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Spatiotemporal Analysis of Acid Adaptation-Mediated *Vibrio cholerae* Hyperinfectivity

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Acid adaptation has previously been shown to increase the infectivity of *Vibrio cholerae* in the infant mouse model. To better understand this phenomenon, we monitored the spatial distribution and temporal changes in the ratios of acid-adapted cells to unadapted *V. cholerae* cells in the small intestine, as well as the timing of virulence factor expression. We found that the competitive advantage afforded by acid adaptation does not become manifest until greater than 3 h postinfection; thus, acid adaptation does not increase *V. cholerae* passage through the gastric acid barrier. Additionally, acid-adapted and unadapted *V. cholerae* cells colonize the same sections of the small intestine and show similar kinetics of transcriptional induction of the virulence genes *tcpA* and *ctxA*. These studies suggest that the increased infectivity of acid-adapted *V. cholerae* is due to a more rapid onset of multiplication and/or to an increased multiplication rate within the infant mouse intestine.

For oral route pathogens, such as *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Vibrio cholerae*, the ability to survive passage through the human gastric acid barrier is a crucial component of the bacterial life cycle. This ability is believed to be linked to an adaptive stress response to acid known as the acid tolerance response (ATR) (7, 9, 11). It is also possible that the ATR may enhance survival and growth within the intestinal tract after passage through the stomach. Analysis of *V. cholerae* has revealed a number of genes that play crucial roles in ATR (8–10). Additionally it was shown that ATR increases bacterial infectivity: specifically, acid-adapted *V. cholerae* outcompeted unadapted *V. cholerae* by 1 order of magnitude after 24 h of coinfection in the infant mouse but did not outcompete the unadapted organism during in vitro growth (9). Mechanistically, this could be explained by increased survival during transit through the stomach, increased fitness within the small intestine, or a combination of the two. In this work, we investigate the nature of ATR-induced *V. cholerae* hyperinfectivity and observe a growth advantage of adapted versus unadapted *V. cholerae* cells at middle to late stages of a 24-h infection. In addition, we evaluate the primary sites of colonization of acid-adapted *V. cholerae* compared to those of unadapted cells and measure the effects of acid adaptation on the kinetics of transcriptional induction of two critical virulence factors, cholera toxin (CT) and toxin-coregulated pilus (TCP).

Gastric survival profile. To determine whether hyperinfectivity of acid-adapted *V. cholerae* was due to an increased ability to survive transit through the gastric barrier, we analyzed the temporal population dynamics during competition assays. In this assay, differentially marked acid-adapted and unadapted strains were coinfected into 5-day-old CD-1 infant mice as previously described (9). Briefly, overnight cultures of isogenic *lacZ*$^+$ and *lacZ* mutant *V. cholerae* strains AC51 and AC168 (9) were subcultured to fresh Luria-Bertani (LB) broth and grown to mid-exponential phase (optical density at 600 nm of 0.2 to 0.3). These cultures were then divided such that 0.1 ml of the culture was resuspended in 1 ml of LB broth, pH 7.0, and 0.9 ml was resuspended in 1 ml of LB broth, pH 5.7. These cultures were incubated for 1 h at 37°C with aeration, subsequently mixed together, and then used for animal infections. Due to growth of bacteria at pH 7.0 but growth arrest at pH 5.7, this treatment results in an approximately 1:1 ratio of acid-adapted to unadapted bacteria in the final mixture. Approximately 10^6 total CFU was inoculated intragastrically into 5-day-old CD-1 mice as described previously (1). At the indicated times postinoculation (Fig. 1), the small intestines of infected animals were removed and homogenized in 2 ml of LB broth supplemented with 20% glycerol. Homogenates were serially diluted and plated on LB agar containing 100 μg of spectinomycin ml^{-1}, 50 μg of ampicillin ml^{-1}, and 40 μg of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) ml^{-1} to determine ratios of LacZ$^+$ to LacZ$^-$ bacteria. As illustrated in Fig. 1, 80% of the inoculum is killed within 1 h, and by 3 h, less than 3% of the inoculum remains in the small intestine. The transit time through the small intestine and into the large intestine is approximately 3 h (1), and therefore the reduction in the number of CFU at 3 h is a combination of bacterial death in the small intestine and passage of bacteria out of the small intestine. Surprisingly, the ratios of adapted to unadapted CFU are virtually identical at these early time points. It was previously found that *V. cholerae* transits the stomach of the infant mouse within 1 h (1); therefore, the results at early time points in Fig. 1 indicate that acid adaptation does not confer a direct survival advantage during passage through the

