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# Cell-Associated Hemolysis Induced by *Helicobacter pylori* Is Mediated by Phospholipases with Mitogen-Activated Protein Kinase-Activating Properties

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**Pathogenic *Helicobacter pylori* strains can selectively activate epithelial mitogen-activated protein kinase (MAPK) signaling pathways linked with disease. We now demonstrate that *H. pylori*-induced hemolysis is strain specific and is mediated by phospholipases PldA1 and PldD. Inactivation of PldD inhibited activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), indicating that *H. pylori* hemolytic phospholipases also harbor MAPK-activating properties.**

*Helicobacter pylori* colonizes the stomachs of at least half of the world's population and is a strong risk factor for gastric adenocarcinoma (24, 26). *H. pylori* can activate epithelial signaling pathways, such as the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinases 1 and 2 (ERK1/2), that lower the threshold for carcinogenesis. The best-studied *H. pylori* virulence factors are the *cag* pathogenicity island (PAI) and VacA, the vacuolating cytotoxin. The *cag* PAI encodes components of a type IV secretion system (T4SS) that can inject bacterial effectors into eukaryotic cells (24, 26). The gene encoding VacA (*vacA*) is found in all strains of *H. pylori*, but not all strains secrete VacA, due to *vacA* allelic variation. However, most persons infected with strains that possess these virulence constituents do not develop disease, indicating that other microbial, host, and/or environmental factors may contribute to pathogenesis (for reviews, see references 5, 8, and 28). Dubois and Boren have recently reported that *H. pylori* not only resides within the gastric mucosa but is also closely associated with red blood cells (10), raising the possibility that this bacterium may exert effects on components of the hematopoietic system.

Synergistic hemolysis was initially described as the extent of hemolysis observed when *Staphylococcus aureus* and *Streptococcus agalactiae* were grown in close proximity on blood agar plates. Regions containing diffusing sphingomyelinase (SMase) from *S. aureus* and the pore-forming toxin of *S. agalactiae* were found to exhibit a higher degree of hemolysis than zones containing only one of these virulence factors (7), a conjoined phenotype commonly known as the CAMP (Christie-Atkins-Munch-Perkins) test. Synergistic hemolysis has subsequently been observed with diffusible factors from organisms as phylogenetically distant as dermatophytes (fungi) and *Listeria ivanovii* (31). Two recent reports have demonstrated that *H. pylori* is a component of a complex gastric microbial ecosystem, which likely promotes dynamic interspecies interactions (6, 16). Here, we examined the ability of *H. pylori* to induce synergistic hemolysis and demonstrate that a microbial constituent which possesses the ability to induce hemolysis can also activate MAPK signaling in gastric epithelial cells.

*S. agalactiae* and *S. aureus* were initially tested for the CAMP reaction on TSAII blood agar plates and reproducibly exhibited synergistic hemolysis (data not shown) as previously reported (4). Since SMases (17) as well as pore-forming toxins (20) have been described within *H. pylori*, we developed a protocol to test for *H. pylori*-induced synergistic hemolysis. Media containing tryptic soy agar with 5% sheep blood (TSBA), TSBA with CAMP factor (from *S. agalactiae* supernatant), or TSBA with SMase (from *S. aureus* filtered supernatant) were prepared, and an assay was performed using *S. aureus* supernatant and *Bacillus cereus* sphingomyelinase C (Sigma) as positive controls for synergistic hemolysis (Fig. 1).

