Vacuolating Cytotoxin of Helicobacter pylori Plays a Role during Colonization in a Mouse Model of Infection

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Helicobacter pylori, the causative agent of gastritis and ulcer disease in humans, secretes a toxin called VacA (vacuolating cytotoxin) into culture supernatants. VacA was initially characterized and purified on the basis of its ability to induce the formation of intracellular vacuoles in tissue culture cells. H. pylori strains possessing different alleles of vacA differ in their ability to express active toxin. Those strains expressing higher toxin levels are correlated with more severe gastric disease. However, the specific role(s) played by VacA during the course of infection and disease is not clear. We have used a mouse model of H. pylori infection to begin to address this role. A null mutation of vacA compromises H. pylori in its ability to initially establish infection. If an infection by a vacA mutant is established, the bacterial load and degree of inflammation are similar to those associated with an isogenic wild-type strain. Thus, in this infection model, vacA plays a role in the initial colonization of the host, suggesting that strains of H. pylori expressing active alleles of vacA may be better adapted for host-to-host transmission.

Helicobacter pylori infection of the human stomach can result in a broad spectrum of disease outcomes ranging from mild gastritis to severe ulcers (8). Additionally, H. pylori is associated with two types of cancer: gastric lymphoid tissue-associated B-cell lymphoma (32, 34) and gastric adenocarcinoma (25). The disease outcome in each infected individual appears to be determined by a combination of host and bacterial factors. The genotypes of H. pylori clinical isolates vary in many genetic loci, including the presence or absence of a pathogenicity island (1, 5) and allelic variation of the vacuolating cytotoxin gene (vacA) (2) and genes encoding adhesion molecules such as BabA2, which binds the Lewisb fucosylated moiety found on human gastric tissue (16). Epidemiological studies suggest that strains expressing the pathogenicity island, those expressing high levels of VacA, and those expressing functional BabA2 correlate with more severe disease (8, 13).

VacA enters eukaryotic cells and exerts its action in the cytoplasm (9, 12). VacA recently was shown to form chloride-conducting channels in both artificial and cellular lipid bilayers (30). Additionally, cells exposed to VacA accumulate vesicles containing rab7, a cellular marker of the late endosome, and lgp110, a marker of lysosomes (21, 24). This VacA-induced alteration of intracellular membranes has been shown to disrupt normal lysosomal degradation of surface receptors in epithelial cells (28) and to interrupt antigen processing in immune cells (22). Comparison of vacA gene sequences among clinical isolates has revealed variability both in the coding region of the signal sequence and in the middle region of the functional protein. Certain alleles of the signal sequence correlate both with higher expression of active toxin and with more severe disease (2). Alleles of the middle region probably act in targeting and internalization of the toxin but do not affect toxin activity once it enters the host cell cytoplasm (23).

The mechanisms by which VacA contributes to infection and disease have remained elusive. Vacuolization of cells in human biopsy samples has been observed (4, 11), and oral administration of partially purified toxin to mice was shown to cause measurable epithelial damage (14). However, isogenic vacA mutants not only colonize but also cause indistinguishable degrees of gastritis in both gnotobiotic pigs (10) and Mongolian gerbils (33). These results, suggesting that vacA is not a virulence factor, contradict the human epidemiology data. This may reflect differences in the animal models relative to the human host or may indicate that VacA is not essential for the establishment or persistence of H. pylori infection. The latter conclusion is particularly unsatisfying since the presence of vacA seems to distinguish H. pylori from Helicobacter species that do not infect humans or interact intimately with the gastric epithelium in their natural hosts (19).

We decided to reexamine the role of VacA in an established mouse model of infection using H. pylori strain SS1 in C57BL/6NTac mice (20). In this model system, we found that isogenic vacA null mutants are severely defective in the ability to establish initial colonization of the host, which profoundly attenuates the virulence potential of these strains.

