Supporting Information

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SI Materials and Methods

Bacterial Strains. *Hp* 60190 (*cag* PAI^+, *vacA* s1/m1; 49503; ATCC) was cultured in bisulfite- and sulfite-free *Brucella* broth (BSFB) containing 5 μg vancomycin/mL (Sigma Aldrich), on a rotary platform shaker for 48 h at 37 °C, under 5% CO_2 and 10% O_2. *Hp* VM022 (*ΔvacA*) (1) and *Hp* VM084 (*ΔvacA::vacA*) were kind gifts from Timothy Cover (Vanderbilt University Medical Center, Nashville, TN) (2). *Hp* 60190-derived strains producing VacA (P9A) and VacA (G14A) were constructed and cultivated as described previously (2, 3). *Hp* 26695 (*cag* PAI^+, *vacA* s1/m1) was obtained from ATCC (700392). *Hp* G27 (*cag* PAI^+, *vacA* s1/m1) (4) was obtained as a kind gift from Karen Guillemin (University of Oregon, Eugene, OR).

Mammalian Cells. AZ-521 human gastric cancer-derived cell line was obtained from Japan Health Science Foundation (3940) and maintained in MEM (Sigma Aldrich), which when supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin sulfate (1 mg/mL), and 10% fetal bovine calf serum, was referred to as "supplemented MEM." HeLa (CCL-2; ATCC), HEp-2 (CCL-23; ATCC), and Madin-Darby canine kidney (MDCK) II cells (CCL-34; ATCC) were maintained in supplemented MEM. AGS cells (CRL-1739; ATCC) were maintained in Ham's F-12 Kaighn's modification medium (Cellgro), which when supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin sulfate (1 mg/mL) (Sigma Aldrich), and 10% fetal bovine calf serum (JRH Biosciences), was referred to as "supplemented Ham's F-12 medium." $bax^{+/+}$ and $bax^{-/-}$ mouse embryonic fibroblasts (MEFs), obtained as a kind gift from Wei-Xing Zong (Stony Brook University, Stony Brook, NY), were maintained in DMEM (Cellgro), which when supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin sulfate (1 mg/mL), and 10% fetal bovine calf serum, was referred to as supplemented DMEM. All cell lines were maintained at 37 °C within a humidified atmosphere and under 5% CO_2.

Plasmids. The plasmids pDsRed2-Mito and pAcGFP1-N1 were obtained from Clontech Laboratories. Plasmids pEGFP-Drp1 and pEGFP-DN-Drp1 (K38A) were obtained as a kind gift from Marina Jendrach (Goethe University; Frankfurt/Main, Germany). The plasmid encoding p34(1-319)-EGFP was obtained as a kind gift from Joachim Rassow (Ruhr Universität, Bochum, Germany).

Transfection. Cells were transfected with the indicated plasmids using Lipofectamine-2000 transfection reagent (Invitrogen), according to the manufacturer's instructions.

***Hp* Infection of Mammalian Cells.** Monolayers of mammalian cells, at 37 °C within a humidified environment under 5% CO_2, were incubated with the indicated *Hp* strains and at the indicated multiplicity of infection (MOI). At the end of incubation, monolayers were washed with PBS pH 7.2 and processed for the indicated analyses.

Analysis of Mitochondrial Fragmentation within AZ-521, AGS, and Mouse Embryonic Fibroblast Cells. Mammalian cells plated in eight-well culture slides (BD Biosciences) that had been transiently transfected with pDsRed2-Mito, a mammalian expression vector that encodes a fusion between red fluorescent protein and the mitochondrial targeting sequence from subunit VIII of the human cytochrome *c* oxidase, were infected with *Hp* (at the indicated MOI), or incubated with VacA (at the indicated concentration) or *Hp* culture filtrate (HPCF) (at the indicated concentration), or mock treated with PBS pH 7.2. At the indicated times, the cells were washed three times with PBS pH 7.2 and then fixed by incubation with paraformaldehyde (4%) at 37 °C for 20 min. Mitochondrial morphology was analyzed by DIC-epifluorescence microscopy. Images were processed using DeltaVision SoftWoRx 3.5.1 software suite (Applied Precision). Mitochondrial lengths were measured using Imaris 5.7 software (Bitplane). Data were rendered as the average mitochondrial lengths obtained by combining data from at least two or three independent experiments. In each independent experiment, 15 cells were analyzed from at least four randomly chosen fields for each treatment. Within each cell, at least three mitochondria were analyzed and mitochondrial lengths measured. Relative mitochondrial lengths were calculated as the fold change in average mitochondrial length measured in cells treated as indicated, relative to those measured in mock-treated cells.

Generation of Polarized Madin-Darby Canine Kidney (MDCK) Monolayers. Polarized MDCK monolayers were generated and maintained, as described previously (5). Briefly, MDCK cells (2.5×10^5 cells/0.5 mL in supplemented MEM) were seeded onto 12 mm, 3.0 μm-pore polyester tissue culture transwell inserts (Corning) and incubated at 37 °C and under 5% CO_2, with 1.5 mL supplemented MEM added in the basal chamber. Cell culture medium in the apical chambers was replaced once, 24 h after seeding, with fresh supplemented MEM, whereas the medium in the basal chamber was replaced with fresh supplemented MEM daily. The plates were maintained at 37 °C in a humidified atmosphere and under 5% CO_2 for at least 4 d before the experiments.

