Mucosal penetration primes *Vibrio cholerae* for host colonization by repressing quorum sensing

Zhi Liu*, Tim Miyashiro[†], Amy Tsou*, Ansel Hsiao*, Mark Goulian[†‡], and Jun Zhu*[§]

Departments of *Microbiology, [†]Physics, and [‡]Biology, University of Pennsylvania, Philadelphia, PA 19104

Edited by R. John Collier, Harvard Medical School, Boston, MA, and approved April 17, 2008 (received for review March 7, 2008)

To successfully infect a host and cause the diarrheal disease cholera, *Vibrio cholerae* must penetrate the intestinal mucosal layer and express virulence genes. Previous studies have demonstrated that the transcriptional regulator HapR, which is part of the quorum sensing network in *V. cholerae*, represses the expression of virulence genes. Here, we show that *hapR* expression is also modulated by the regulatory network that governs flagellar assembly. Specifically, FliA, which is the alternative σ-factor (σ^{28}) that activates late-class flagellin genes in *V. cholerae*, represses *hapR* expression. In addition, we show that mucin penetration by *V. cholerae* is sufficient to break flagella and so cause the secretion of FlgM, the anti-σ factor that inhibits FliA activity. During initial colonization of host intestinal tissue, *hapR* expression is repressed because of low cell density. However, full repression of *hapR* expression does not occur in *fliA* mutants, which results in attenuated colonization. Our results suggest that *V. cholerae* uses flagellar machinery to sense particular intestinal signals before colonization and enhance the expression of virulence genes by modulating the output of quorum sensing signaling.

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera, an acute dehydrating diarrheal disease that is still endemic in many developing countries (1). To survive in its various habitats, *V. cholerae* must be able to sense and respond to changing environmental signals (2). One such signal is the local *V. cholerae* cell density, which is sensed through a process known as quorum sensing (3). *V. cholerae* measures its population density by producing, secreting, and monitoring the concentration of at least two autoinducers (4). The sensory information provided by autoinducer concentration is channeled through the response regulator LuxO. In its phosphorylated state, LuxO represses *hapR* transcription by activating the expression of several small regulatory RNAs (5, 6). At high cell density, LuxO is not phosphorylated, so production of HapR increases. HapR controls a number of cellular functions and indirectly regulates the expression of multiple virulence genes (6, 7). As *V. cholerae* colonizes the small intestine and multiplies, it activates a cascade of regulatory proteins that leads to the production of an array of virulence factors [supporting information (SI) Fig. S1]. The membrane-localized ToxRS and TcpPH regulatory complexes respond to host environmental signals to initiate this cascade, which culminates in the production of ToxT, which directly up-regulates virulence genes encoding Cholera Toxin and the Toxin Coregulated Pilus (TCP) (8). Expression of *tcpPH* requires the transcriptional regulator AphA (9). Quorum sensing and pathogenesis are coupled through the action of HapR, which represses the transcription of *aphA* and thus inhibits optimal virulence factor production (10) (see Fig. S1). Because high cell densities are common during the late stage of infection, HapR-mediated repression of colonization and virulence genes is thought to help *V. cholerae* to detach to find a new site of infection or exit the host and initiate a new infectious cycle (7, 11). Although the action of HapR links quorum sensing and virulence gene regulation, the pathways by which quorum sensing is regulated in the host and the exact roles played by quorum sensing during *V. cholerae* infection have not been elucidated.

In addition to the production of virulence factors, for *V. cholerae* to colonize the villus epithelial cells within the small intestine, bacteria must swim through a protective mucus glycocalyx (12). It has been proposed that the motility conferred on *V. cholerae* by its single polar flagellum is necessary for this process (12, 13). Flagellar biogenesis is complex and involves a combination of transcriptional, translational, and posttranslational regulation (14). Flagellar biosynthesis genes can be categorized into three classes (early, middle, and late) based on their order of activation. In *V. cholerae*, FlrA and the σ^{54}-holoenzyme transcribe early genes, including those that encode the Motor/Switch ring and export components. The middle genes, encoding structural and assembly proteins that form the hook-basal-body (HBB) of the flagellum, are activated by FlrC and the σ^{54}-holoenzyme. After formation of the HBB, the anti-σ^{28} factor (FlgM) is secreted from the cell, allowing σ^{28} (FliA) to activate transcription of late genes, which encode the flagellin proteins and motor components (15, 16).

In this study, we identify components of flagellar biosynthesis that also control quorum sensing via regulation of *hapR* expression, suggesting a link between regulation of motility and regulation of quorum sensing in *V. cholerae*. This combination efficiently prepares bacteria for accessing colonization sites and, at the same time, allows for the maximal production of virulence genes.

Results

Identification of Additional Regulatory Factors Involved in Quorum Sensing. To identify factors that regulate virulence genes through the quorum sensing pathway in *V. cholerae*, we designed an antibiotic-coupled transposon screen. We fused the zeocin-resistance gene *sh ble* (17) to the ToxT-activated promoter of the TCP subunit gene *tcpA* and integrated the resulting cassette into the *lacZ* locus of wild-type, *luxO* and *hapR* strains. Wild-type and *hapR* strains containing this cassette are resistant to zeocin after growth in AKI medium, which induces the expression of virulence genes (18). However, the corresponding *luxO* mutant is sensitive to zeocin when grown in AKI medium (Table S1). The latter result is consistent with low-virulence gene expression caused by the high levels of HapR in a *luxO* mutant (7, 10) (Fig. S1). We performed transposon mutagenesis in this *luxO lacZ*::P_{tcpA}-*sh ble* strain (LZV8) using the mariner transposon TnAraOut (pNJ17), which contains *araC* and a P_{BAD} promoter that transcribes away from the transposon (19). The addition of arabinose derepresses the P_{BAD} promoter by removing AraC repression, leading to the transcription of any downstream genes. We reasoned that if the transposon disrupts a gene required for

Author contributions: Z.L., T.M., A.H., M.G., and J.Z. designed research; Z.L., T.M., A.T., A.H., and J.Z. performed research; M.G. contributed new reagents/analytic tools; Z.L., T.M., M.G., and J.Z. analyzed data; and Z.L., T.M., A.H., and J.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[§]To whom correspondence should be addressed. E-mail: junzhu@mail.med.upenn.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0802241105/DCSupplemental.