MATERIALS AND METHODS

Bacterial and cell culture. The mouse-adapted H. pylori strain SS1 was used for these studies (20). H. pylori was grown on solid media on horse blood agar (HB) plates, containing 4% Columbia agar base (Oxoid), 5% defibrinated horse blood (HemoStat Labs), 0.2% β-cyclodextrin (Sigma), 10 μg of vancomycin (Sigma) per ml, 5 μg of cefuroxim (Sigma) per ml, 2.5 U of polyoxymen B (Sigma) per ml, 50 μg of cycloheximide (Sigma) per ml, 5 μg of trimethoprims (Sigma) per ml, and 8 μg of amphotericin B (Sigma) per ml, under microaerobic conditions at 37°C. A microaerobic atmosphere was generated either by using a CampyGen sachet (Oxoid) in a gas pack jar or by incubating the culture in an incubator equilibrated with 10% CO2 and 90% air. For liquid culture, H. pylori was grown in brucella broth (Difco) containing 10% fetal bovine serum (Gibco/BRL) (BB10) with shaking in a microaerobic atmosphere. Escherichia coli growth and...
manipulations were performed as specified by standard laboratory protocols (3). AGS cells were grown in Dulbecco modified Eagle medium with high glucose, t-glutamine, sodium pyruvate, and pyridoxine hydrochloride (Gibco/BRL) supplemented with 10% fetal bovine serum.

Construction of vacA and cagA mutant strains and restored derivatives. The AuvacA-aphA3 (∆V) derivative of SS1 was made by transforming SS1 with 2 μg of genomic DNA prepared from strain 342Δv (provided by Marta Marchetti), using natural transformation (http://www.metazoa.com/PLP3244). 342ΔV contained the Campylobacter coli aphA3 gene, conferring kanamycin resistance, inserted at nucleotide 1392 (amino acid 296) of the vacA coding sequence (31). Kanamycin-resistant colonies were isolated on HB plates containing kanamycin (30 μg/ml). The C-capCα derivative of SS1 was made by subcloning the C. coli aphA3 gene from pILL520 (17) into the NotI site at position 1053 of the cagA gene in pBSsCagA (7).

To make an independent vacA mutant in SS1, we first amplified the entire coding region of vacA from strain NCTC 11638 (accession number U07145) (26) using PCR with primers VN (CGCTTGTGATGACACCCCCACA) and VC (GC GATCCTGCGATGATAAG) in reaction mixtures containing 4 μM (each) primers and 20 ng of NCTC 11638 genomic DNA. The resulting reaction product was gel purified and cloned into the TopoXO vector using the TopoXL kit (Invitrogen) as specified by the manufacturer. Plasmids from two resulting clones, TV2 and TV7, that contained the expected 4-kb inserts were sequenced to confirm that they contained the expected gene. DNA sequencing revealed that both clones contained several mutations. However, the three mutations in pTV7 were confined to a SpfI fragment which contained no mutations in the pTV2 clone. Therefore, the SpfI fragment from pTV2 was subcloned into pTV7. Sequencing revealed that the new clone, pTV2/7, contained the entire vacA coding region with no mutations. pVacKanSacB was made by subcloning an XhoI-Smal fragment from pBSFII (6) containing the aphA3 gene, conferring kanamycin resistance, and the sacB gene, conferring sucrose sensitivity, into pTV2/7 that had been digested with SpfI, removing the DNA between positions 1332 and 2340 of the coding sequence. SS1 was then transformed with 10 μg of pVacKanSacB using natural transformation, and kanamycin-resistant clones were selected as described above. Several of the resulting colonies were screened for sucrose sensitivity (from the sacB gene) by plating on HB plates containing 6% sucrose. One kanamycin-resistant, sucrose-sensitive clone VS3 was further analyzed. PCR amplification with primers flanking the SpfI sites (VS4 [GATAAAACACTCCTAAAG] and VS5 [TTAGCTTGATGACTAGCT]) within the aphA3 gene (Aph3out [GGCGTATAACATAGTAGCTAC]) was used to confirm that a single crossover event had occurred. The vacA locus was then restored to the wild-type sequence by transforming VS3 with pTV2/7 and selecting sucrose-resistant colonies. Clone 3.1 was additionally found to be kanamycin sensitive, suggesting a double crossover, and again PCR was used to confirm the double crossover as described above.