To assess the integrity of polarized monolayers, transwell inserts with MDCK cells or mock-seeded transwell inserts (incubated with PBS pH 7.2 alone) were incubated at 37 °C and under 5% CO_2 with biotin-albumin (50 μg/mL, 1.5 mL; Sigma Aldrich) in supplemented MEM in the transwell basal chamber and supplemented MEM alone (0.5 mL) in the apical chamber. After 1 h, medium from the apical and basal chambers was collected and resolved by SDS gel electrophoresis, followed by Western blot analysis to probe for biotin-albumin using streptavidin-HRP (GE Healthcare). The presence of biotin-albumin was detected by chemiluminescence using the Supersignal West Femto chemiluminescence detection kit (Thermo Scientific).

Analysis of Mitochondrial Fragmentation within HeLa, HEp-2, and Polarized MDCK Cells. HeLa and HEp-2 cells plated in eight-well culture slides, and transmembrane polyester inserts containing polarized MDCK monolayers were washed twice with PBS pH 7.2 and fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization in PBS pH 7.2 containing Triton-X 100 (0.1%) for 10 min at 4 °C. After washing twice with PBS pH 7.2, cells were probed for the mitochondrial marker Tom-20 by incubation at 4 °C overnight with anti-Tom 20 mAb (BD Biosciences) in PBS pH 7.2 containing 3% BSA (Sigma Aldrich), followed by incubation at 25 °C for 1 h with mouse anti-IgG conjugated to Alexa Fluor 488 (Invitrogen) in PBS pH 7.2 containing 3% BSA. The cells were washed three times with PBS pH 7.2 and mitochondria were visualized by DIC-epifluorescence microscopy.

Preparation of HPCF. The indicated *Hp* strains were grown in 200 mL bisulfite- and sulfite-free *Brucella* broth (BSFB) containing 5 μg vancomycin/mL, in 1-L culture flasks, on a rotary platform shaker at 37 °C, under 5% CO_2 and 10% O_2. After 48 h, *Hp* cultures were harvested by centrifugation at 8,000 *g* for 30 min at 4 °C. The supernatants were collected, and pellets were decontaminated by autoclaving. The supernatants were cooled to 4 °C, and the total protein was precipitated by slowly dissolving ammonium sulfate (Sigma Aldrich) to 90% saturation with stirring, followed by stirring overnight at 4 °C. The precipitates were collected by centrifugation at 8,000 *g* for 30 min at 4 °C, and the pellets were resuspended in 10 mM sodium phosphate buffer pH 7.0. The samples were dialyzed at 4 °C into 10 mM sodium phosphate buffer pH 7.0 using the Spectra/Por membrane (molecular weight cutoff (MWCO) 50,000 Da; Spectrum Laboratories), concentrated approximately fivefold using an Amicon Ultra centrifugal filter unit (MWCO 50,000 Da; Sigma Aldrich), and filter sterilized using a 0.2-μm vacuum filtration unit (Corning) to obtain the final HPCF.

The presence of full-length VacA within the HPCFs was confirmed by Western blot analysis, using VacA rabbit antiserum (Rockland Immunochemicals), followed by incubation with HRP-conjugated antirabbit IgG secondary antibody (Cell Signaling Technology). The presence of VacA was detected by chemiluminescence using the Supersignal West Femto chemiluminescence detection kit. VacA concentrations were normalized using densitometry analysis (UN-SCAN-IT gel analysis software; Silk Scientific) to compare the total pixels of each band against those obtained using known concentrations of purified VacA. The HPCFs were used within several days of preparation, during which time there was no detectable loss of VacA-induced vacuolation of AZ-521 cells, as determined by quantification of cellular vacuolation (6, 7).

Heat Inactivation of HPCF. HPCFs (5 mg/mL) were incubated in a 37 °C or 95 °C water bath for 30 min, followed by incubation for 10 min at 37 °C. The HPCFs were immediately activated by adding 0.1 vol/vol 300 mM HCl to HPCF preparation and incubation for 30 min at 37 °C, followed by neutralization with the same volume of 300 mM NaOH. Activated HPCFs were incubated with AZ-521 cells at a final concentration of 0.05 mg/mL. At the indicated times, the AZ-521 cells were analyzed for changes in mitochondrial morphology relative to cells that were mock intoxicated with PBS pH 7.2.

Intoxication of Mammalian Cells with VacA or HPCF. Mammalian cells were incubated at 37 °C and within a humidified environment under 5% CO_2 with purified VacA or HPCF (both at the indicated concentrations). At the indicated times, the cells were processed for specific analyses.