HapR function, virulence gene expression would be derepressed, and P_{tcpA}-*sh ble* will be induced and confer zeocin resistance. Similarly, if the transposon inserts into the promoter region of a gene that either represses *hapR* expression or bypasses the HapR repression of *aphA*, then zeocin resistance will arise in the presence of arabinose. To distinguish between mutations that affect HapR directly and those that affect regulation downstream of HapR, we included the *Vibrio harveyi luxCDABE* reporter construct, which is activated directly by HapR (6).

From 10 transposon libraries each containing $\approx 10^9$ cells, we obtained ≈500 zeocin-resistant colonies. Table S1 summarizes the locations of the transposon insertions and their related phenotypes. More than 50% of these mutants have transposons inserted in the *hapR* locus and exhibited the rugose-colony phenotype associated with a mutation in *hapR* (20, 21). Because disruption of *hapR* relieves the repression of the *aphA* promoter by HapR, strains containing transposon insertions within *hapR* are zeocin-resistant. Many transposon insertions occurred upstream of the P_{tcpA}-*sh ble* construct resulting in overexpression of the zeocin-resistance gene in the presence of arabinose. We obtained mutants with insertions upstream of *aphA*, *tcpP*, or *toxT* that are resistant to zeocin in the presence of arabinose, further confirming that HapR inhibits the expression of virulence genes by repressing *aphA*. Disruption of the H-NS-like DNA-binding protein VicH (VC1130) also resulted in zeocin resistance; studies have shown that deletion of *vicH* results in high expression of the genes encoding cholera toxin, TCP, and ToxT (22). However, the *lux* expression pattern of *vicH* mutants was the same as that of the parental *luxO* mutant, indicating that the increased *tcpA* expression was not due to a regulatory defect in quorum sensing. Similarly, mutations that disrupt either VC2305 (*ompK*) or VC2271 (*ribD*) restore both cholera toxin and TCP production in the *luxO* mutant without changing *lux* expression. The mechanism that enables these two mutants to increase the expression of virulence genes independent of quorum sensing is the subject of another study.

Interestingly, we obtained a high number of mutants with transposon insertions in genes involved in flagellar biosynthesis (Table S1). Disruption of the *flgBCD* genes, which encode flagellar rod proteins (23), activated P_{tcpA}-*sh ble* in the *luxO* mutant. We also obtained a number of arabinose-dependent, zeocin-resistant mutants with transposon insertions located upstream of *fliA*, which encodes the σ-factor required for activation of certain flagellin genes by RNA polymerase (RNAP) (16). All of these flagellar mutants produce CT and TCP (Table S1). In addition, *luxO* mutants that harbor such flagellar mutations exhibit reduced *lux* expression (Table S1), suggesting that quorum sensing regulation is affected. We selected these mutants for further study.

Deletion of *flgD* Enhances the Expression of Virulence Genes Through *hapR*. To investigate how flagellar synthesis affects the expression of virulence genes, we constructed strains with an in-frame deletion of *flgD*, which encodes one of the flagellar rod proteins. As expected, *flgD* mutants are nonmotile ((Fig. S4*A*). Although TcpA and CT production was abolished in the *luxO* mutant, wild-type levels of TcpA and CT were detected in both *flgD* and *flgD luxO* mutants (Fig. S2*A*). To determine whether the deletion of *flgD* alters *aphA* expression in the *luxO* mutant, we measured the corresponding activity levels of an *aphA-lacZ* transcriptional reporter. As expected, deletion of *luxO* results in repression of *aphA* (Fig. S2*B*). However, mutation of *flgD* not only results in wild-type levels of *aphA* transcription but also restores *aphA* expression in the *luxO* mutant. This explains why the inhibition of the virulence regulon in the *luxO* mutant was restored in the flagellar mutants. To further characterize the role of *flgD* in quorum sensing, we transformed wild-type and *flgD* strains with pBB1, which contains the *luxCDABE* operon from

