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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 of c-Met expression with small interference RNA suppressed both cell invasion and MMP activity. Studies with different *H. pylori* strains revealed that cell invasion, c-Met tyrosine phosphorylation, and increased MMP-2 and MMP-9 activity were all dependent on the presence of a functional bacterial T4SS, but not on VacA cytotoxicity. Our findings demonstrate that *H. pylori* strains with a functional T4SS stimulate gastric epithelial cell invasion through a c-Met-dependent signaling pathway that comprises an increase in 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).

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

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[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.

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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% CO_2 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 mM, 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, lithocholic 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'Étoile, 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* (60190Δ*cagA*), *cagE* (60190Δ*cagE*) or *vacA* (60190Δ*vacA*) 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, 1×10^8 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 & Schüell, 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% CO_2 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 (1×10^5) 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, 5×10^4 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′-UUCUCCGAACGUGUCACGU-3′ and antisense: 5′-ACGUGACACGUUCGGAGAA-3′) were used as negative control. Prior to transfection, 50% confluent AGS monolayers platted 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 nondenaturing 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. 1*A*) and Matrigel-coated filters (Fig. 1*B*).

To test whether the proportion of bacteria:cell used in the experiments would influence the cellular phenotype, invasion was assessed using different MOIs (Fig. 1*A*). 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.

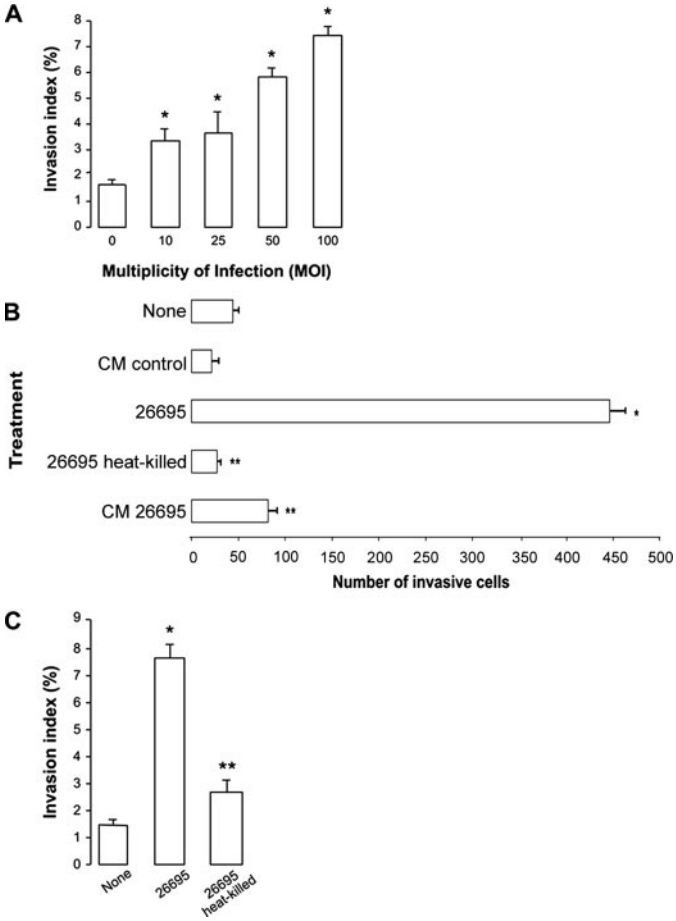

FIGURE 1. ***H. pylori* stimulates AGS cell invasion, and for that direct contact between bacteria and cells is required.** Invasion assays of AGS cells infected with *H. pylori* 26695 for 24 h on collagen type I gels at different MOI (*A*), or with heat-killed bacteria (26695 heat-killed), conditioned medium of *H. pylori* 26695 (*CM 26695*), or control-conditioned medium (*CM control*) on Matrigel-coated filters (*B*) and on collagen type I gels (*C*). 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 intact *H. pylori* 26695.