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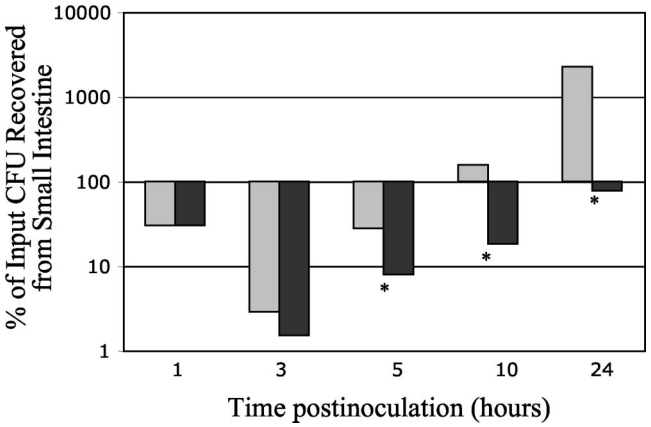

FIG. 1. Percentages of recovery of input CFU of competing acid-adapted and unadapted strains from the infant mouse small intestine. At various times listed on the *x* axis, *V. cholerae* was recovered and the ratio of acid-adapted (light bars) to unadapted (dark bars) cells was determined as described in the text. Each data column represents the geometric mean of results from three independent experiments using one or two mice per time point in each experiment. The *y* axis reflects the percentages of input CFU recovered. The asterisks indicate a *P* of <0.1 (Student's two-tailed *t* test) where acid-adapted bacteria outcompete unadapted bacteria at the times indicated.

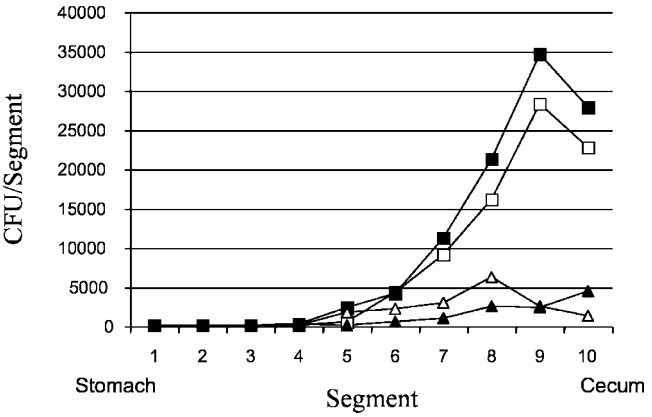

FIG. 2. Recovery of acid-adapted (squares) or unadapted (triangles) *V. cholerae* organisms after 10-h single-strain infections. The *x* axis represents individual segments of small intestine starting immediately distal to the stomach and continuing for a total of 10 cm (numbers 1 to 10) until reaching but not including the cecum. Open and filled symbols represent the results of independent experiments from which each symbol represents the average for two mice.

stomach of the infant mouse. However, at 5 h postinoculation, a statistically significant difference is observed between the numbers of adapted and unadapted *V. cholerae* bacteria. This difference is greater by 10 h, and by 24 h the ratio of adapted to unadapted cells is 30-fold greater (9). These results suggest that acid adaptation does not increase the survival of *V. cholerae* during passage through the stomach. Instead, it appears that acid adaptation either enhances survival during later stages of infection or allows for replication to commence sooner. The latter supposition is supported by the fact that there is on average a 10-fold increase in the absolute number of acid-adapted bacteria between 3 and 5 h but only on average a 3-fold increase in the absolute number of unadapted *V. cholerae* cells during the same time period (Fig. 1).