Heat Inactivation of *Hp*. *Hp* 60190 (12.5×10^8 cfu/mL in 1 mL PBS pH 7.2) were incubated in a 37 °C or 65 °C water bath for 30 min and then further incubated for 10 min at 37 °C. Immediately, 37 °C- or 65 °C-pretreated *Hp* was incubated with AZ-521 cells (MOI 100) or, alternatively, enumerated by serially diluting in PBS pH 7.2, followed by spread plating onto fresh F-12 agar plates (supplemented with 5% FBS and 5 μg vancomycin/mL), and incubating the plates at 37 °C, and under 5% CO_2 and 10% O_2. After 72 h, cfu/mL were determined by direct counting of colonies on the F-12 plates and back calculating the appropriate dilution factor.

Analysis of *Hp* Association with AZ-521 Cells. Monolayers (85–95% confluence) of AZ-521 cells, plated at 0.75×10^5 cells per well, were incubated with *Hp* 60190, *Hp* VM022 (*ΔvacA*), or *Hp* VM084 (*ΔvacA::vacA*) (all at MOI 100) at 37 °C and under 5% CO_2 and 10% O_2. After 8 h, the cell monolayers were washed twice with PBS pH 7.2. Each monolayer was gently lysed by incubating with 0.1% Triton X-100 in PBS pH 7.2 (50 μL) for 3 min on ice and collected in 950 μL PBS pH 7.2. The cell-associated *Hp* was plated on F-12 media plates supplemented with 10% FBS and 5 μg vancomycin/mL and incubated at 37 °C under 5% CO_2 and 10% O_2. After 72 h, the cfu/mL was determined by the direct counting of colonies on F-12 plates and back calculating using the appropriate dilution factor.

Purification of VacA. VacA (s1m1) from *Hp* 60190 was purified and activated, as previously described (8). *Hp* culture filtrate (from *Hp* broth culture) was prepared as previously described (9).

Heat Inactivation of VacA. Purified VacA (4 μM) was incubated in a 37 °C or 95 °C water bath, followed by further incubation of both samples at 37 °C for 10 min. Immediately, VacA was activated as described previously (3, 8, 10) and incubated with AZ-521 cells (at a final concentration of 250 nM). At the indicated times, the AZ-521 cells were analyzed for changes in mitochondrial morphology relative to cells that were mock intoxicated with PBS pH 7.2.

Inactivation of VacA with Anti-VacA Antibody. Purified VacA (0.1 mg/mL) was preincubated on ice with VacA rabbit antiserum (2 mg/mL), a nonspecific rabbit antiserum against *Haemophilus ducreyi* cytolethal distending toxin A (CdtA; 2 mg/mL) or PBS pH 7.2. After 30 min, the VacA-containing samples were activated as described previously (3, 8, 10) and immediately incubated at 37 °C with AZ-521 cells (250 nM VacA in each sample). At the indicated times, the AZ-521 cells were analyzed for changes in mitochondrial morphology relative to cells that were mock intoxicated with PBS pH 7.2.

Expression and Purification of Recombinant VacA Proteins. The recombinant VacA fragments, p33 (residues 1–312) and p55 (residues 312–821), were expressed in *Escherichia coli* (DH5α) and purified as described previously (11).

Analysis of Drp1 Localization to Mitochondria. Mitochondrial localization of Drp1 was analyzed as described previously (12). Mammalian cells that had been transiently transfected with pDsRed2-Mito and plated in eight-well culture slides, were incubated with *Hp* (at the indicated MOI), or purified VacA (at the indicated concentrations), or *Hp* culture filtrates (at the indicated concentrations). At the indicated times, the monolayers were washed three times with PBS pH 7.2, fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, permeabilized by incubation with PBS pH 7.2 containing Triton-X 100 (0.1%) for 10 min at 4 °C, immunostained for Drp1 using anti-Drp1 mAb (BD Biosciences), and, finally, incubated at 25 °C for 1 h with mouse anti-IgG conjugated to Alexa Fluor 647 (Invitrogen). The cells were imaged using DIC-epifluorescence microscopy. Drp1 localization to mitochondria was quantified using the colocalization module of the DeltaVision SoftWoRx 3.5.1 software suite. Results were expressed as the colocalization index, derived from calculating Pearson's coefficient of correlation, which in this study was a measure of colocalization between Drp1 and mitochondria in each *z* plane of the cell. For each cell, images from an average of 10–20 *z* planes at a thickness of 0.2 μm were collected. A colocalization index of 1.0 indicates 100% colocalization of Drp1 to mitochondria, whereas a colocalization index of 0.0 indicates the absence of detectable colocalization between Drp1 and mitochondria. Data were rendered as the average colocalization index obtained from analyzing 30 cells from over the course of three independent experiments. In each independent experiment, 10 cells were analyzed from at least four randomly chosen fields for each treatment.