To investigate the ability of *H. pylori* to induce synergistic hemolysis, strains ($n = 6$, $\sim 10^7$ CFU) were spotted on medium and incubated overnight. Hemolysis developed directly under areas of bacterial growth (Fig. 2A), consistent with previous observations that hemolytic factors are likely not secreted by *H. pylori* (3). *H. pylori* strains exhibited different levels of hemolysis on either control, CAMP-containing, or SMase-containing plates (Table 1; Fig. 2A). For example, strains J99 and 98-18 did not exhibit hemolysis when grown on TSBA plates alone; however, synergistic hemolysis developed on both SMase- and CAMP-containing plates, highlighting the utility of this approach for examining strain-specific hemolytic differences (Fig. 2A). The degree of hemolysis was significantly greater ($P = 0.0067$) when *H. pylori* strains were grown on CAMP-containing plates than on control plates. We also examined potential roles exerted by the *cag* PAI by use of *cag* PAI deletion mutations in strains 7.13 and 26695; however, no difference was found between wild-type strains and their corresponding mutants (data not shown).

HP0499 encodes a membrane-bound phospholipase A (PldA1) in *H. pylori* (9). HPAG1_0184 was identified as a "phospholipase D-family protein" (http://cmr.jcvi.org/tigr-scripts/CMR/shared/GenePage.cgi?locus=NTL03HP0185), and HP0190 of strain 26695 is predicted to be 94% identical to HPAG1_0184 at the protein level. An analysis of HP0190 with NCBI's Conserved Domain Database (19) revealed phospholipase D (PldD) active-site

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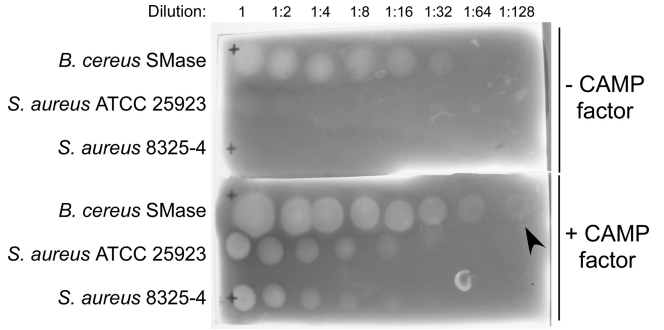


**FIG 1** Synergistic hemolysis on blood agar medium. The sensitivity of synergistic hemolysis was estimated using medium containing CAMP factor (16 μg/ml) from *S. agalactiae* filter-sterilized culture supernatant. *B. cereus* SMase (top row) or *S. aureus* culture supernatants from strain ATCC 25923 (middle row) or 8325-4 (bottom row) were applied at 2-fold dilutions as indicated. The area of hemolysis on the top row in the lower slide (arrowhead) indicates that the limit of detection for this assay is 0.00165 units of sphingomyelinase activity.

motifs. To define potential roles of these genes in synergistic hemolysis, we inactivated HP0499 and HP0190 individually and in tandem (HP0499 HP0190 mutant) in strains 7.13 and J166 (see Tables S1 and S2 in the supplemental material) (25). Wild-type strains and isogenic mutants were then tested for hemolytic ability on TSBA plates with or without CAMP factor (Fig. 2B). Single phospholipase mutants of strain J166 or 7.13 were similar to wild type (Fig. 2B). Double mutants of both strains were nonhemolytic, indicating that both phospholipases mediate synergistic hemolysis. We also examined strain B128, the parental strain of 7.13, and found that this induced less hemolysis than did strain 7.13.

*H. pylori* can activate MAPK pathways in gastric epithelial cells in a strain-specific manner, and activation of ERK1/2 may play a role in pathogenesis (15). *H. pylori* encodes SMases that catalyze the conversion of host cell sphingomyelin to ceramide, which can activate MAPK (17, 34). We therefore determined if phospholipases HP0499 and HP0190 were required for activation of ERK1/2 signaling in gastric epithelial cells. AGS gastric epithelial cells were grown to confluence, then cultured in medium for 24 h, and then cocultured with *H. pylori* for specified times at a multiplicity of infection (MOI) of 100. *H. pylori*-infected and uninfected AGS cells were lysed in RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 1% Triton X-100; 0.1% SDS), and protein concentrations were quantified by the bicinchoninic acid (BCA) assay (Pierce). Proteins (30 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation, Ann Arbor, MI). Levels of phosphorylated or total ERK1/2 were detected using an anti-phospho-ERK1/2 antibody (Promega) (1:5,000) or an anti-total-ERK1/2 antibody (Cell Signaling) (1:5,000). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer) according to the manufacturer's instructions. Western blots were imaged and band intensities were quantified using the ChemiGenius Gel Bio Imaging System (Syngene) (22). Our previous studies determined that a bacterium/cell ratio of 100:1 and a time course ranging from 5 to 60 min were optimal to evaluate the ability of *H. pylori* wild-type and mutant strains