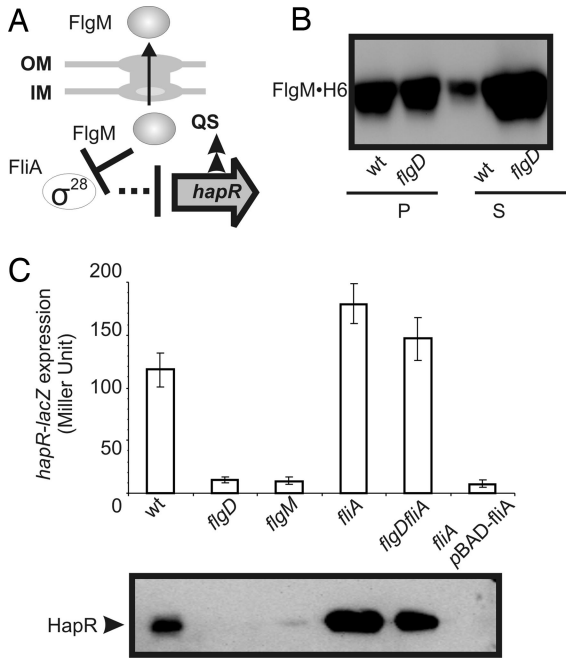

Fig. 1. FliA represses quorum sensing in *flgD* mutants. (*A*) Proposed model for repression of the *hapR* promoter by secretion of FlgM. (*B*) FlgM secretion is increased in the *flgD* mutant. Strains containing a plasmid expressing a functional *flgM-his*$_6$ were grown to midlog. Culture pellets (P) and TCA-precipitated supernatants (S) were isolated and subjected to Western blot analysis using anti-His-6 antiserum. All samples were normalized to contain 10^9 bacterial cells. (*C*) Reduced production of HapR is due to *hapR* repression by FliA. Strains harboring *hapR-lacZ* transcriptional fusions were grown to midlog in LB and harvested to measure β-galactosidase activity (*Upper*). Results, reported in Miller units, are means of three experiments ± standard deviations. Whole-cell extracts were subjected to Western blot analysis using anti-HapR antiserum (*Lower*). All samples were normalized to contain 10^9 bacterial cells.

V. harveyi that can be regulated directly by HapR (6). Although *lux* expression depends on cell density in both cases, overall expression is lower in the *flgD* strain than in the wild-type strain (Fig. S2*C*). We attribute this reduction in *lux* expression to lower levels of HapR because the expression of a *hapR-lacZ* transcriptional reporter was also inhibited in the *flgD* mutant (Fig. S2*D*). Taken together, these results indicate that flagellar synthesis modulates the quorum sensing output, including the expression of virulence factors, by regulating *hapR* transcription.

The Alternative σ-Factor FliA (σ^{28}) Represses *hapR* Expression. The regulatory hierarchy that determines the order for expressing flagellar genes in *V. cholerae* ends with the σ^{28}-dependent activation of particular flagellar genes (e.g., *flaBCD*) (16). Because constitutive expression of *fliA*, which encodes σ^{28}, inhibits regulation by quorum sensing (Table S1), we hypothesized that the repression of *hapR* observed in *flgB*, *flgC*, and *flgD* mutants is due to increased activity of FliA (Fig. 1*A*). The activity of FliA increases upon secretion of the anti-σ^{28} factor FlgM through the flagellar export apparatus (15, 16). To determine whether this process plays a role in the altered *hapR* regulation of the *flgD* mutant, we compared the level of FlgM in supernatants of wild-type and *flgD* cultures. The level of FlgM present in the supernatant of the *flgD* mutant is higher than that of wild-type *V. cholerae* (Fig. 1*B*), suggesting that disrupting flagellar rod assembly leads to higher FliA activity by increasing FlgM secretion. Consistent with this hypothesis, the expression of the FliA-activated gene *flaD* was enhanced in a *flgD* mutant (Fig. S3). To further investigate the relationship between FliA–FlgM

interaction and *hapR* expression, we measured *hapR* transcription and HapR production in various mutants grown to midlog phase. Whereas *hapR* expression is inhibited in the *flgM* and *flgD* mutants, *hapR* is overexpressed in the *fliA* mutant (Fig. 1*C*). More importantly, deletion of *fliA* in the *flgD* mutant restores HapR production, indicating that the effect of *hapR* repression in *flgD* mutants is mediated through FliA. Furthermore, a *fliA* mutant complemented by a plasmid that constitutively expresses *fliA* restores the repression of *hapR*. Mutations in *flaD* and *motY*, which are known to be regulated by FliA (16), did not affect FlgM secretion or *hapR* expression (data not shown). Taken together, these results suggest that the high activity of FliA in either *flgM* or *flgD* mutants inhibits *hapR* expression. At this time, we do not know whether FliA regulates *hapR* directly or through another regulatory mechanism. The alternative σ-factor σ^{28} could activate the expression of unknown repressor to repress the *hapR* expression or activate small RNAs to regulate *hapR* mRNA. In fact, *hapR* has been shown to be regulated by RNA-binding protein Hfq and a set of sRNAs activated by LuxO and σ^{54} (5). However, if sRNAs are involved in σ^{28}-mediated *hapR* repression, they are different from those sRNAs induced by LuxO because σ^{28} represses *hapR* in a *luxO* mutant (Fig. S2). Furthermore, because σ^{28} also represses *hapR* in an *hfq* mutant, this regulation is independent of Hfq (data not shown).

Repression of Quorum Sensing by FliA Is Important for Proper Intestinal Colonization. Flagella are thought to help *V. cholerae* swim through mucosal layers to colonize the intestinal surface (13). Consistent with this hypothesis, *flgD*, *fliA*, and *flgM* mutants, which have reduced motility (15) (Fig. S4*A*), do not colonize the intestines of infant mice as well as motile wild-type cells (Fig. 2*A*). The observation that a *fliA* mutant displays a severe colonization defect was intriguing because FliA represses *hapR* expression (see Fig. 1). Colonization efficiency was significantly increased in a *fliA hapR* mutant (Fig. 2*A*) as compared with the *fliA* single mutant (P value <0.01). Consistent with previous reports (7), the *hapR* mutant colonized mice as well as wild-type. In addition, an *in vivo* colonization competition assay with a *fliA* single mutant and a *fliA hapR* double mutant demonstrated that the double-mutant colonizes ≈6-fold better than the *fliA* mutant (Fig. S4*B*). These results suggest that the colonization defect of the *fliA* mutant is partially due to insufficient repression of *hapR*.

To provide further *in vivo* evidence that *hapR* expression is higher in *fliA* mutants, we colonized infant mice using strains that contain a *hapR*-K_m^r transcriptional fusion, which confers kanamycin resistance to cells expressing *hapR* (24). At 4 h, *hapR* expression in wild-type cells was low, presumably because of the small number of cells that had colonized the small intestine (Fig. 2*B*). However, by 18 h, the number of cells in the small intestine had increased, and *hapR* expression was high. The *hapR* expression patterns of *flgD*, *fliA*, and *flgM* mutants *in vivo* were similar to those *in vitro* (compare Figs. 1*C* and 2*B*), with significantly higher *hapR* expression in *fliA* mutants early in colonization (Fig. 2*B*). Taken together, these results suggest that the repression of *hapR* by FliA is required for proper colonization of host intestines.