Flow Cytometry Assay for Determination of Total Cellular and Activated Bax. AZ-521 cells were detached from tissue culture wells by mild trypsinization for 3 min at 37 °C with trypsin EDTA (Cellgro), fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization on ice with saponin (0.1%) in PBS pH 7.2 containing BSA (0.5%) and anti-Bax 2D2 mAb (1 μg/mL; BD Biosciences) to stain for total cellular Bax. Alternatively, the cells were fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization on ice with saponin (0.1%) in PBS pH 7.2 containing BSA (0.5%) and anti-Bax Clone 3 mAb to stain for activated Bax. After 45 min, the cells were washed three times with PBS pH 7.2, followed by incubation with mouse anti-IgG conjugated to Alexa Fluor 488 (1 μg/mL) in PBS pH 7.2 containing saponin (0.1%) and BSA (0.5%) for 30 min on ice and in the dark. As a negative control, cells were incubated in the presence of mouse anti-IgG conjugated to Alexa Fluor 488 (1 μg/mL) alone. Cells were washed in PBS pH 7.2 containing saponin (0.1%) and BSA (0.5%) and resuspended in PBS pH 7.2. Alexa Fluor 488 fluorescence was quantified by flow cytometry in the FL1 channel (525/40 nm band pass filter). A total of 10,000 cells were analyzed for each sample.

Microscopic Analysis of Intracellular Activated Bax. Cells were fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, followed by permeabilization on ice with saponin (0.1%; Sigma Aldrich) in PBS pH 7.2 containing BSA (0.5%) and anti-Bax (Clone 3) mAb (1 μg/mL; BD Biosciences) to stain for activated Bax. After 45 min, the cells were washed three times with PBS pH 7.2, followed by incubation with mouse anti-IgG conjugated to Alexa Fluor 647 or Alexa Fluor 488 (1 μg/mL) in PBS pH 7.2 containing saponin (0.1%) and BSA (0.5%) for 1 h on ice in the dark. The cells were visualized by DIC-epifluorescence microscopy, with visible fluorescence indicative of activated Bax. To enumerate the number of cells displaying active Bax, imaging was carried out at a constant time of exposure. All images were deconvolved using SoftWoRx constrained iterative deconvolution tool (ratio mode) to remove out-of-focus signal. Following deconvolution, cells displaying visible fluorescence were considered to contain active Bax, as opposed to the absence of Bax activation in cells that did not display fluorescence. Data were rendered as the percentage of cells displaying active Bax within the entire population and were obtained by analyzing over 700 cells from randomly chosen fields over the course of two independent experiments.

Analysis of Cyt *c* Release. Cyt *c* release was analyzed as described previously (13). Briefly, mammalian cells were fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, permeabilized by incubation with PBS pH 7.2 containing Triton-X 100 (0.1%) for 10 min at 4 °C, immunostained for Cyt *c* by incubating with anti-Cyt *c* mAb (BD Biosciences), and then further incubated with one of two secondary antibodies. To visualize Cyt *c* within cells intoxicated with VacA in the presence or absence of mdivi-1, cells were further incubated with mouse anti-IgG conjugated to Alexa Fluor 488. Alternatively, to visualize Cyt *c* within VacA-intoxicated cells overexpressing either EGFP-Drp1 or EGFP-DN-Drp1 (K38A), cells were further incubated with mouse anti-IgG conjugated to Alexa Fluor 647. The cells were imaged using DIC-epifluorescence microscopy. Cells with diffuse, nonlocalized fluorescence were scored as having released (cytosolic) Cyt *c*, whereas cells with punctate fluorescence localized in the perinuclear regions were scored as having mitochondrial-localized Cyt *c*.

Cell Death. Cell death was measured by flow cytometry, using the Live-Dead viability/cytotoxicity assay kit (Invitrogen) according to the manufacturer's instructions.

Analysis of VacA Localization to Mitochondria. AZ-521 cells plated in eight-well culture slides were incubated with purified VacA (100 nM). After 30 min, the monolayers were washed three times with PBS pH 7.2, fixed by incubation with paraformaldehyde (4%) for 20 min at 37 °C, permeabilized by incubation with PBS pH 7.2 containing Triton-X 100 (0.1%) for 10 min at 4 °C, immunostained for VacA using anti-VacA rabbit polyclonal Ab and mitochondria using anti-Tom 20 mAb, and incubated at 25 °C for 2 h. The cells were washed three times with PBS pH 7.2 followed by incubation with rabbit anti-IgG conjugated to Alexa Fluor 568 and mouse anti-IgG conjugated to Alexa Fluor 488 for 1 h at 25 °C. The cells were washed three times with PBS pH 7.2 and imaged using DIC-epifluorescence microscopy.

Analysis of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$). AZ-521 cells were incubated with Tetramethylrhodamine ethyl ester perchlorate (TMRE) (50 nM; Sigma Aldrich) for 30 min before the end of each experiment. The cells were detached by mild trypsinization for 3 min at 37 °C with trypsin-EDTA and washed two times with PBS pH 7.2. TMRE fluorescence was quantified by flow cytometry in the FL2 channel (575/30-nm band pass filter). A total of 10,000 cells were analyzed for each sample.