At 24 h, the unadapted *V. cholerae* organisms reach a cell number that is approximately equal to the original input. This result is in contrast to the population dynamics seen in single-strain infections, wherein unadapted wild-type *V. cholerae* El Tor has a cell count at 24 h postinoculation that is typically 10-fold greater than the initial input (1). It has previously been hypothesized that there are a limited number of colonization sites within the infant mouse small intestine (2). The differences between the competition data presented in Fig. 1 and our previous single-strain infection data (1) support this hypothesis, at least for the later times of infection. At these later times, but not necessarily at early times in the infection, presumably the unadapted bacteria are being outcompeted by the acid-adapted bacteria either for sites of attachment or for nutrients.

Spatial distribution of colonization sites. Although the results in Fig. 1 show that increased gastric survival is not responsible for the hyperinfectivity phenotype, they do not preclude the possibility that acid-adapted *V. cholerae* strains are capable of colonizing additional regions of the small intestine, for instance, colonization sites closer to the stomach. To test this possibility, two infant mice were infected with either acid-adapted or unadapted *V. cholerae* bacteria, and at 10 h postinoculation we determined the number of CFU in individual 1-cm-long segments starting just below the stomach and extending to the cecum. The results of two independent experiments are shown in Fig. 2 and reveal that while the acid-adapted *V. cholerae* organisms attain higher numbers than the unadapted organisms, the sites of colonization at a gross level remain similar. That is, it appears that acid adaptation does not allow for colonization of previously uninhabited sections of the small intestine.

Virulence gene expression. Two major virulence factors of *V. cholerae* are CT (13) and TCP (12). TCP, a type IV bundle-forming pilus, has been shown to be crucial for colonization in infant mice and humans (3, 12) and is believed to be important for adherence to the intestinal epithelial layer and/or bacterial cell-cell adherence and potentially for secretion of virulence factors (5). CT is the major cause of the profuse, watery diarrhea that is the hallmark of cholera (4). Considering the importance of TCP for a successful infection, it is possible that earlier expression of TCP provides a competitive advantage in vivo. Additionally, early expression of CT and the resulting chloride secretion could lead to a local growth advantage, i.e., as a result of its close proximity to the bacteria that are producing CT. Previous studies have monitored the transcriptional induction of the TCP and CT subunit genes, *tcpA* and *ctxA*, respectively, at various times during an infant mouse infection (6). To determine if acid adaptation has an effect on the expression of these critical virulence factors, we independently monitored the temporal patterns of the transcriptional induction of *tcpA* and *ctxA* as previously described (6). Briefly, to monitor *tcpA* induction, we utilized *V. cholerae* C6709 containing a *res1-tet-res1* cassette inserted into *lacZ* and a *tcpA*::*tnpR135* fusion (6). *ctxA* induction was measured in a similar fashion using a strain containing a *ctxA*::*tnpR135* fusion (6). For the experimental results shown in Fig. 3, the relevant strains were either acid adapted or unadapted and individually inoculated into infant mice as described above. At 1-h intervals, the small intestine was removed from infected animals