***V. cholerae* Loses Its Flagellum and Represses Regulation by Quorum Sensing During Mucosal Penetration.** The results described above strongly suggest a link between the regulatory networks of quorum sensing and flagellar assembly during intestinal colonization by *V. cholerae*. We hypothesized that the cross-regulation may occur while colonizing bacteria cross the mucosal layer of the intestinal surface, because flagella are thought to be important for mucus penetration. To approximate this stage of infection, we used an *in vitro* assay based on the migration of cells through a column of mucin (25, 26). Wild-type cells migrated through the mucin column significantly faster than nonmotile

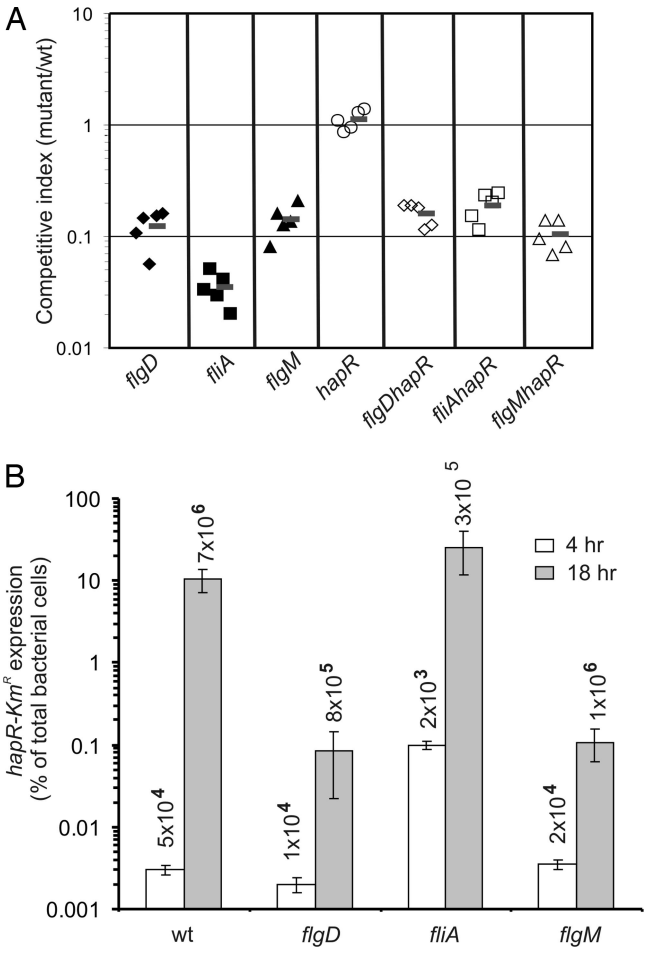

Fig. 2. The expression of *hapR* is repressed by FliA during the colonization of infant mice. (*A*) *In vivo* competition assays. Infant CD-1 mice were orally inoculated with ≈10^6 wild-type and mutant bacteria. For each mutant, the competitive index is defined as the number of colony-forming units (CFU) for the mutant compared with the corresponding CFU number for a wild-type strain recovered from the intestines 18 h after inoculation. (*B*) Expression of *hapR* during colonization. Infant CD-1 mice were orally inoculated with ≈10^6 bacteria that contain a *hapR*-K_m^r reporter. At each time point, mouse intestines were homogenized, treated with or without kanamycin (500 μg/ml) for 10 min, and plated on LB plates. The number of colonized bacteria per mouse (above each bar) was calculated based on the number of CFU recovered from samples not treated with kanamycin. Expression of *hapR* is defined as the percentage of K_m-resistant CFU of the total CFU. Results are means from experiments with three to five mice, and the bars represent the corresponding standard deviations.

strains (i.e., *flgD* and *flaA* mutants) (Fig. 3*A* and data not shown). In addition, antibiotic-killed bacteria failed to penetrate the mucin column (data not shown). These data suggest that flagella may help *V. cholerae* swim through mucosal layers. Surprisingly, both flagellar staining and electron microscopy revealed that the majority of wild-type cells (>80%) had lost their flagella while migrating through a column containing 1% mucin (Fig. 3*B*). It should be noted that the absence of flagellar structures observed in these experiments is not due to the interference of mucin with flagellar staining or microscopy, because flagellar structures are apparent when mucin and bacteria are mixed and then stained for flagella (data not shown). To determine whether bacteria are motile after penetrating mucin, we deposited a suspension of cells onto a transwell containing a thin layer of mucin on top of a 3-μm filter and monitored the motility of the cells that passed through the filter. Consistent with our electron microscopy

