RpoS Controls the *Vibrio cholerae* Mucosal Escape Response

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Vibrio cholerae **causes a severe diarrhoeal disease by secreting a toxin during colonization of the epithelium in the small intestine. Whereas the initial steps of the infectious process have been intensively studied, the last phases have received little attention. Confocal microscopy of *V. cholerae* O1-infected rabbit ileal loops captured a distinctive stage in the infectious process: 12 h post-inoculation, bacteria detach from the epithelial surface and move into the fluid-filled lumen. Designated the "mucosal escape response," this phenomenon requires RpoS, the stationary phase alternative sigma factor. Quantitative in vivo localization assays corroborated the *rpoS* phenotype and showed that it also requires HapR. Expression profiling of bacteria isolated from ileal loop fluid and mucus demonstrated a significant RpoS-dependent upregulation of many chemotaxis and motility genes coincident with the emigration of bacteria from the epithelial surface. In stationary phase cultures, RpoS was also required for upregulation of chemotaxis and motility genes, for production of flagella, and for movement of bacteria across low nutrient swarm plates. The *hapR* mutant produced near-normal numbers of flagellated cells, but was significantly less motile than the wild-type parent. During in vitro growth under virulence-inducing conditions, the *rpoS* mutant produced 10- to 100-fold more cholera toxin than the wild-type parent. Although the *rpoS* mutant caused only a small over-expression of the genes encoding cholera toxin in the ileal loop, it resulted in a 30% increase in fluid accumulation compared to the wild-type. Together, these results show that the mucosal escape response is orchestrated by an RpoS-dependent genetic program that activates chemotaxis and motility functions. This may furthermore coincide with reduced virulence gene expression, thus preparing the organism for the next stage in its life cycle.**

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Introduction

Vibrio cholerae, an autochthonous microbe of aquatic ecosystems, causes an illness characterized by infection of the small bowel and the production of liquid stools. Infection follows the ingestion of *V. cholerae* in water or food and then proceeds according to the following scenario. The organism transits the gastric acidity barrier of the stomach and the bile-rich duodenum and proximal jejunum. Upon entering the ileum of the small intestine, it swims through mucus to reach and colonize villous epithelial cells, a process that appears to be mediated by chemotaxis and flagella-dependent motility [1–3]. Induction of a virulence program follows, including the coordinated expression of genes encoding cholera toxin (CT) and the toxin co-regulated pilus (TCP) [4]. Bacterial replication ensues and CT produced by surface-attached bacteria results in the secretion of water and electrolytes. Diarrhea occurs when the volume of the fluid produced exceeds the absorptive capacity of the small bowel and colon. Severely affected patients pass many liters of "rice water" stool containing *V. cholerae* in concentrations that can exceed 10^8 bacteria per ml [4]. In regions without sanitation, much of this output is returned to the environment. Thus, from an ecological perspective, the principal role of the intestinal phase of the *V. cholerae* life cycle is to greatly boost its biomass in aquatic reservoirs and increase its spread through human populations. The initial steps of the infectious process, particularly colonization of the small bowel and induction of the virulence program, have been the subject of many reports. By contrast, the later steps, characterized by detachment of bacteria from epithelial surfaces and their movement into the fluid-filled lumen of the bowel, have received comparatively little attention, even though this is seemingly crucial for the organism's dissemination to other hosts and environmental sites.

In this report we focus on the last phase of the infectious process, seeking to learn how and why *V. cholerae* leave its niche as an attached community on the mucus membranes of the small intestine. We test the idea that this process is mediated by a genetic program that causes a near-synchronous detachment of bacteria from epithelial surfaces and activation of chemotaxis and motility functions. This notion comes from previously reported scanning electron micrographs (SEM) of *V. cholerae*-infected rabbit ileal loops showing

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Abbreviations: CT, cholera toxin; GFP, green fluorescent protein; LB, Luria Bertani; SEM, scanning electron microscopy; TCP, toxin co-regulated pilus

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Synopsis

Vibrio cholerae, a pathogenic microbe, causes a severe diarrhoeal disease mainly in Third World countries. Although the pathogenicity of this organism has been intensively studied for more than a century, most research has focused on the initial stages of the infection, especially colonization of the intestine and virulence gene expression. However, the last stages of the infectious process have received very little attention. In the present manuscript, the authors use the rabbit ileal loop model of cholera to show how this organism, late in the infection, detaches from the epithelial surface and migrates into the luminal fluid, a process the authors termed the "mucosal escape response." This study identifies, for the first time, how the alternative starvation sigma factor RpoS regulates this process. Features of this genetic program include the dramatic induction of genes involved in motility and chemotaxis functions. This study furthermore identifies RpoS as an important regulator of virulence gene expression and shows that the mucosal escape response may coincide with diminished virulence gene expression. This work is essential for understanding a key and under-appreciated step in the life cycle of this important human pathogen: its exit from the intestine and how this serves to prepare it for transmission into environmental reservoirs or to new human hosts.