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. 1*B*), 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

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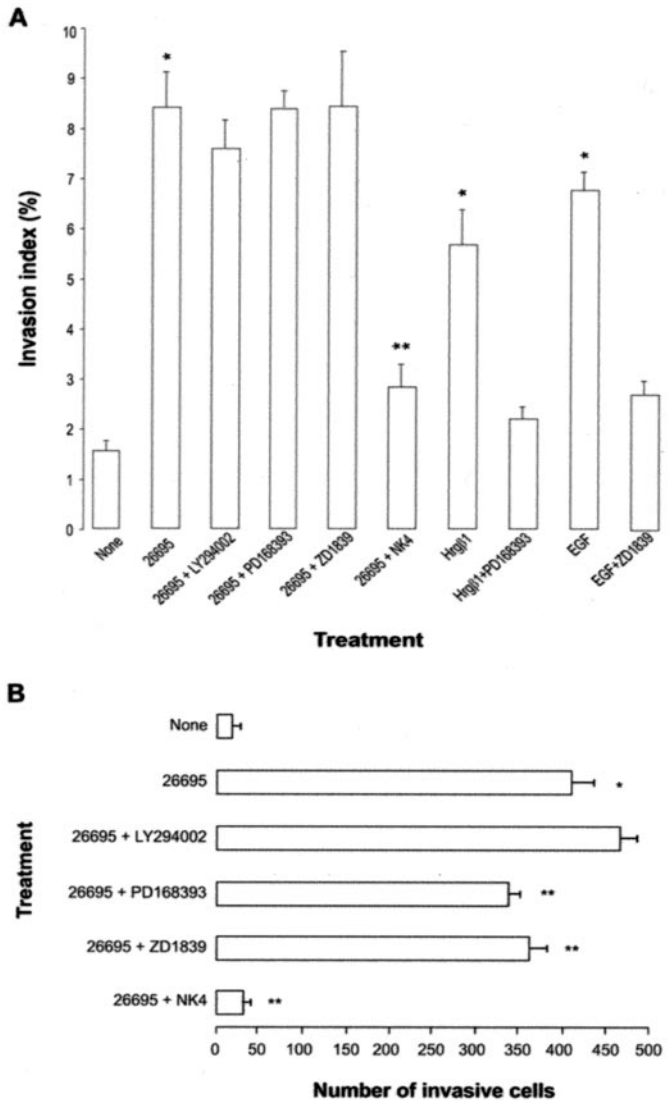

FIGURE 2. ***H. pylori* stimulation of AGS cell invasion is blocked by NK4, a c-Met inhibitor.** Invasion assays of AGS cells infected for 24 h with *H. pylori* 26695, with EGF or with Heregulin β1 (*Hrgβ1*) and with a PI3K inhibitor (*LY294002*, 100 mM), an EGFR/ErbB2 inhibitor (*PD168393*, 2 μM), an 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.

invasion 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. 2*A*). The other inhibitors, although used at concentrations known to block invasion by specific stimulators, such as EGF, Heregulin β1 (Hrgβ1) (Fig. 2*A*), 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. 2*B*). 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. 3*A*, *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 3*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. 3*C*). 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, *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 such cultures were assessed for proteolytic activity on gelatin or β-casein substrates. We observed that AGS cells constitutively secrete MMP-2 and MMP-9, and that supernatants of AGS cells infected with *H. pylori* 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. 4*C*). 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 trypan 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* strain 60190 were assessed for proteolytic activity on a gelatin substrate and for invasion on Matrigel. By abrogating the expression and activity of MMP-2 and MMP-9 the ability of *H. pylori* to induce