MICROBIOLOGY

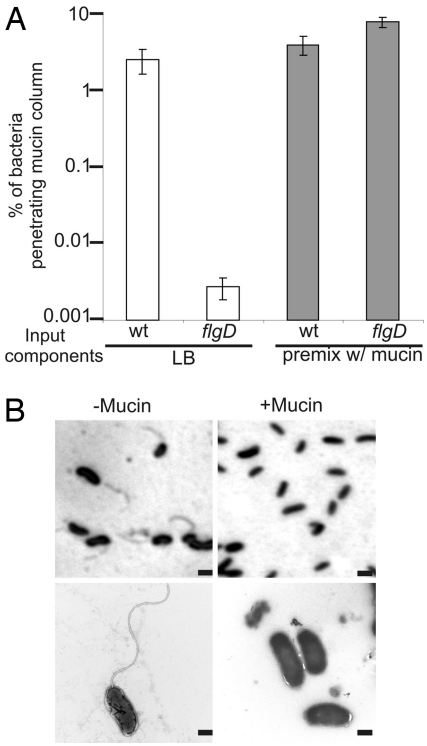

Fig. 3. *V. cholerae* cells lose flagella during mucin penetration. (*A*) Flagellar mutants swim more slowly through mucin than do wild-type cells. Midlog cultures (100 μl) of wild-type *V. cholerae*, the *flgD* mutant (white bars), or cultures premixed with 100 μl of 1% mucin were loaded into a column containing 1 ml of 1% mucin. After incubation for 30 min at 37°C, 500 μl were collected from the bottom of the column and plated on LB medium. (*B*) Images of *V. cholerae* during mucin column penetration. Flagellar staining (*Upper*) and transmission electron microscopy (*Lower*) of *V. cholerae* cells in the presence or absence of mucin. [Scale bars : 2 μm (*Upper*) and 500 nm (*Lower*).]

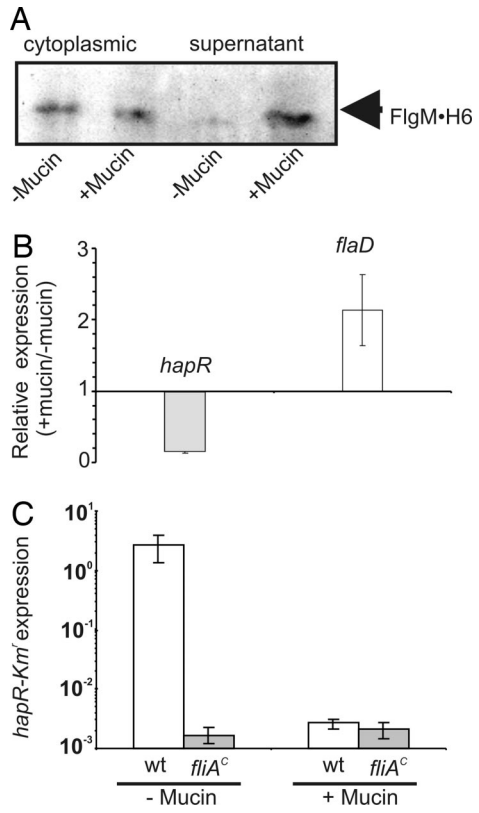

Fig. 4. Quorum sensing is repressed when *V. cholerae* penetrate mucin. (*A*) Secretion of FlgM increases during mucin penetration. Midlog cultures of wild-type bacteria expressing *flgM-his*$_6$ were incubated for 30 min in LB medium with or without 1% mucin. Cytoplasmic and cell-free supernatant fractions were subjected to Western blot analysis. All samples were normalized to contain 10^8 bacterial cells. (*B*) Real-time RT-PCR analysis of *hapR* expression. Mucin penetration assays were performed as described above. RNA was extracted, and real-time RT-PCR was performed for *hapR* and *flaD* transcripts. Transcript levels were normalized by 16S RNA. Results for each promoter are reported as the ratio of transcript level in the presence of mucin to the transcript level in the absence of mucin. (*C*) Expression of *hapR* in *V. cholerae* cells on the surface of HEp-2 cells. Confluent HEp-2 cell monolayers were submerged in 1 ml of 1% mucin or LB. Midlog cultures of wild-type (wt) and *fliA*-overexpressing (*fliA*^{C}) strains of *V. cholerae* that contain the *hapR-K*$_m^r$ reporter were inoculated onto HEp-2 cell cultures. After 2-hr incubation, the bacteria bound to HEp-2 cells were collected, treated with or without kanamycin (500 μg/ml) for 10 min, and plated onto LB agar. The *hapR-K*$_m^r$ expression was defined as the percentage of CFU that survive treatment with kanamycin of the total CFU. All results are means of three experiments ± standard deviations.

results, we found that bacteria that passed through the mucin layer and reached the outer chamber were not motile (see MovieS1 and MovieS2).

The above experiments indicate that *V. cholerae* cells use flagella to penetrate mucin layers but lose them during the process. Because we did not observe intact flagella on cells within mucin, we hypothesized that *V. cholerae* uses an alternative mechanism to translocate through the mucin. To test this, we compared the rates of migration through a 1% mucin column for wild-type and *flgD* mutant cells that had been premixed with mucin. Consistent with our hypothesis, we found that the cells migrated through the mucin column at similar rates (Fig. 3*A Right*). Based on these data, we speculate that *V. cholerae* flagella are important for intestinal colonization only during the initial penetration of mucin. The exact mechanism used by *V. cholerae* to cross mucosal layers is currently unknown and may involve flagellum-independent motility used by *V. cholerae* (27) and other bacteria (28).

To determine whether the loss of flagella from the exposure of cells to mucin leads to increased FlgM secretion, we measured FlgM levels in the cytoplasmic and extracellular fractions of cells grown in the presence of mucin. The level of FlgM in the supernatant of cultures supplemented with mucin was higher than that of cultures without mucin (Fig. 4*A*), suggesting that FliA activity increases during interaction between *V. cholerae* and mucin. Consistent with our observation of *hapR* repression by FliA (Figs. 1*C* and 2*B*), we also found that *hapR* expression is repressed when *V. cholerae* penetrates mucin (Fig. 4*B*). Importantly, this repression depends on FliA because *hapR* is not repressed in a *fliA* mutant as well as in a *fliF* mutants, which is required for FlgM secretion (15) (data not shown). Furthermore, transcription of the FliA-activated gene *flaD* increased when *V. cholerae* penetrates mucin (Fig. 4*B*). Taken together, these results suggest that exposure to mucin is sufficient to activate FliA, which inhibits regulation of quorum sensing by repressing *hapR* expression.