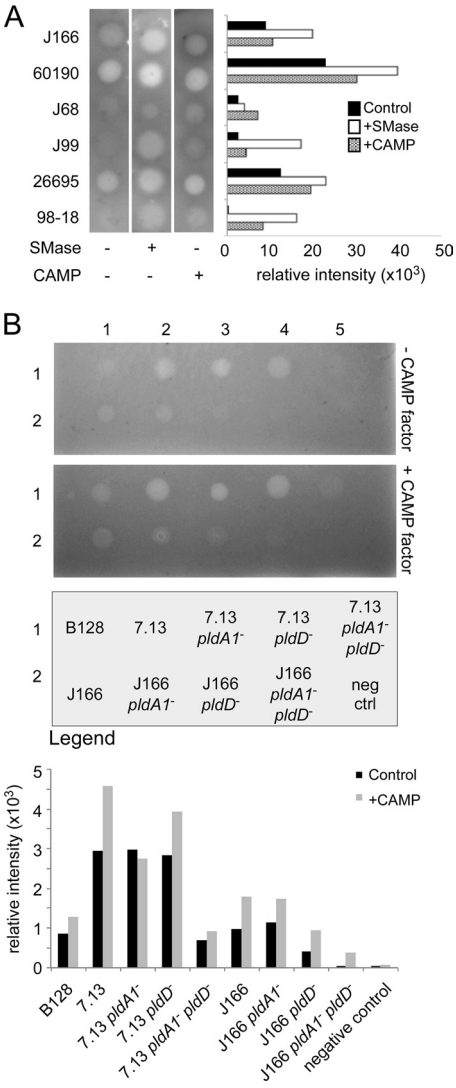


**FIG 2** Variability of hemolysis among strains of *H. pylori.* (A) Hemolytic ability of *H. pylori* strains was determined by inoculating 3 to 4 μl ($10^7$ CFU) onto TSB agar containing 140 mM NaCl and crude preparations of either SMase or CAMP factor. Hemolysis under each condition was quantified as relative intensity using NIH ImageJ and is shown in the bar graph on the right. Data shown are from an experiment representative of several replicates. (B) Indicator plates estimating SMase activity in wild-type strains J166 and 7.13 and mutants in phospholipase genes in J166 and 7.13 backgrounds as well as the parental clinical strain B128 are shown. The lower plate contains CAMP factor. Row 1, *H. pylori* B128, 7.13, 7.13 *pldA1* mutant, 7.13 *pldD* mutant, and 7.13 *pldA1 pldD* mutant; row 2, *H. pylori* J166, J166 *pldA1* mutant, J166 *pldD* mutant, J166 *pldA1 pldD* mutant, and broth (negative control). Hemolysis under each condition was quantified as relative intensity using NIH ImageJ and is shown in the bar graph.

to activate MAPK using AGS gastric epithelial cells (15); therefore, *H. pylori* or *B. cereus* SMase C (1 unit/ml) was added to gastric cells and ERK activation was detected 5 to 60 min later (Fig. 3). *B. cereus* SMase induced robust activation of ERK. Coculture of strain J166 with AGS cells led to gradual ERK activation over 60 min. Inactivation of *pldA1* did not affect this activation; however, loss of *pldD* led to reduced ERK activation, peaking at a much lower level by 30 min (Fig. 3). The double *pldD pldA* mutant also activated ERK1/2 less potently than did the wild-type strain.