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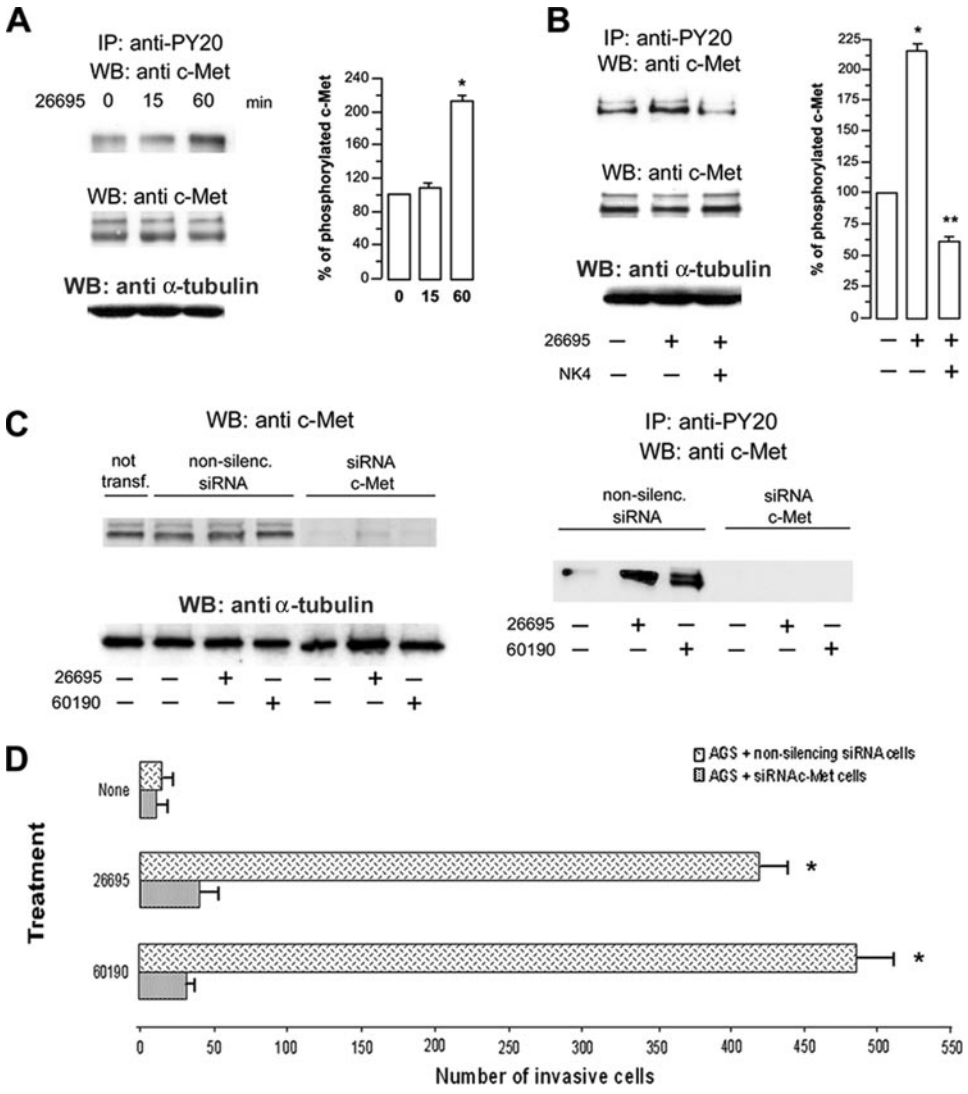

FIGURE 3. ***H. pylori* stimulation of AGS cell invasion occurs through a c-Met-dependent mechanism.** AGS cells were infected with *H. pylori* 26695 for 0, 15, or 60 min (*A*) or with *H. pylori* 26695 and NK4 (100 ng/ml) for 60 min (*B*). AGS cells or AGS cells transfected with non-silencing siRNA (*non-silenc. siRNA*) or with siRNA to c-Met (*siRNA c-Met*) were infected with *H. pylori* 26695 or 60190 for 60 min (*C*). After infection, cells were lysed, immunoprecipitated with an antibody against tyrosine-phosphorylated residues (*PY-20*), and immunoblotted with an anti-c-Met antibody. In parallel, total cell lysates were immunoblotted with an anti c-Met and reblotted with an anti-α-tubulin antibody. Cultures with c-Met siRNA-transfected cells were incubated on Matrigel filters and assessed for invasion (*D*). Graphics represent the variation on c-Met tyrosine phosphorylation in comparison to the endogenous phosphorylation levels of untreated cells. Data correspond to the mean value ± S.D. and are representative of three independent experiments. *, significantly different from untreated cells or cells transfected with non-silencing siRNA; **, significantly different from cells infected with *H. pylori* 26695.

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. 4*A*). In contrast, MMP-3 activity was not affected by c-Met silencing (Fig. 4*B*). 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. 5*A*). 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 cell 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. 5*A*).