To further test the *in vivo* effect of the repression of *hapR* by exposure of cells to mucin, we turned to a cell-culture infection model. HEp-2 cells were grown to confluence and either covered with a layer of 1% mucin or left untreated. The expression of *hapR* was lower in those HEp-2-bound *V. cholerae* that had to pass through mucin to reach the epithelial cell monolayer (Fig. 4*C*). However, for strains overexpressing *fliA*, *hapR* expression remained low whether or not mucin was present. Consistent with the colonization assays and mucin-penetration assays described

above, these results indicate that regulation of quorum sensing is repressed through σ^{28} during mucosal penetration.

Discussion

The facultative human pathogen *V. cholerae* must rapidly adapt to different environments, such as from its natural aquatic habitats to the human digestive system, during its life cycle. To do this, *V. cholerae* uses complex signal transduction pathways that modulate its gene expression in response to various environmental cues. The quorum sensing regulatory network monitors the level of small autoinducer molecules as a measure of cell density; however, other components also regulate quorum sensing by modulating transcription of the regulator HapR (29–31). In this study, we discovered that flagellar components also regulate *hapR* expression. Specifically, we show that *hapR* transcription is repressed by *fliA*, which encodes the alternative σ-factor involved in flagellar synthesis (σ^{28}). Similarly, removal of flagellar rod proteins, which results in high levels of active σ^{28} because of increased secretion of the anti-σ^{28} protein FlgM, inhibits *hapR* transcription.

Why does *V. cholerae* integrate motility and quorum sensing regulatory pathways? One possibility is to further derepress the virulence genes that are repressed by quorum sensing. Unlike some enteric pathogens, which successfully infect hosts with a low starting dose [e.g., *Shigella* (32)], *V. cholerae* requires $\approx 10^6$ cells to induce symptomatic cholera (33). However, the number of *V. cholerae* cells that reach the small intestine is reduced by the hostile compounds found in host environments, such as gastric acid and bile salts (2). This reduction in cell number decreases the concentration of autoinducer, which, in conjunction with a HapR negative-feedback loop (34), represses *hapR* transcription. Our results suggest that this reduction in cell number may not be sufficient to completely inhibit HapR-mediated repression of virulence gene expression.

At their sites of colonization at mucosa, *V. cholerae* must penetrate the thick glycocalyx of mucins that cover and protect the intestinal epithelium. However, in penetrating this barrier, FlgM is secreted (Fig. 4). This leads to derepression of FliA and further repression of *hapR* through the activation of FliA. Through *hapR* repression, *V. cholerae* cells are primed for intestinal colonization, which is an important step leading to the onset of cholera. This model is illustrated in Fig. S5. Consistent with this model is our observation that a *fliA hapR* double mutant colonizes infant mice better than a *fliA* single mutant (Fig. 2). Furthermore, the higher *hapR* expression (as compared with wild-type levels) observed early in infection associated with a *fliA* mutant also supports this model. Interestingly, a recent report suggests that *Salmonella* can sense the wetness of its surrounding environment by controlling the amount of FlgM secreted through its flagellar apparatus (35). Similarly, our model implies that *V. cholerae* uses flagella to sense host mucosal signals to regulate genes that allow the cell to adapt to host intestinal environment.

Previous studies suggest that the ability of the current pandemic *V. cholerae* strains to swim into the mucosal layer is important for colonizing the intestinal surface (13, 36). Consistent with these reports, we found that nonmotile strains colonize 10–25 times less efficiently than wild-type strains (Fig. 2*A*). Furthermore, using mucin column-penetration assays, we found that nonmotile strains penetrate mucin layers more slowly than wild-type *V. cholerae* (Fig. 3*A*). However, many *V. cholerae* cells lose their flagella while penetrating mucin layers (Fig. 3*B*), suggesting that intact flagella are not required for the cells to migrate through mucin once they have passed the initial interface. One possibility is that flagella initially help *V. cholerae* swim through mucin, but once flagella are lost in this process, other processes assist in the passage of bacteria through the glycocalyx to the epithelium. Indeed, when flagellar mutants were premixed with mucin, they migrated as fast as wild-type cells (Fig. 3*A Right*). Another possibility is that bacteria continuously regenerate flagella to replace those lost while penetrating mucus layers. In fact, cells that encounter mucin secrete FlgM and so begin to express FliA-regulated genes such as *flaD* (Fig. 4 *A* and *B*). However, a recent study reported that FlgM/σ^{28}-dependent gene expression in *Salmonella enterica* remains unchanged by flagellar shearing despite the rapid regeneration of flagella (37). Whether the cellular response to mechanically sheared flagella is similar to that of breaking flagella during mucus penetration is not clear.

The ability of *V. cholerae* to colonize and cause disease requires tight control over the expression of multiple virulence factors. However, pathogenesis and the associated genetic regulatory events in the host are not a series of disconnected cascades nor do they depend only on the activation of virulence regulators. Instead, the complex infection cycle of this pathogen depends on a variety of genetic regulatory strategies, including the repression of genes that inhibit colonization. It has already been shown that *V. cholerae* reciprocally regulates the transcription and biogenesis of virulence determinants and that of the type IV MSHA pili to evade host immune defenses (26, 38). In this study, we show that another reciprocal regulatory mechanism serves to allow this pathogen to maximize virulence during early colonization. *V. cholerae* cells use their flagella to penetrate the mucosal barrier protecting the cells of the small-intestinal epithelium. In the process, the flagella are lost, which leads to repression of *hapR*, which encodes a negative regulator of virulence genes. Thus, *V. cholerae* is capable of "dual use" of necessary virulence processes such as flagellar motility and TCP biogenesis to position and equip itself for colonization while simultaneously inhibiting anticolonization factors such as HapR and MSHA. This enables *V. cholerae* to access efficiently its preferred colonization niches during the critical early phase of infection, where a small number of bacterial cells must attach to and establish themselves at the epithelium. It is this regulatory flexibility that has made *V. cholerae* a potent human pathogen, and a striking example of the complexities possible in bacterial virulence regulation in disparate environments.

