Occurs via a c-Met-dependent Mechanism—To further explore the relationship between c-Met inhibition and H. pylori-induced AGS cell invasion, we investigated the effect of H. pylori on the tyrosine phosphorylation status of the c-Met receptor. After infection with H. pylori, cells were lysed, immunoprecipitated with an antibody against tyrosine-phosphorylated residues (PY-20), and immunoblotted with an anti-c-Met antibody. As observed in Fig. 3A, H. pylori significantly increased the tyrosine phosphorylation level of c-Met. Similar results to those obtained with H. pylori strain 26695 were observed with H. pylori 60190.

Because NK4 suppressed the invasion of AGS cells stimulated by H. pylori, we also examined the effect of this inhibitor on H. pylori-induced c-Met tyrosine phosphorylation. For that, AGS cells were cultured for 60 min with H. pylori and with NK4. NK4 blocked not only H. pylori-mediated AGS cell invasion, but also c-Met tyrosine phosphorylation (Figs. 2, A and B and 3B), suggesting that H. pylori-mediated AGS cell invasion requires phosphorylation of the c-Met receptor. Neither H. pylori nor NK4 affected the expression levels of c-Met. 

FIGURE 2. H. pylori stimulation of AGS cell invasion is blocked by NK4, a c-Met inhibitor. Invasion assays of AGS cells infected for 24 h with H. pylori 26695, with EGF or with Heregulin β1 (Hrgβ1) and with a PI3K inhibitor (LY294002, 100 μm), an EGFR/ErbB2 inhibitor (PD168393, 2 μm), an EGFR inhibitor (ZD1839, 1 μm), or an HGF antagonist (NK4, 100 ng/ml) on collagen type I gels (A) and on Matrigel-coated filters (B). Data correspond to the mean value ± S.D. and are representative of three independent experiments. *, significantly different from untreated cells; **, significantly different from cells infected with H. pylori 26695.
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cell invasion was significantly reduced (Fig. 4, C and D). This demonstrates that the activity of both MMP-2 and MMP-9 is essential for H. pylori-mediated cell invasion.

H. pylori Induces MMP-2 and MMP-9 Activity through a c-Met-dependent Mechanism—Because H. pylori stimulates cell invasion via the c-Met receptor we sought to determine whether c-Met was involved in the increase of the gelatinolytic and caseinolytic activities elicited by H. pylori. Therefore, AGS cells transfected with siRNA to c-Met and infected with H. pylori 60190 were tested for MMP activity. After abrogating c-Met expression, H. pylori was no longer able to increase MMP-2 and MMP-9 activity (Fig. 4A). In contrast, MMP-3 activity was not affected by c-Met silencing (Fig. 4B). These results show that the increase in MMP-2 and MMP-9 activity occurs through a c-Met-dependent mechanism.

H. pylori-mediated AGS Cell Invasion, c-Met Tyrosine Phosphorylation, and MMP-2 and MMP-9 Activity Are Dependent on a Functional Bacterial T4SS—To assess the role of the bacterial cag PAI-encoded T4SS, CagA, and VacA in AGS cell invasion, a series of experiments was performed using different H. pylori strains. The vacuolating, cag PAI-positive, and cagA-positive H. pylori strains 26695 and 60190 were used. In addition, mutants for cagE (that do not express a functional T4SS), cagA (that do not express CagA), and vacA (that do not express VacA), were studied together with their parental wild-type strain 60190. Furthermore, we used the naturally occurring, non-vacuolating, and cag PAI-negative Tx30a strain.

AGS cells were infected with the different H. pylori strains in the Matrigel invasion assay and assessed for the number of invasive cells (Fig. 5A). As observed for strain 26695, strain 60190 was also able to stimulate cell invasion. When these two strains were compared with H. pylori Tx30a, we observed that the latter was unable to induce an invasive phenotype in AGS cells.

To further define the relative contributions of the cag PAI-encoded T4SS, CagA, and VacA to cell invasion, we compared the effects of the wild-type strain 60190 with those of the respective mutants. The vacA mutant induced similar levels of invasion to those of the wild-type strain, indicating that VacA is not involved in H. pylori-mediated cell invasion. The cagA mutant induced significantly lower levels of invasion than its parental wild-type strain, indicating a role for CagA in cell invasion. The cagE mutant also induced lower levels of invasion than the wild-type 60190 strain and than the cagA mutant, indicating that a functional T4SS is required for H. pylori-mediated cell invasion, and suggesting that bacterial molecules other than CagA are involved in this process. Strain Tx30a did not stimulate AGS cell invasion, further suggesting that cell invasion is dependent on the cag PAI-encoded T4SS (Fig. 5A).

c-Met tyrosine phosphorylation levels of AGS cells infected with the same panel of H. pylori strains were also studied. H. pylori strains 26695, 60190, and the vacA mutant, all containing a...
with a functional T4SS, we also assessed their ability to induce MMP-2 and MMP-9. Gelatinolytic activity was maximal in supernatants of cultures with \textit{H. pylori} strain 60190, whereas supernatants obtained from cultures with the \textit{cagA} and \textit{cagE} mutants, that were also less effective on c-Met tyrosine phosphorylation and cell invasion, induced lower levels of MMP-2 and MMP-9 activity (Fig. 4A). \textit{H. pylori}-mediated MMP-3 activity was not affected in cells cultured with the \textit{cagA} mutant, but was abolished by infection with the \textit{cagE} mutant (Fig. 4B).