**TABLE 1** List of bacterial strains with a description of clinical outcome, *cag* status, and hemolytic ability

| Strain | Genotype/relevant characteristics and hemolytic ability (if applicable) | Reference(s) or source |
|---|---|---|
| *H. pylori* | | |
| J166 | Duodenal ulcer, *cagA*$^+$ strain of *H. pylori*; hemolytic, synergistic hemolysis | 25 |
| 60190 | Lab derivative of ATCC 49503, *cagA*$^+$; hemolytic, enhanced synergistic hemolysis on SMase plate relative to CAMP plate | |
| J68 | Duodenal ulcer, *cagA* mutant; minimally hemolytic, enhanced synergistic hemolysis on CAMP plate compared to SMase plate | |
| J99 | Duodenal ulcer, *cagA*$^+$; sequenced strain; minimally hemolytic; synergistic hemolysis on both plates, maximum enhancement on SMase plate | 2 |
| 26695 | Gastritis, *cagA*$^+$; sequenced strain; hemolytic, synergistic hemolysis | 1, 33 |
| 26695 Δ*cag* | 26695 with the *cag* PAI deleted; hemolytic, synergistic hemolysis | 11 |
| 98-18 | Gastric ulcer, *cagA*$^+$; minimally hemolytic, enhanced synergistic hemolysis on SMase plate compared to CAMP plate | 27 |
| J166 *pldA1* | Mutant in *pldA1*; kanamycin resistant; hemolytic, synergistic hemolysis on CAMP plate | This work |
| J166 *pldD* | Mutant in *pldD*; chloramphenicol resistant; hemolytic, synergistic hemolysis on CAMP plate | This work |
| J166 *pldA1 pldD* | Double mutant in *pldA1* and *pldD*; kanamycin and chloramphenicol resistant; nonhemolytic, no synergistic hemolysis on CAMP plate | This work |
| B128 | Gastric ulcer; *cagA*$^+$; minimally hemolytic, synergistic hemolysis | 13 |
| 7.13 | Carcinogenic strain of *H. pylori* recovered after infection of a gerbil with strain B128; hemolytic, synergistic hemolysis on CAMP plate | 12 |
| 7.13 Δ*cag* | Mutant of 7.13 lacking the *cag* PAI; hemolytic, enhanced synergistic hemolysis on CAMP plate relative to SMase plate | Lab strain |
| 7.13 *pldA1* | Mutant in *pldA1*; kanamycin resistant; hemolytic, synergistic hemolysis on CAMP plate | This work |
| 7.13 *pldD* | Mutant in *pldD*; chloramphenicol resistant; hemolytic, synergistic hemolysis on CAMP plate | This work |
| 7.13 *pldA1 pldD* | Double mutant in *pldA1* and *pldD*; kanamycin and chloramphenicol resistant; nonhemolytic, no synergistic hemolysis on CAMP plate | This work |
| *E. coli* | | |
| DH5α | *E. coli* K-12; F$^-$ *ϕ*80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_K^-$ $m_K^-$) *phoA supE44 thi-1 gyrA96 relA1* mutant; strain used for cloning | Lab strain |
| BL21 | *E. coli* B; F$^-$ *dcm ompT hsdS*($r_B^-$ $m_B^-$) *gal*; Dam$^+$ Dcm$^-$ | Stratagene |
| Other bacteria | | |
| *Streptococcus agalactiae* ATCC 12386 | Secretes CAMP factor into culture medium | American Type Culture Collection |
| *Staphylococcus aureus* subsp. *aureus* ATCC 25923 | Secretes sphingomyelinase D into culture medium, observed to cause hemolysis to a greater extent than strain 8325-4 (below) | American Type Culture Collection |
| *Staphylococcus aureus* 8325-4 | Secretes sphingomyelinase D into culture medium | 21 |