Methods

Strains, Plasmids, and Culture Conditions. All *V. cholerae* strains used in this study were derived from E1 Tor C6706 (39). Strain and plasmid constructions are described in *SI Text*.

Transposon Screen for *hapR* Repression. LZV8 (*lacZ*::*tcpA-sh ble*, Δ*luxO*) was mutagenized with the TnAraOut mariner transposon (19). The resulting libraries were inoculated into AKI medium containing 0.05% arabinose and incubated without shaking at 37°C for 4 h until the OD_{600} was ≈0.1. Cultures were treated with zeocin (25 μg/ml) for 30 min at 37°C and plated on LB agar without zeocin. Arbitrary PCR (19) and DNA sequencing were performed to identify transposon insertion sites.

***hapR* Transcription Measurements.** The *hapR-lacZ* transcriptional reporter was integrated into the chromosomes of various flagellar mutants. β-Galactosidase assays were performed as described (7).

In the mouse colonization and mucin-penetration assays, *hapR* expression was measured by using strains harboring the *hapR-*K_m^r reporter, which is at the *hapR* locus and maintains an intact copy of *hapR* (24). Bacteria isolated from mouse intestines or mucin columns were added to fresh LB in the presence or absence of 500 μg/ml kanamycin and incubated for 10 min at 37°C. This treatment is sufficient to kill 100% of *V. cholerae* cells that do not carry any K_m^r gene or that contain the *hapR-*K_m^r construct and a constitutively active LuxO (40). After treatment, the samples were plated onto LB. Expression of *hapR* was defined as the number of kanamycin-resistant CFU normalized by the number of total CFU.

For the mucin-penetration assays, *hapR* transcription was measured by quantitative real-time PCR. Total RNA was isolated from column samples by using TRIzol reagent (Invitrogen) and cleaned with the RNeasy kit (Qiagen). RNA reverse transcription was performed by using the SuperScript II kit

MICROBIOLOGY

(Invitrogen) with 200 ng of RNA sample. Quantitative real-time PCR using primers specific for *hapR* was performed on an Opticon 2 system (MJ Research). The 16S ribosomal RNA was used for an internal control in all reactions.

Infant Mouse Colonization Assay. The infant mouse colonization assay was performed as described (41). Briefly, 10^6 *V. cholerae* cells were inoculated into 6-day-old CD-1 suckling mice. At different time points, intestines were homogenized, serially diluted, and plated onto LB agar. Bacterial numbers were determined from the number of CFU.

Mucin-Penetration Assay and Flagella Visualization. Mucin columns were prepared by adding different concentrations of bovine submaxillary mucin (Sigma) to 1-ml syringes. Midlog bacterial cultures (100 μl)or 100 μl of cells premixed with 1% mucin were loaded on the top of the mucin columns and allowed to settle for 30 min at 37°C. Fractions (500 μl) were collected from the bottom of the mucin columns. Bacteria numbers were measured by serially diluting samples, plating onto LB agar, and counting CFU. A drop of the collected fractions was placed on slides to detect flagella by using the flagella stain kit (Remel, Lenexa) or by transmission electron microscopy (University of Pennsylvania Bioimaging Core).

FlgM Detection. To detect FlgM protein, a plasmid containing the P_{BAD}-*flgM-his6* was introduced into various strains of *V. cholerae*. All samples were normalized to contain 10^9 (or 10^8 in the case of samples treated with mucin) bacterial cells. Supernatants were concentrated by using TCA precipitation (10% trichloroacetic acid). Both extracellular and cytoplasmic proteins were separated by SDS/PAGE, transferred to a nitrocellulose membrane, and immunoblotted with affinity purified anti-6× His rabbit antiserum (Rockland).

Measurement of *hapR* Expression in Bacteria Bound to HEp-2 Cells. HEp-2 human epithelial cells were propagated in DMEM supplemented with 10% FBS (Sigma) in $<$5% CO_2 at 37°C. Before infection, the cell cultures were overlaid with 1 ml of LB with or without 1% mucin. Midlog cultures of wild-type and *fliA*-overexpressing strains of *V. cholerae* that contain the *hapR*-K_m^r reporter were then inoculated onto HEp-2 cell cultures at moi = 100. After incubation for 2 h at 37°C with 5% CO_2, the medium was removed, and epithelial cells were washed three times with PBS buffer to remove unbound bacteria. HEp-2 cells were lysed by using 0.1% Triton X-100. Bound bacterial cells were collected and subjected to the kanamycin treatment described above.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health (NIH) Grant R01AI072479 (to J.Z.), National Science Foundation Grant MCB0615957 (to M.G.), an American Heart Association predoctoral fellowship (to T.M.) and a NIH T32 Bacteriology training grant (to A.T.).