Flow Cytometry. Analytical flow cytometry was carried out using a BD FACSCanto II flow analyzer (BD Biosciences) located at the R. J. Carver Biotechnology Center Flow Cytometry Facility (University of Illinois at Urbana–Champaign). The flow cytometer was equipped with a 70-μm nozzle, 488-nm line of an air-cooled argon-ion laser, and 400 mV output. The band pass filters used for analysis were 525/40 nm, 575/30 nm, and 675/30 nm. Cell analysis was standardized for scatter and fluorescence by using a suspension of fluorescent beads (Beckman Coulter). Events were recorded on a log fluorescence scale and the geometric mean and percent events were determined using FCS Express analysis software (De Novo Software). Forward and side scatter properties were considered to exclude noncellular (debris) events from viable and (or) dead cell populations.

DIC-Epifluorescence Microscopy. Fluorescence and DIC images were collected using a Delta Vision RT microscope (Applied Precision), EX 490/20 and EM 528/38, EX 555/28 and EM 617/73, EX 640/20 and EM 685/40 using Olympus Plan Apo 60× oil objective with NA 1.42 and working distance of 0.17 nm. Images were processed using DeltaVision SoftWoRx 3.5.1 software suite.

Statistical Analysis. Unless otherwise indicated, each experiment was performed at least three independent times. For those data requiring statistical analysis, data were combined from two or three independent experiments, as indicated, with each independent experiment carried out in triplicate. Statistical analyses were performed using Microsoft Excel (version 11.0). Unless otherwise noted, error bars represent SD. All P values were calculated with the Student's t test using paired, two-tailed distribution. $P < 0.05$ indicates statistical significance.

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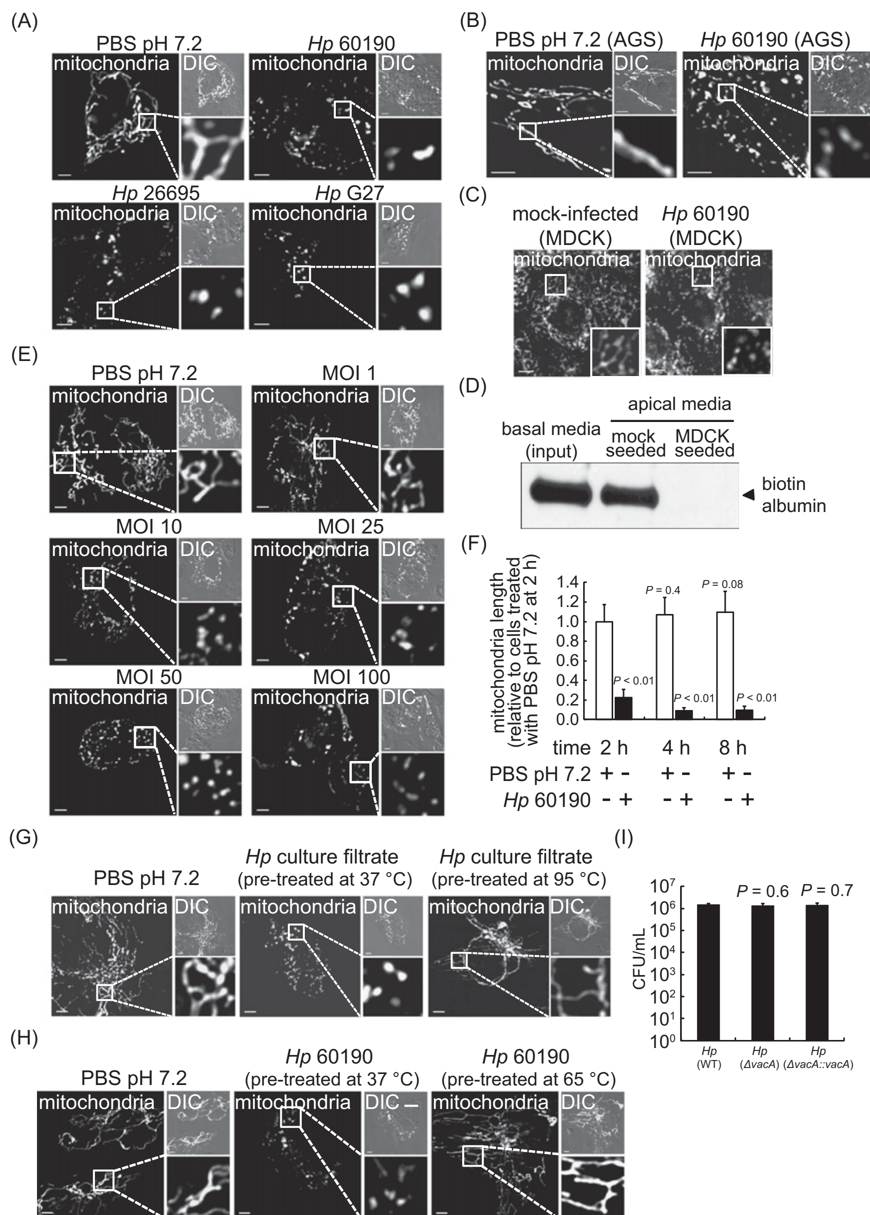