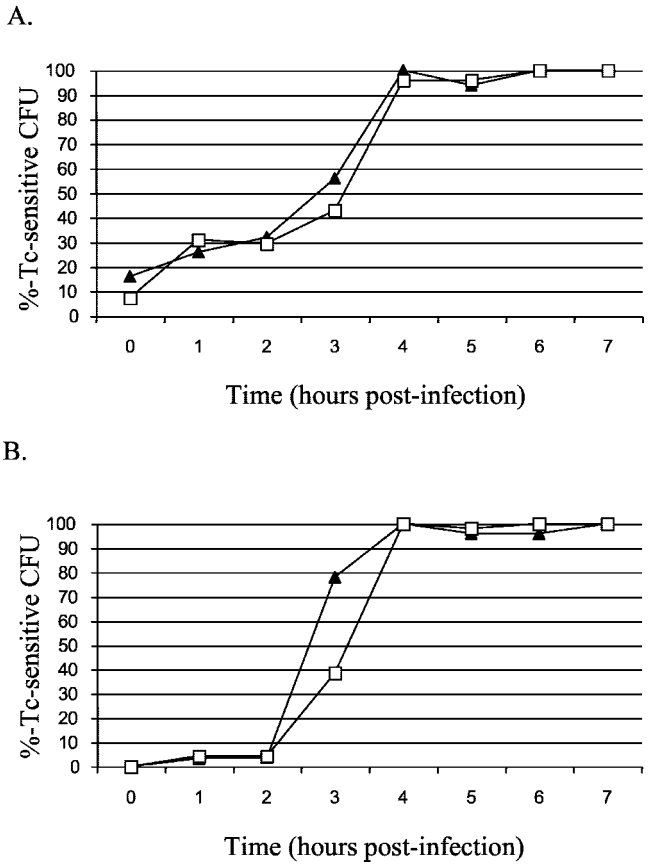

FIG. 3. Kinetics of *tcpA*::*tnpR* (A) and *ctxA*::*tnpR* (B) transcriptional induction in acid-adapted (open squares) and unadapted (filled triangles) *V. cholerae* organisms during infection. The *x* axis indicates times postinoculation that *V. cholerae* bacteria were recovered from the infant mouse small intestine. The percent tetracycline (Tc) sensitivity was determined by replica plating and is plotted on the *y* axis. Each symbol represents the average for two mice.

and the resolution of the tetracycline resistance marker was measured by first plating serial dilutions onto LB agar and subsequently replica plating colonies to LB agar containing tetracycline. There was no significant difference in the temporal patterns of *tcpA*::*tnpR135* or *ctxA*::*tnpR135* induction between acid-adapted and unadapted *V. cholerae* cells. Note that the apparent difference in *ctxA*::*tnpR135* induction at 3 h was not reproducible in repeat experiments. Figure 3A and B demonstrate that acid adaptation has no discernible effect on the timing of the transcription of either *tcpA* or *ctxA*, respectively, in vivo. Thus, altered kinetics of expression of these two virulence factors does not account for the infectious advantage of acid-adapted *V. cholerae*.

Our results clearly show that the hyperinfectious state of acid-adapted *V. cholerae* is not due to an increased ability to survive transit through the stomach of the infant mouse. It is formally possible that the lumen of this organ in 5-day-old infant mice is not very acidic and may not accurately represent the human gastric barrier to *V. cholerae* passage. However, the large degree of killing of *V. cholerae* bacteria observed during the early stages of infection suggests that the infant mouse gastrointestinal tract is a hostile environment. Our spatial analysis of regions of colonization reveals that although there are preferred segments of the small intestine for colonization, acid-adapted and unadapted *V. cholerae* cells have similar preferences for these sites. Finally, analysis of the temporal patterns of induction of the major virulence genes *tcpA* and *ctxA* shows that adapted and unadapted *V. cholerae* cells have virtually identical patterns of induction in vivo. Taken together, our data suggest that the mechanism by which acid-adapted cells outcompete unadapted *V. cholerae* cells in vivo is likely due to the ability to begin multiplication earlier and/or to multiply with a faster doubling time. However, we were not able to reproduce this phenomenon in vitro using either rich or minimal medium (data not shown). Alternatively, it is possible that acid adaptation confers increased survival at later stages of infection, i.e., after the organism has passed through the stomach and upper small bowel. However, such a temporally and spatially delayed survival advantage is difficult to explain given our current understanding of the rapid adaptive abilities of bacteria.

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