1. Faruque SM, Albert MJ, Mekalanos JJ (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* 62:1301–1314.
2. Reidl J, Klose KE (2002) *Vibrio cholerae* and cholera: out of the water and into the host. *FEMS Microbiol Rev* 26:125–139.
3. Waters CM, Bassler BL (2005) Quorum sensing: Cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* 21:319–346.
4. Higgins DA, *et al.* (2007) The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* 450:883–886.
5. Lenz DH, *et al.* (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* 118:69–82.
6. Miller MB, Skorupski K, Lenz DH, Taylor RK, Bassler BL (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* 110:303–314.
7. Zhu J, *et al.* (2002) Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 99:3129–3134.
8. Hase CC, Mekalanos JJ (1998) TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 95:730–734.
9. Skorupski K, Taylor RK (1999) A new level in the *Vibrio cholerae* ToxR virulence cascade: AphA is required for transcriptional activation of the tcpPH operon. *Mol Microbiol* 31:763–771.
10. Kovacikova G, Skorupski K (2002) Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the aphA promoter. *Mol Microbiol* 46:1135–1147.
11. Nielsen AT, *et al.* (2006) RpoS controls the *Vibrio cholerae* mucosal escape response. *PLoS Pathog* 2:e109.
12. Freter R, Jones GW (1976) Adhesive properties of *Vibrio cholerae*: Nature of the interaction with intact mucosal surfaces. *Infect Immun* 14:246–256.
13. Butler SM, Camilli A (2005) Going against the grain: chemotaxis and infection in *Vibrio cholerae*. *Nat Rev Microbiol* 3:611–620.
14. Aldridge P, Hughes KT (2002) Regulation of flagellar assembly. *Curr Opin Microbiol* 5:160–165.
15. Correa NE, Barker JR, Klose KE (2004) The *Vibrio cholerae* FlgM homologue is an anti-sigma28 factor that is secreted through the sheathed polar flagellum. *J Bacteriol* 186:4613–4619.
16. Prouty MG, Correa NE, Klose KE (2001) The novel sigma54- and sigma28-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. *Mol Microbiol* 39:1595–1609.
17. Bostock JM, Miller K, O'Neill AJ, Chopra I (2003) Zeocin resistance suppresses mutation in hypermutable *Escherichia coli*. *Microbiology* 149:815–816.
18. Iwanaga M, *et al.* (1986) Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. *Microbiol Immunol* 30:1075–1083.
19. Judson N, Mekalanos JJ (2000) TnAraOut, a transposon-based approach to identify and characterize essential bacterial genes. *Nat Biotechnol* 18:740–745.
20. Yildiz FH, Schoolnik GK (1999) *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc Natl Acad Sci USA* 96:4028–4033.
21. Zhu J, Mekalanos JJ (2003) Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell* 5:647–656.
22. Nye MB, Pfau JD, Skorupski K, Taylor RK (2000) *Vibrio cholerae* H-NS silences virulence gene expression at multiple steps in the ToxR regulatory cascade. *J Bacteriol* 182:4295–4303.
23. McCarter LL (2001) Polar flagellar motility of the Vibrionaceae. *Microbiol Mol Biol Rev* 65:445–462.
24. Liu Z, Stirling FR, Zhu J (2007) Temporal quorum-sensing induction regulates *Vibrio cholerae* biofilm architecture. *Infect Immun* 75:122–126.
25. Sheikh J, *et al.* (2002) A novel dispersin protein in enteroaggregative *Escherichia coli*. *J Clin Invest* 110:1329–1337.
26. Hsiao A, Liu Z, Joelsson A, Zhu J (2006) *Vibrio cholerae* virulence regulator-coordinated evasion of host immunity. *Proc Natl Acad Sci USA* 103:14542–14547.
27. Brown II, Hase CC (2001) Flagellum-independent surface migration of *Vibrio cholerae* and *Escherichia coli*. *J Bacteriol* 183:3784–3790.
28. Murray TS, Kazmierczak BI (2008) *Pseudomonas aeruginosa* exhibits sliding motility in the absence of type IV pili and flagella. *J Bacteriol*, DOI:10.1128/JB.01620-07.
29. Lenz DH, Bassler BL (2007) The small nucleoid protein Fis is involved in *Vibrio cholerae* quorum sensing. *Mol Microbiol* 63:859–871.
30. Lenz DH, Miller MB, Zhu J, Kulkarni RV, Bassler BL (2005) CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol Microbiol* 58:1186–1202.
31. Liu Z, Hsiao A, Joelsson A, Zhu J (2006) The transcriptional regulator VqmA increases expression of the quorum-sensing activator HapR in *Vibrio cholerae*. *J Bacteriol* 188:2446–2453.
32. Leclerc H, Schwartzbrod L, Dei-Cas E (2002) Microbial agents associated with waterborne diseases. *Crit Rev Microbiol* 28:371.
33. Colwell RR, *et al.* (2003) Reduction of cholera in Bangladeshi villages by simple filtration. *Proc Natl Acad Sci USA* 100:1051–1055.
34. Svenningsen SL, Waters CM, Bassler BL (2008) A negative feedback loop involving small RNAs accelerates *Vibrio cholerae*'s transition out of quorum-sensing mode. *Genes Dev* 22:226–238.
35. Wang Q, Suzuki A, Mariconda S, Porwollik S, Harshey RM (2005) Sensing wetness: a new role for the bacterial flagellum. *EMBO J* 24:2034–2042.
36. Lee SH, Butler SM, Camilli A (2001) Selection for *in vivo* regulators of bacterial virulence. *Proc Natl Acad Sci USA* 98:6889–6894.
37. Rosu V, Hughes KT (2006) σ28-dependent transcription in *Salmonella enterica* is independent of flagellar shearing. *J Bacteriol* 188:5196–5203.
38. Hsiao A, Toscano K, Zhu J (2008) Post-transcriptional cross-talk between pro- and anti-colonization pili biosynthesis systems in *Vibrio cholerae*. *Mol Microbiol* 67:849–860.
39. Joelsson A, Liu Z, Zhu J (2006) Genetic and phenotypic diversity of quorum sensing systems in clinical and environmental isolates of *Vibrio cholerae*. *Infect Immun* 74, 1141–1147.
40. Vance RE, Zhu J, Mekalanos JJ (2003) a constitutively active variant of the quorum-sensing regulator luxo affects protease production and biofilm formation in *Vibrio cholerae*. *Infect Immun* 71:2571–2576.
41. Gardel CL, Mekalanos JJ (1996) Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence factor expression. *Infect Immun* 64:2246–2255.