Fig. S1. Analysis of *Hp*-dependent mitochondrial fragmentation. AZ-521 cells (*A* and *E–H*) or AGS cells (*B*) that had been previously transfected with pDsRed2-Mito or polarized MDCK cells (*C*) were incubated at 37 °C and under CO_2 with *Hp* 60190 at MOI 100 (*A–C* and *F*) or the indicated MOI (*E*), or incubated with *Hp* 26695 (*A*) or *Hp* G27 (*A*) (all at MOI 100). In *G*, monolayers were incubated with HPCF that had been preincubated at either 37 °C or 95 °C for 30 min. In *H*, monolayers were incubated with *Hp* 60190 (MOI 100) that had been preincubated at either 65 °C or 37 °C for 30 min. Additionally, cells were mock infected with PBS pH 7.2 (*A–C* and *E–H*). After 8 h (*A–C*, *E*, *G*, and *H*) or at the indicated times (*F*), the cells were fixed (*A*, *B*, and *E–H*) or fixed, permeabilized, and immunostained for Tom-20 as a mitochondrial marker (*C*). The cells were evaluated for mitochondrial fragmentation using DIC-epifluorescence microscopy. The images reveal the morphology of fluorescently stained mitochondria (Scale bar, 5 μm.) and are representative of those collected from two (*A* and *C*) or three (*B* and *E–H*) independent experiments. Mitochondrial lengths were measured using Imaris 5.7 (Bitplane) software. (*D*) The integrity of the polarized MDCK monolayer was evaluated by monitoring the passage of biotin-BSA (50 μg/mL) from the transwell basal chamber through either the MDCK monolayer or mock-seeded transwell inserts (in the absence of cells) into the apical chamber. The presence of biotin-BSA within the apical or basal chamber was assessed by Western blot analysis, using the streptavidin-HRP conjugate and chemiluminescent signal development. The Western blot data are representative of those collected from two independent experiments, each with two independent MDCK monolayers. (*I*) Association of $vacA^+$ and $vacA^-$ *Hp* strains with AZ-521 cells was evaluated. AZ-521 cells were incubated at 37 °C and under CO_2 with *Hp* 60190, *Hp* VM022 (*ΔvacA*), or *Hp* VM084 (*ΔvacA::vacA*) (all at MOI 100) or mock infected with PBS pH 7.2. After 1 h, the monolayers were washed two times with PBS pH 7.2, lysed, and *Hp* associated with AZ-521 cells in each treatment was quantified by dilution plating and direct cfu counting. Data are rendered as the average cfu/mL obtained from combining data collected from two independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for differences in cfu/mL between *Hp* 60190 (WT) and *Hp* VM022 (*ΔvacA*) or *Hp* VM084 (*ΔvacA::vacA*).

Fig. S2. Characterization of VacA induced mitochondrial fragmentation. AZ-521 cells (*A* and *D*) or AGS cells (*B*) that had been previously transfected with pDsRed2-Mito or HeLa (*B*), HEp-2 (*B*) and polarized MDCK cells (*C*), were incubated at 37 °C and under CO_2 with VacA (250 nM), AZ-521 (*A* and *D*), and AGS (*B*) or 500 nM HeLa (*B*), HEp-2 (*B*), and MDCK (*C*). In *A*, cells were also incubated with VacA (250 nM) that had been pretreated for 30 min at 37 °C or 95 °C or for 30 min on ice with VacA antiserum (2 mg/mL) or a nonspecific *H. ducreyi* CdtA antiserum (2 mg/mL). Additionally, the cells were mock intoxicated with PBS pH 7.2 (*A*–*D*). After 4 h AZ-521 (*A*), AGS (*B*), and MDCK (*C*) or 8 h HeLa (*B*) and HEp-2 (*B*), the cells were fixed AZ-521 (*A*), AGS (*B*), or fixed, permeabilized, and immunostained for Tom-20 as a mitochondrial marker HeLa (*B*), HEp-2 (*B*), and MDCK (*C*), and cellular mitochondria were visualized by DIC-epifluorescence microscopy. (*D*) Mitochondria within each representative cell of AZ-521 monolayer that was previously intoxicated with VacA or mock treated with PBS pH 7.2 were monitored for the indicated time periods using live cell imaging. The images reveal the morphology of fluorescently stained mitochondria and are representative of those collected from two (*C* and *D*) or three (*A* and *B*) independent experiments. (Scale bar, 5 μm.)

Fig. S3. Evaluation of VacA-dependent Drp1 localization to mitochondria. AZ-521 cells that had been previously transfected with pDsRed2-Mito were incubated at 37 °C and under CO_2 with VacA (at the indicated concentrations) or mock intoxicated with PBS pH 7.2. After 4 h, the cells were fixed, permeabilized, and immunostained for cellular Drp1. Localization of Drp1 to mitochondria was determined using DIC-epifluorescence microscopy followed by colocalization analysis. The data are rendered as the average Drp1-mitochondrial colocalization obtained by combining data from two independent experiments. In each independent experiment, 15 randomly chosen, pDsRed2-Mito transfected cells were analyzed. Error bars indicate SD. Statistical significance was calculated for differences in colocalization indices between cells incubated with VacA at the indicated concentrations and those cells mock intoxicated with PBS pH 7.2.