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

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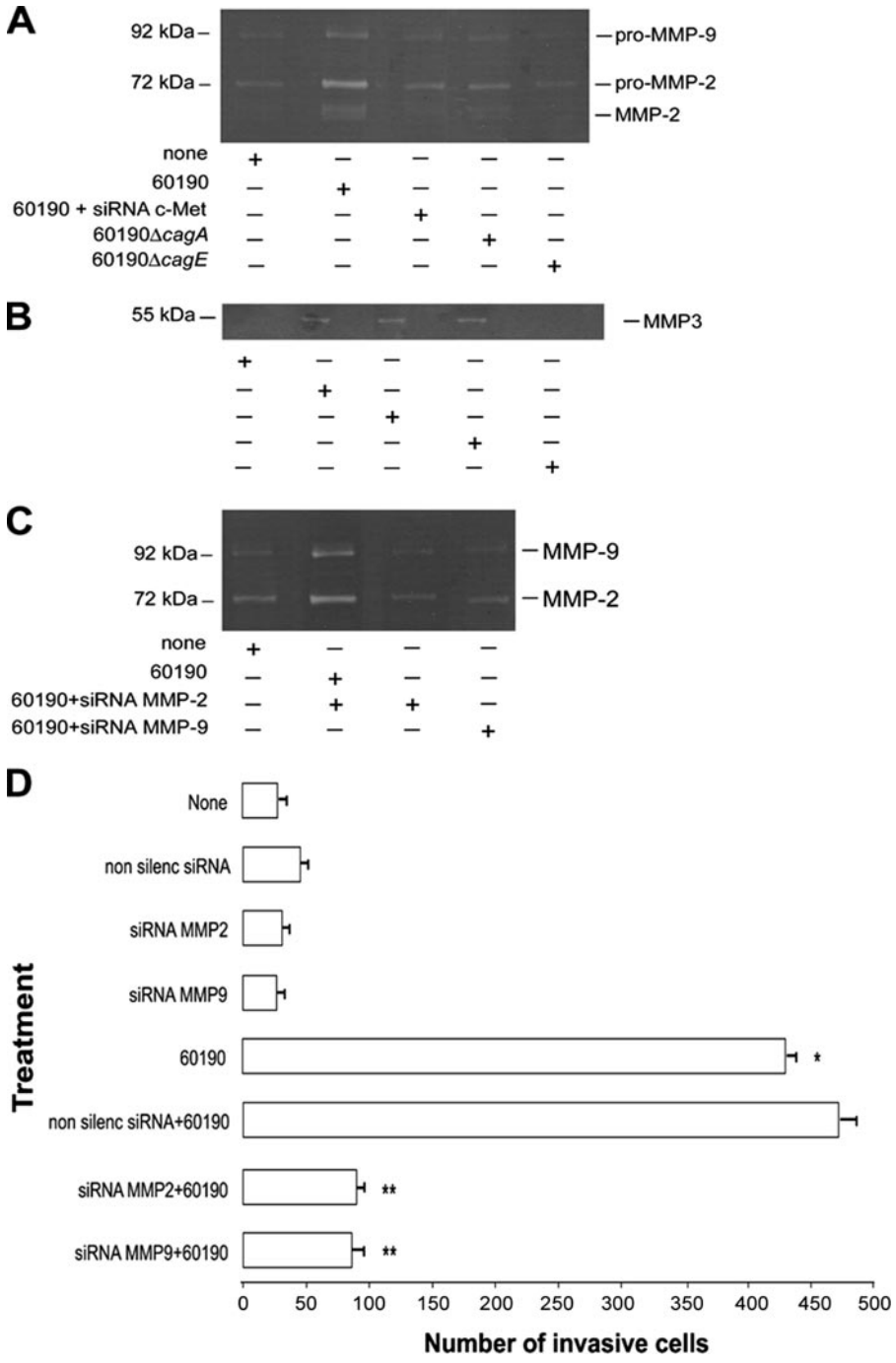