Immunoblot detection of VacA. The presence of the VacA protein in bacterial extracts was determined by resuspending half of a blue inoculating loop (Nunc) full of plate-grown bacteria in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (3). This sample was fractionated by SDS-PAGE (10% polyacrylamide), and the proteins were transferred to a Hybond-P membrane (Amersham). The resulting blot was incubated with rabbit anti-VacA polyclonal serum (1:10,000) (provided by Antonello Covacci [31] in TBS-T (50 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Tween 20) followed by a secondary anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (Amersham) at the same dilution. The immunoreactive bands were visualized using the ECL-Plus detection system as specified by the manufacturer. Plasmids from two resulting clones, TV2 and TV7, were gel purified and cloned into the TopoXL vector using the TopoXL kit (Invitrogen). Determination of ID50. To determine the 50% infective dose (ID50), mice were infected with serial dilutions of bacteria as described above. The inoculum was diluted and plated to determine the actual bacterial dose. After 1 month the mice were sacrificed and the stomachs were cultured as above to determine the number of animals infected at each dose. The Reed-Muench calculation was then used to determine the ID50 (27). Mixed infection in vitro. Bacteria were resuspended in BB10 after 1 day of growth on plates, and the OD600 was determined. A mixture containing approximately 3×109 of both VS3 (vacA::aphA3::SacB) and 3.1 (vacA) bacteria were inoculated into 5 ml of BB10 for the 0.5-day time point. A parallel 5-ml culture was made by diluting this culture 10-fold for the 1-day time point. For subsequent time points, 3×108 bacteria from that day’s culture were diluted into 5 ml of fresh BB10 and grown for 24 h. All cultures were grown with shaking in a microaerobic atmosphere. At 0, 0.5, 1, 2, 3, 4, and 5 days, the cultures were plated on both plain plates and plates supplemented with kanamycin to distinguish VS3 (KanR) from 3.1 (KanR). The percentage of wild-type bacteria was determined using the above equation.

RESULTS

Wild-type H. pylori outcompete vacA mutant bacteria in the mouse stomach. To assess the role of VacA in colonization, we made a vacA mutant derivative (ΔV) of the mouse-adapted H. pylori strain, SS1 (20), by natural transformation with genomic DNA from H. pylori strain 342Δv, which contained the aphA3 gene from C. coli, conferring kanamycin resistance, inserted into the vacA coding region and selection of a kanamycin-resistant clone. First we tested the infecting potential of a high dose (9×108 CFU) of wild-type and mutant bacteria alone and in a co-infection experiment with a 50:50 mixture of the two strains. In the mixed infection, we could distinguish mutant bacteria from wild-type bacteria on the basis of the kanamycin resistance gene used to generate the vacA mutation. Each strain could infect mice, and the infections resulted in a similar bacterial load after 1 month (Table 1). In the mixed infection, however, only wild-type bacteria were recovered. We repeated this experiment using both 50:50 and 90:10 ratios of mutant to wild-type bacteria. In both cases, we could recover only wild-type bacteria from infected animals (Table 2).

The vacA mutant has a 320-fold-higher ID50 than the wild-type strain dose. We next tested both the wild-type and vacA mutant (ΔV) strains to determine the precise ID50. In this experiment, we infected mice with 10-fold serially diluted bacteria and determined the number of animals colonized at each dose. The Reed-Muench calculation was used to determine the number of bacteria required to obtain colonization of 50% of the animals (27). Table 3 shows that the ID50 for the wild-type strain was less than 5×107 bacteria while the vacA mutant had an ID50 of 1.6×109. This is at least a 320-fold difference. To rule out possible effects of the presence of the aphA3 gene, which confers kanamycin resistance in the mutant strain, we determined the ID50 of another mutant, generated by insertion of the aphA3 gene into its coding region, the cagA gene, shown previously to colonize animal models of infection (33).
We also observed good colonization of the cagA mutant both alone and in competition with wild-type bacteria (data not shown). The ID_{50} of the cagA mutant was 5.4 \times 10^5, similar to that of the wild-type bacteria. We also determined the localization and density of infection by staining histological sections of infected animals with the Warthin-Starry stain to visualize the bacteria. In animals infected with both the wild-type and vacA mutant bacteria both strains were localized primarily in the stomach antrum, with some bacteria being found in the cardia at the junction of the forestomach and the stomach. The density of infection observed by histological examination appeared similar for the two strains, consistent with the culture results. Examination of the stomachs with hematoxylin and appeared similar for the two strains, consistent with the culture density of infection observed by histological examination at the junction of the forestomach and the stomach. The bacteria. In animals infected with both the wild-type and of infected animals with the Warthin-Starry stain to visualize the bacteria. We could also observed good colonization of the cagA mutant both alone and in competition with wild-type bacteria (data not shown).