Fig. S4. Effects of inhibiting Drp1 GTPase activity on mitochondrial fragmentation. AZ-521 cells (*A*, *C*, and *D*) or AGS cells (*B*), previously transfected with pDsRed2-Mito (*A*–*C*) and cotransfected with either pEGFP-Drp1 or pEGFP-DN-Drp1 (K38A) (*D*), were incubated at 37 °C and under CO_2 with VacA (250 nM) (*A*, *B*, and *D*) or infected with *Hp* 60190 (MOI 100) (*C*) or mock treated with PBS pH 7.2 (*A*–*D*), both in the absence or presence of mdivi-1 (50 μM) (*A*–*C*). After 4 h (*A*, *B*, and *D*) or 8 h (*C*), the cells were fixed and cellular mitochondria were visualized by DIC-epifluorescence microscopy. The images reveal the morphology of fluorescently stained mitochondria and are representative of those collected from three independent experiments. (Scale bar, 5 μm.)

Fig. S5. Relationships between Drp1 GTPase activity, Bax activation, and mitochondrial Cyt *c* release. AZ-521 cells (*A*, *C*, and *E*), AZ-521 cells that had been transfected with pEFGP-Drp1 or pEGFP-DN-Drp1 (K38A) (*B* and *F*) or $bax^{+/+}$ or $bax^{-/-}$ MEFs that had been previously transfected with pDsRed2-Mito (*D*) were incubated at 37 °C and under CO_2 with VacA (250 nM) (*A*–*C*, *E*, and *F*) 500 nM (*D*), as preliminary studies indicated that MEFs are less sensitive to VacA than AZ-521 cells). (*A*) Studies were conducted in the absence or presence of mdivi-1 (50 μM). After 8 h (*A*, *B*, *D*, and *F*) or at the indicated times (*C* and *E*), the monolayers were evaluated for total cellular Bax using flow cytometry (*A*), activated Bax using DIC-epifluorescence microscopy (*B* and *C*), mitochondrial fragmentation (*D*), or Cyt *c* release (*E* and *F*). For *A*, the data were rendered as the fold change in total cellular Bax between cells incubated with VacA and those mock intoxicated with PBS pH 7.2, both in the presence or absence of mdivi-1 and were obtained by combining data from two independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for fold differences in Bax levels between cells incubated with PBS pH 7.2 alone versus cells pretreated with mdivi-1 or cells treated with VacA in the presence or absence of mdivi-1. For *B* and *C*, the cells were fixed, permeabilized, and immunostained for activated Bax. Intracellular-activated Bax within each transfected cell was visualized by DIC-epifluorescence microscopy. (Scale bar, 5 μm.) (*B*) Images are representative of those collected from three independent experiments. (*C*) Data were rendered as the fraction of the entire population of cells incubated with VacA or PBS pH 7.2 that display active Bax, at the indicated time periods. Data were obtained by analyzing over 700 cells from randomly chosen fields over the course of two independent experiments. Error bars indicate SD. Statistical significance was calculated for differences in fraction of total cells that display Bax activation between cells treated with VacA and those treated with PBS pH 7.2 or between cells intoxicated with VacA for indicated time periods versus those intoxicated for 1 h. For *D*, the cells were fixed, and cellular mitochondria were visualized using DIC-epifluorescence microscopy. The images reveal the morphology of fluorescently stained mitochondria and are representative of those collected from three independent experiments. (Scale bar, 5 μm.) For *E* and *F*, the cells were fixed, permeabilized, and immunostained for Cyt *c*. Cellular localization of Cyt *c* within each transfected cell was visualized by DIC-epifluorescence microscopy. (Scale bar, 5 μm.) For *E*, data were rendered as the fraction of the entire population of cells incubated with VacA or PBS pH 7.2 that display Cyt *c* release, at the indicated time periods. Data were obtained by analyzing over 1,000 cells from randomly chosen fields over the course of two independent experiments. Error bars indicate SD. Statistical significance was calculated for differences in fraction of total cells that display Cyt *c* release between cells intoxicated with VacA and those treated with PBS pH 7.2 or between cells intoxicated with VacA for indicated time periods versus those intoxicated for 1 h. (*F*) Images are representative of those collected from three independent experiments.

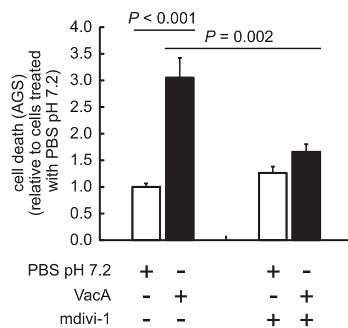

Fig. S6. Drp1 GTPase activity and VacA induced cell death in AGS cells. AGS cells were incubated at 37 °C and under CO_2 with VacA (250 nM) or mock treated with PBS pH 7.2, both in the absence or presence of mdivi-1 (50 μM). After 24 h, cell viability was determined with the Live-Dead viability/cytotoxicity assay kit, using flow cytometry. The data were rendered as the fold increase in dead cells following VacA intoxication relative to cells mock treated with PBS pH 7.2, obtained by combining data collected from two independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for fold differences in cell death between cells intoxicated with VacA versus cells treated with PBS pH 7.2 in the presence or absence of mdivi-1.