FIGURE 4. ***H. pylori*-mediated AGS cell invasion requires MMP-2 and MMP-9 activity, downstream c-Met receptor activation.** AGS cells transfected or not with siRNA to c-Met (*siRNA c-Met*) were infected for 48 h with *H. pylori* 60190, 60190Δ*cagA* or 60190Δ*cagE*, on collagen type I gels. Filtered culture supernatants were run on gelatin (*A*) or β-casein (*B*) zymograms. AGS cells transfected or not with siRNA to MMP-2 (*siRNA MMP-2*) or to MMP-9 (*siRNA MMP-9*) were infected for 48 h with *H. pylori* 60190. Filtered culture supernatants were run on a gelatin zymogram to detect MMP activity (*C*). The invasion ability of the transfected cells was tested on Matrigel assays (*D*). Proteolytic bands were revealed in white on a Coomassie Blue-stained background. 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* 60190.

functional T4SS, induced an increase in c-Met tyrosine phosphorylation levels, in contrast to what was observed for strain Tx30a and for the *cagA* and *cagE* mutants (Fig. 5*B*). None of the strains affected c-Met expression.

Because both cell invasion and c-Met tyrosine phosphorylation levels were maximal in the presence of *H. pylori* strains 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 *H. pylori* strain 60190, whereas supernatants obtained from cultures with the *cagA* and *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. 4*A*). *H. pylori*-mediated MMP-3 activity was not affected in cells cultured with the *cagA* mutant, but was abolished by infection with the *cagE* mutant (Fig. 4*B*).

Altogether these results show that *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 *H. pylori* T4SS.

DISCUSSION

We have demonstrated that *H. pylori* infection causes *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 *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 *in vitro* assays we used contain some of the elements of the *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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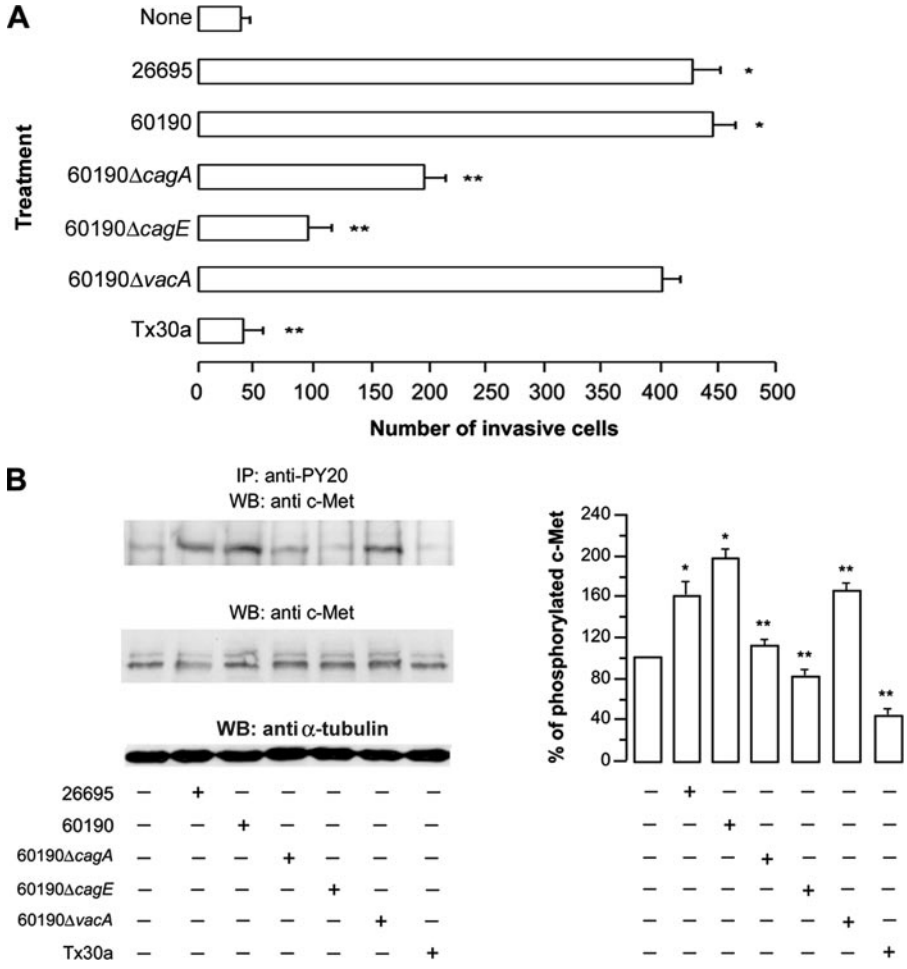