Altogether these results show that \textit{H. pylori} induces cell invasion and MMP-2 and MMP-9 activity through a mechanism that depends on both host c-Met tyrosine phosphorylation and on a functional \textit{H. pylori} T4SS.

**DISCUSSION**

We have demonstrated that \textit{H. pylori} infection causes \textit{in vitro} invasion of gastric epithelial cells, in a bacterial T4SS-dependent manner, involving the phosphorylation of the host cell c-Met receptor, and the activation of MMP-2 and MMP-9. The collagen type I and the Matrigel invasion assays used in this study are by no means the equivalent of invasion in the \textit{in vivo} situation. We and others have repeatedly emphasized that all elements of the ecosystem need to be taken into account when analyzing the phenotype of cells. Still, the \textit{in vitro} assays we used contain some of the elements of the \textit{in vivo} ecosystem of invasion, such as collagen type I, collagen type IV, laminin, and fibronectin. Furthermore, these assays also cover some of the cellular activities implicated in invasion, like three-dimensional migration through matrices, and proteolysis. Our choice for these experimental systems was based on the fact that they: (a) allow the precise localization of cells with an error less than 0.1 μm; (b) provide a numerical evaluation; and (c) are reproducible when evaluated by the same or different observers (31, 32). Moreover, both collagen type I and Matrigel assays have been useful in finding molecules and pathways relevant for invasion in human cancer (29, 33, 34).
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One of the most important findings of this study is that the c-Met receptor is involved in H. pylori-mediated gastric cell invasion. c-Met is a receptor tyrosine kinase with an important and well-documented participation in cell invasion (35–37). Upon binding of HGF, c-Met undergoes conformational changes leading to phosphorylation of specific tyrosine residues at the receptor intracellular domains, which act as docking sites for downstream signaling molecules. This results in the phosphorylation and binding of adaptor proteins and activation of signal transducers such as PI3K, eventually leading to cell phosphorylation and binding of adaptor proteins and activation sites for downstream signaling molecules. This results in the dures at the receptor intracellular domains, which act as docking

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One of the most important findings of this study is that the c-Met receptor is involved in H. pylori-mediated gastric cell invasion. c-Met is a receptor tyrosine kinase with an important and well-documented participation in cell invasion (35–37). Upon binding of HGF, c-Met undergoes conformational changes leading to phosphorylation of specific tyrosine residues at the receptor intracellular domains, which act as docking sites for downstream signaling molecules. This results in the phosphorylation and binding of adaptor proteins and activation of signal transducers such as PI3K, eventually leading to cell invasion (37). It has been recently reported that H. pylori CagA protein targets and phosphorylates the c-Met receptor, enhancing the motogenic response (10). In the present study, we show that in cells treated with the c-Met inhibitor NK4 or transfected with siRNA to c-Met, H. pylori was not able to stimulate cell invasion, thus confirming the importance of c-Met on this cell phenotype. Previous studies have shown that H. pylori also activates tyrosine kinase receptors EGFR, and ErbB-2 in gastric epithelial cells (8–10). On the collagen type I invasion assay the EGFR and ErbB2 inhibitors did not reduce H. pylori-induced cell invasion. On the Matrigel assay however, there was a decrease in cell invasion when EGFR and/or ErbB2 where inhibited. Thus, we cannot completely exclude the participation of EGFR and/or ErbB2 in H. pylori-mediated cell invasion. Using a pharmacological inhibitor we showed that PI3K is not involved in this process. Therefore, H. pylori induces cell invasion by activation of the c-Met receptor and independently of PI3K activation. This is in contrast with other models of cancer cell invasion induced by bacteria that occur via PI3K downstream ErbB2/ErbB3 activation (27).

Increased MMP levels may facilitate the process of invasion by degradation of matrix components, cleavage of cell surface receptors that act as signal transducers in invasion pathways, or through ectodomain shedding of proinvasive fragments from transmembrane receptors (12). Previous work highlighted the participation of H. pylori on the up-regulation of several MMPs (13–15, 38). In this study, we evidenced an increase in MMP-2, MMP-9, and de novo MMP-3 activity in the supernatants of cells cultured with H. pylori. After silencing MMP-2 and MMP-9 expression, a decrease in MMP-2 and MMP-9 activity as well as a significant decrease on cell invasion were observed. The simultaneous effect on MMP-2 and MMP-9 expression and activity by treatment with different exogenous factors has been reported in vitro and in vivo (12). In our study, siRNA directed to MMP-9 decreased MMP-2 activity and vice versa. Using specific antibodies directed to MMP-2 we could confirm by Western blot analysis that the siRNA directed to MMP-9 did affect MMP-2 activity but not its expression (data not shown). One possible explanation could be that the decreased expression of one MMP could affect the expression of molecules involved in the regulation of other MMPs activity, such as tissue inhibitors of metalloproteases (TIMPs). Further studies should be performed to evaluate the mechanism behind such observation.

A decrease in MMP-2 and MMP-9 activity was also observed after silencing c-Met expression. MMP-2 and MMP-9 are type IV collagenases considered to play key roles in the invasive ability of tumor cells, as basement membrane is composed primarily of type IV collagen. In gastric carcinoma, overexpression of MMP-2 and MMP-9 has also been shown to correlate with poor
could lead to extracellular matrix degradation and subsequent invasion of cancer cells, suggesting a role for \textit{H. pylori} in later stages of gastric carcinogenesis. The elucidation of \textit{H. pylori}-host interactions may provide further insight on \textit{H. pylori} pathogenesis and on the mechanisms relevant to gastric carcinoma development.

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