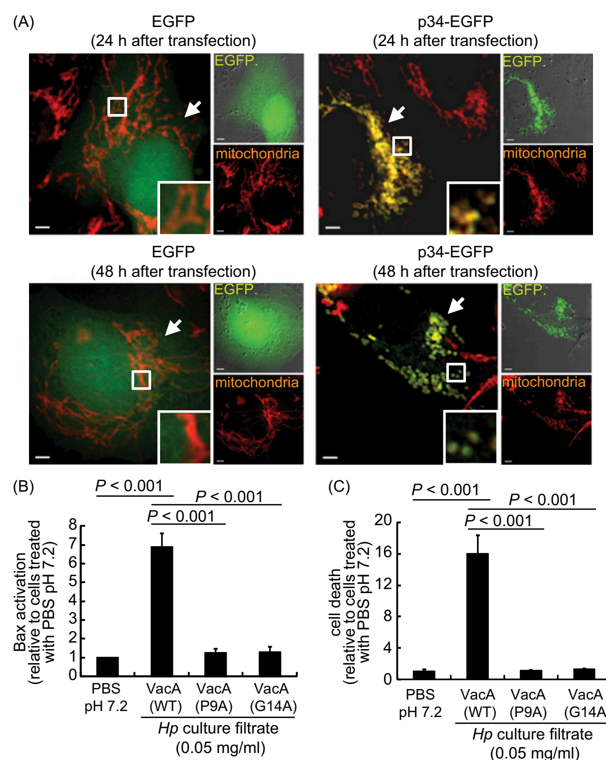

Fig. S7. Role of VacA anion channel function in mitochondrial morphology changes, Bax activation, and cell death within AZ-521 cells. AZ-521 cells were transfected with plasmids encoding p34-EGFP (residues 1–319) or EGFP (*A*) or incubated at 37 °C and under CO_2 with HPCFs (0.05 mg/mL) prepared from *Hp* 60190 or *Hp* strains expressing VacA (P9A) or VacA (G14A) (*B* and *C*), which are mutant forms of VacA deficient in membrane channel activity (1, 2). (*A*) After 24 h or 48 h the cells were fixed, permeabilized, and immunostained for Tom-20 as a mitochondrial marker, followed by visualization of cellular mitochondria and p34-EGFP and EGFP using DIC-epifluorescence microscopy. Images include, as indicated, mitochondria (red), p34-EGFP, or EGFP (green). Arrows (white) indicate cells positive for p34-EGFP or EGFP expression. Images are representative of those collected over the course of two independent experiments ($n = 60$). (Scale bar, 5 μm.) (*B*) After 18 h, the cells were fixed, permeabilized, and immunostained for activated Bax. The data were rendered as the fold change in activated Bax levels between cells incubated with the indicated HPCF compared with monolayers mock intoxicated with PBS pH 7.2, obtained by combining data collected from three independent experiments, each conducted in triplicate. (*C*) After 24 h, the percentage of dead cells was determined using the Live-Dead viability/cytotoxicity assay kit. The data were rendered as the fold increase in dead cells between monolayers incubated with the indicated HPCF relative to monolayers mock intoxicated with PBS pH 7.2, obtained by combining data collected from three independent experiments, each conducted in triplicate. Statistical significance was calculated for differences in fold-activated Bax (*B*) or cell death (*C*) between cells mock intoxicated with PBS pH 7.2 and those cells incubated with HPCF, HPCF-VacA (P9A), or HPCF-VacA (G14A).

Fig. S8. VacA localizes to mitochondria. AZ-521 cells were incubated with VacA (100 nM) at 37 °C and under CO_2. After 30 min, the cells were fixed, permeabilized, and immunostained for VacA, and Tom-20 as a mitochondrial marker, and analyzed for localization of VacA to mitochondria. Image includes, as indicated, mitochondria (red), VacA (green), and VacA colocalized to mitochondria (yellow). Image is representative of those collected over the course of three independent experiments. (Scale bar, 5 μm.)

Fig. S9. The effects of inhibiting Drp1 GTPase activity on VacA-dependent dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$). AZ-521 cells were incubated at 37 °C and under CO_2 with VacA (250 nM) or mock intoxicated with PBS pH 7.2, both in the absence or presence of mdivi-1 (50 μM). After 4 h, the relative $\Delta\Psi_m$ was determined using flow cytometry analysis of TMRE (50 nM)-stained cells. The data are rendered as the fold change in TMRE uptake in cells intoxicated with VacA relative to cells mock intoxicated with PBS pH 7.2, obtained by combining data collected from three independent experiments, each conducted in triplicate. Error bars indicate SD. Statistical significance was calculated for differences in $\Delta\Psi_m$ between cells mock intoxicated with PBS pH 7.2 versus cells incubated with mdivi-1 or cells incubated with VacA in the presence or absence of mdivi-1.