FIGURE 5. **Maximal *H. pylori* stimulation of AGS cell invasion requires a functional bacterial T4SS.** AGS cells were infected with *H. pylori* 26695, 60190, 60190Δ*cagA*, 60190Δ*cagE*, 60190Δ*vacA*, or Tx30a and assessed after 24 h for invasion on Matrigel. (*A*) Cells were infected with *H. pylori* for 60 min, lysed, and immunoprecipitated with an antibody against tyrosine-phosphorylated residues (*PY-20*) and immunoblotted with an anti c-Met antibody (*B*). In parallel, total cell lysates were immunoblotted with an anti c-Met and re-blotted with an anti-α-tubulin antibody. Graphics represent the variation on c-Met tyrosine phosphorylation in comparison to the endogenous phosphorylation levels of untreated cells. 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* 60190.

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

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survival (39, 40). These facts are in accordance to our results, pointing to a role for MMP-2 and MMP-9 downstream c-Met activation in *H. pylori*-mediated cell invasion. In contrast to what was observed for MMP-2 and MMP-9, after silencing c-Met expression we did not register any changes in MMP-3 activity. One possibility for this observation is that *H. pylori* induction of MMP-3 does not occur downstream c-Met activation. Another possible explanation is that the MMP-3 activity detected in the conditioned media of our cultures is just reflecting *H. pylori* MMP-3-like activity (13).

The vast majority of host cell changes in response to *H. pylori* are dependent on the presence of a functional T4SS (41). Our experiments with different *H. pylori* strains clearly show that maximal stimulation of cell invasion by *H. pylori* requires a functional T4SS, because strains lacking or containing a non-functioning T4SS induced limited or no cell invasion and were also not able to increase c-Met tyrosine phosphorylation, MMP-2 and MMP-9 activity. It is possible that the *cag* PAI encoded T4SS triggers signaling leading to cell invasion by establishing close contact with the host cell membrane. Our experiments using conditioned medium from *H. pylori* suggest that direct contact between bacteria and cells is necessary for stimulation of invasion. In addition to the direct contact effect of the T4SS, it is also possible that bacterial molecules translocated into the host cell by the T4SS are responsible for cell invasion. CagA is a bacterial effector molecule translocated via T4SS into the host cell (18). After translocation into the host cell, tyrosine-phosphorylated CagA modulates cellular functions by interacting intracellularly with the c-Met receptor, and by triggering morphological changes and motility similar to those induced by HGF (10, 17, 42). Our observations that *cagA* mutants did not alter c-Met tyrosine phosphorylation and were not fully competent in inducing cell invasion are in keeping with the hypothesis that CagA is important for maximal stimulation of cell invasion. These observations are in accordance with recent data showing that kidney canine cells transfected with *cagA* displayed an invasive phenotype (43). Nevertheless, the *cagA* mutants were able to stimulate cell invasion, although to a lesser extent than that of the parental wild-type strain. This suggests that additional effector molecule(s) other than CagA might be translocated into the host cell by the T4SS. Previous work from other authors had also pointed to this possibility, as *H. pylori* can activate a number of signaling pathways in a T4SS dependent, but CagA-independent manner (41, 44, 45). Recently Viala *et al.* (7) demonstrated that the *cag* PAI-encoded T4SS was also responsible for delivery of soluble compounds of peptidoglycan into host cells. It is therefore possible that the T4SS can translocate other so far unknown bacterial molecules that play a role in cellular invasion.

In accordance with recent studies (46, 47), our work with bacterial mutants also demonstrated a role for the T4SS in the up-regulation of MMPs, supporting the idea that *H. pylori* T4SS-dependent increased MMP-2 and MMP-9 activity are functionally important host cell responses associated with cell invasion.

In conclusion, we have demonstrated that *H. pylori* strains with a functional T4SS induce an increase in c-Met tyrosine phosphorylation and in MMP-2 and MMP-9 activity. This could lead to extracellular matrix degradation and subsequent invasion of cancer cells, suggesting a role for *H. pylori* in later stages of gastric carcinogenesis. The elucidation of *H. pylori*-host interactions may provide further insight on *H. pylori* pathogenesis and on the mechanisms relevant to gastric carcinoma development.

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

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