### TABLE 1. Colonization of wild-type and vacA::aphA3 H. pylori in mice after 1 month alone and in competition

<table>
<thead>
<tr>
<th>Input</th>
<th>WT of stomach (g)</th>
<th>Log (CFU/g) of wild-type plus mutant</th>
<th>Log (CFU/g) of mutant</th>
<th>Log CFU/g</th>
<th>% Colonization</th>
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<tr>
<td>WT + ΔV (50:50)</td>
<td>5.8</td>
<td>NC</td>
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<td>WT + ΔV (50:50)</td>
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<td>WT + ΔV (50:50)</td>
<td>5.2</td>
<td>NC</td>
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<tr>
<td>WT + ΔV (90:10)</td>
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<td>NC</td>
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<td></td>
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<tr>
<td>WT + ΔV (90:10)</td>
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<td>NC</td>
<td></td>
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<tr>
<td>WT + ΔV (90:10)</td>
<td>5.9</td>
<td>NC</td>
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</table>

### TABLE 2. Colonization of wild-type and vacA::aphA3 H. pylori strains in mice

<table>
<thead>
<tr>
<th>Dilution (CFU/ml)</th>
<th>No. of mice after 1 mo.</th>
<th>Total no. infected</th>
<th>Total no. uninfected</th>
<th>% Infected</th>
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<td>Wild type</td>
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<td>18</td>
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<tr>
<td></td>
<td>5 \times 10^6</td>
<td>5</td>
<td>0</td>
<td>13</td>
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<td></td>
<td>5 \times 10^5</td>
<td>5</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5 \times 10^4</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>ΔvacA::aphA3</td>
<td>5 \times 10^6</td>
<td>3</td>
<td>3</td>
<td>1</td>
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<tr>
<td></td>
<td>5 \times 10^5</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
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<td></td>
<td>5 \times 10^4</td>
<td>0</td>
<td>4</td>
<td>0</td>
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<tr>
<td>ΔcagA::aphA3</td>
<td>7.9 \times 10^6</td>
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<td>0</td>
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<td>2</td>
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<td></td>
<td>7.9 \times 10^2</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

### TABLE 3. ID_{50} determination for wild-type, vacA::aphA3, and cagA::aphA3 strains in mice

- Individual mice were infected with wild-type SS1 bacteria or mutant derivatives of SS1 at the indicated dilutions. Reed-Muench ID_{50} calculations were <5 \times 10^5 for wild type, 1.6 \times 10^5 for ΔvacA::aphA3, and 5.4 \times 10^4 for ΔcagA::aphA3.
- The number of mice infected or uninfected at each dilution after 1 month.
- Cumulative total of mice infected from the lowest dilution.
- Cumulative total of mice uninfected from the highest dilution.
- Percentage of total mice infected.
distinguish the \textit{vacA} mutant, VS3, from the strain restored to wild type, 3.1, because VS3 contains a kanamycin resistance cassette, allowing selection on kanamycin-containing plates. After 1 month of infection, both strains were able to infect mice. The bacterial loads in the stomachs of mice infected with each strain were similar (VS3, $1.6 \times 10^5$ CFU/g; 3.1, $6.9 \times 10^5$ CFU/g). However, as above, only the restored wild-type bacteria could be recovered from a mixed infection (Fig. 2). Thus, strain 3.1, containing a reconstituted \textit{vacA} gene, was indistinguishable from the wild type in its capacity to colonize animals and to outgrow a \textit{vacA} mutant strain.

**Kinetics of mixed infection.** To determine when the \textit{vacA} mutant bacteria were disappearing from the infection, we infected 24 mice with a 50:50 mixture of \textit{vacA} mutant (VS3) and wild-type (3.1) bacteria. Four mice were sacrificed on each of days 0, 1, 2, 7, 14, and 28, and the viable counts of both strains were determined. The initial inoculum contained $2 \times 10^8$ bacteria of each strain. Immediately after infection, approximately $10^8$ total organisms could be recovered (Fig. 3A). After 1 day, bacteria could be recovered from only one mouse, which had 1,600 CFU/g. On day 2, approximately $2 \times 10^5$ organisms were recovered from all four animals. By day 7, the bacterial load reached approximately $5 \times 10^5$ CFU/g for all four animals and remained at this level for the 14- and 28-day time points. We also determined the percentage of wild-type and mutant bacteria in each of the animals (Fig. 3B). At the time zero, approximately equal numbers of wild-type and mutant bacteria were recovered (the average percent wild type was 48%). The single infected animal at the 1-day time point had 70% wild-type bacteria and only 30% mutant bacteria. By 2 days, three mice were infected with 100% wild-type bacteria while one mouse had 9% mutant bacteria and 91% wild-type bacteria. At the later time points, all the mice were infected with only wild-type bacteria, except for one mouse at the 14-day time point, in which 33% of the bacteria recovered carried the \textit{vacA} mutation.