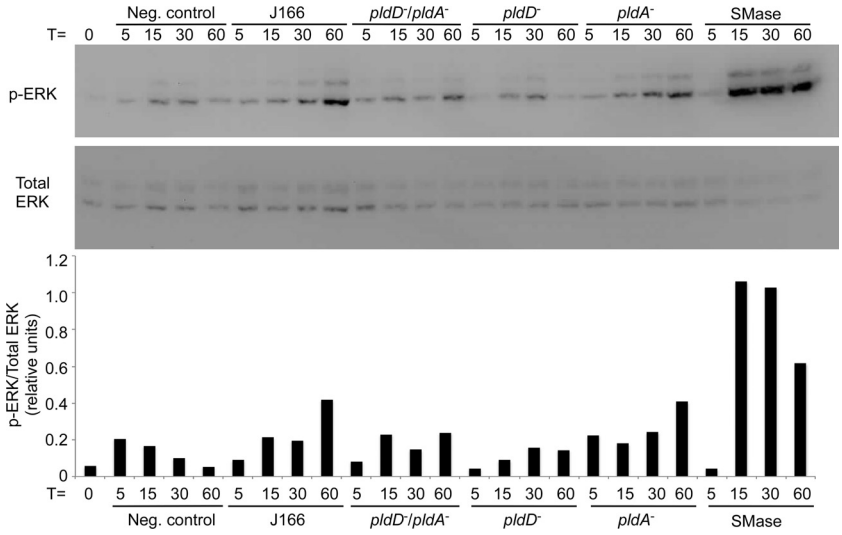


**FIG 3** Western blot assays to evaluate ERK1/2 activation in AGS cells. AGS cells were incubated with *B. cereus* SMase (1 unit/ml), wild-type *H. pylori* J166, or its isogenic single or double phospholipase mutants from 0 to 60 min. The blot was probed for phosphorylated ERK1/2 (top panel), stripped, and reprobed for total ERK1/2 (bottom panel). Densitometric analysis of phospho-ERK1/2 is shown below the Western blot.

In this study, we developed assays that incorporated cell-free culture supernatants containing CAMP factor or SMase into growth medium to permit testing for synergistic hemolysis in slow-growing fastidious bacteria. This allowed identification of cell-associated hemolytic factors, whereas previous studies detected only secreted hemolytic factors. We also demonstrate that both *H. pylori* phospholipases analyzed mediate synergistic hemolysis in combination with the *S. agalactiae* CAMP factor. PldA1 is highly conserved among *H. pylori* strains, but variation in *pldA1* sequences exists among strains from different geographic locales (35). Our current experiments also indicate that *H. pylori* PldD can activate ERK1/2 in AGS cells and thus represent a novel function of *H. pylori* phospholipases.

Phospholipases are ubiquitous throughout the living world and have been implicated as virulence factors in several bacterial species such as *Staphylococcus aureus* (23), *Pseudomonas aeruginosa* (30), *Yersinia pseudotuberculosis* (14), *Listeria monocytogenes* (32), and *Mycobacterium tuberculosis* (29). The current work highlights an important issue regarding the effect of colonization by multiple bacterial species (as commensals, symbionts, or pathogens) on the host. The ability of *H. pylori* phospholipases to participate in synergistic hemolysis with a pore-forming toxin from *S. agalactiae* indicates that membrane-active factors of phylogenetically distant organisms can exhibit cooperative effects. Within the gastric niche, *H. pylori* is a resident of a complex microflora, containing >120 distinct phylotypes (6, 16). A recent report consistent with our findings (18) demonstrated that the severity of skin lesions in mice was exacerbated by the cooperative effect of the SMase of *S. aureus* and a CAMP factor from *Propionibacterium acnes*, an abundant commensal found on human skin. Neutralization of the CAMP factor and immunization against the SMase reduced the severity of lesions during coinfection of mice. Thus, complementary functions of membrane-damaging agents produced by unrelated microbial species may be a common theme in microbial biology.

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