In a parallel experiment, we checked the growth of a similar mixed culture in vitro at 0, 0.5, 1, 2, 3, 4 and 5 days. At all time points, the culture consisted of approximately 50% wild-type and 50% mutant bacteria (Fig. 3B). This is in stark contrast to the in vivo experiment, where the mutant bacteria represented only 30% of the population after 1 day and were essentially absent after 2 days. Thus, the \textit{vacA} mutation seems to confer a specific disadvantage for colonization rather than growth.

**DISCUSSION**

In contrast to previous studies (10, 33), we have shown that two independent mutations in the \textit{vacA} gene of \textit{H. pylori} strain SS1 resulted in bacteria that could not survive in mice in the presence of competing wild-type bacteria. The fact that a bio-
logical effect could be revealed only in a competition experiment might suggest a subtle virulence phenotype for this gene. However, the \textit{vacA} mutant had an ID$_{50}$ fully 2 log units higher than that of the wild-type bacteria. The reasons why no role for \textit{vacA} could be demonstrated in previous studies may reflect differences in the animal models used. Alternatively, the lack of phenotype may have resulted from the general practice of using very high inocula for animal infections, usually several log units above the ID$_{50}$, and multiple rounds of infection. Interestingly, by using a competition experiment, we were able to see a phenotype, even at a dose above the ID$_{50}$ for the mutant strain. This should provide an improved protocol for testing new potential virulence genes.

We were able to localize the defect of the \textit{vacA} mutant to a role in colonization by determining the kinetics of infection during coinfection with the wild-type bacteria. After an initial inoculation of an equal mixture of $2 \times 10^8$ mutant and wild-type organisms, the challenged animals cleared most of the bacteria. On day 1, few viable or cultivable organisms could be detected. However, by day 2, significant numbers of bacteria were detected, and this number increased on day 7, after which the bacterial load remained relatively constant over 1 month of observation. Examination of the genotype of the bacteria recovered from the mouse stomach revealed that a significant proportion of the mutant bacteria were present only immediately after inoculation. By day 2 postinfection, generally only wild-type bacteria could be recovered from the stomach. That 2 of 16 mice showed a small percentage of mutant bacteria may

FIG. 3. Kinetics of mixed infection with a 50:50 mixture ($2 \times 10^8$ CFU each) of \textit{vacA} mutant (VS3) and restored wild-type (3.1) strains in vivo and in vitro. (A) Total number of bacteria recovered from the stomachs of each of four mice on days 0, 1, 2, 7, 14, and 28 after infection. (B) Percentage of wild-type bacteria recovered at each time point during infection of mice (diamonds) or during growth in vitro (squares).
reflect the residual ability of this mutant to establish infection on its own or, since VacA is a secreted protein, trans-translation from nearby wild-type bacteria.

The mechanism by which VacA facilitates colonization is not known; however, VacA host cell toxicity has been well documented. This toxicity may stimulate host cell turnover, presenting a new cell type to which H. pylori can adhere. We examined the ability of the vacA mutant to adhere to a gastric epithelial cell line but found no obvious differences (data not shown). Further studies with primary gastric epithelial cells may shed more light on this hypothesis. Alternatively, VacA may cause tissue damage that alters the local environment by releasing nutrients or altering the local pH, allowing H. pylori to survive. Local or global hypochlorhydria in the stomach has been postulated to have a significant impact on H. pylori colonization and to play a role in the expression of disease. Prolonged H. pylori infection can induce the host to produce autointimies against the Lewis' antigen found on parietal cells, resulting in loss of this acid-secreting cell type (15, 29). In the mouse model, inhibitors of acid secretion can change the distribution of H. pylori from a localized infection of the antrum to colonization of the entire stomach (18). It is possible that VacA plays a role in the local inhibition of acid secretion due to cell damage, which then facilitates the ability of H. pylori to establish infection.

VacA may increase the ability of H. pylori to be transmitted from host to host. There is considerable sequence variation in the vacA gene in different clinical isolates. These different alleles vary in VacA expression levels. Epidemiological studies show that strains expressing high levels of the vacA correlate more highly with ulcer disease. This could result from direct effects of the toxin or could occur because VacA promotes higher levels of sustained colonization in the stomach, possibly in a precise anatomic region of the stomach. The presence of low-expressing alleles could reflect a delicate balance between high levels of the toxin allowing successful transmission but also causing too much damage to the host.

The mouse model of H. pylori infection appears to reflect primarily the capacity to colonize animals and does not accurately reflect the inflammatory response seen during natural human infection. Hence, we may be assessing only one aspect of the contribution of H. pylori virulence. Nevertheless, our findings do present an interesting and unexpected facet of the effect of VacA on the biology of H. pylori infection and disease.

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