that bacteria detach from the epithelial surfaces late in the infection [2,5]. Microscopic and gene expression studies of rice water stools from cholera patients furthermore show large numbers of actively swimming organisms [6–8] and a strong expression of chemotaxis and motility genes [9]. We also test the idea that this event coincides with termination of virulence gene expression, a supposition prompted by microarray expression studies of *V. cholerae* in rice water stools showing low transcript abundance for genes coding for TCP and CT [10,11]. *V. cholerae* in rice water stools also appears to be physiologically transformed to a state of enhanced infectivity during passage through the gastrointestinal tract [10]. Thus, observations from the rabbit ileal loop model of cholerae and the study of stool samples from cholera patients point to a genetic program that separates the microbe from mucus membranes, activates motility functions, suppresses the expression of virulence determinants, and prepares the organism for infection of the next host.

Little is known about conditions at the epithelial surface that might elicit these responses during the later stages of the infection. One possible condition is an increase in the number of attached bacteria owing to rapid replication during earlier stages of the infection. The accumulation of quorum-sensing signals produced by the expanding population of attached bacteria would increase the production of HapR [12], a transcription factor that represses virulence gene expression and biofilm formation and induces production of the hemagglutinin/protease HapA [13–17], a proteolytic enzyme shown to be involved in detachment of *V. cholerae* from cultured epithelial cells [18,19]. Such a quorum-sensing response would likely affect all bacteria at the same site and thus might serve to coordinate a response by the entire population. Another condition, nutrient limitation, likely occurs as bacterial numbers increase owing to an imbalance between the metabolic demands of the bacterial population and substrate availability. The slowing of growth that likely ensues might resemble, in part, the entry of in vitro grown bacteria into stationary phase. Finally, the epithelial surface may become increasingly inhospitable to attached bacteria as the infection progresses owing to H_2O_2 production by villous epithelial cells [20]. CT also stimulates nitric oxide production by small bowel mucous membranes [21] and the release of defensins from Paneth cells in the villous crypt (A. Nielsen, unpublished data). Together these factors—high cell density, nutrient limitation, and the presence of H_2O_2, nitric oxide, and antimicrobial peptides—might explain why bacteria detach and swim away.

Conditions likely to be encountered by *V. cholerae* in the intestinal environment, including amino acid starvation, osmotic stress, and growth deceleration, are known in *Escherichia coli* to result in the accumulation of RpoS, the stationary phase alternative sigma factor [22–26]. *V. cholerae* and *E. coli,* both enteric members of the proteo-gamma bacteria, share conserved regulatory elements [27]. Although little is known about the regulation of RpoS in *V. cholerae,* it is likely to be similar to *E. coli* in many respects. In *E. coli,* scarcity of amino acids causes a build-up of the nucleotide ppGpp and subsequent induction of the stringent response. If this is associated with a reduction in the transcription of rRNA, then the stringent response results in a strong induction of *rpoS* [25,28]. In turn, RpoS confers resistance in *E. coli* and in *V. cholerae* to several environmental stresses including hydrogen peroxide [29]. The accumulation of RpoS in *V. cholerae,* when combined with activation of the quorum-sensing circuit in dense aggregates of bacteria on the epithelial surface, would powerfully upregulate *hapR* expression leading to repression of virulence gene expression [14] and increased production of HapA [30,31], as discussed above.

Two observations indicate that RpoS does accumulate late in the infection and that the bacteria are transitioning from exponential growth to stationary phase. First, bacteria recovered from rice water stools from cholera patients show a clear induction of *rpoS* when compared to bacteria grown exponentially in vitro (see original microarray data from [10], summarized in Table S6). Second, viable plate counts of *V. cholerae* in the rabbit ileal loop model show initial rapid replication of the organism followed by the onset of bacteriostasis later in the infection [32], a period that coincides with the departure of bacteria from the epithelial surface (this report) and with the increased expression of *rpoS* as determined by microarray expression profiling (this report). Together, these observations have led us to examine the possible role of RpoS during the last step of the infectious process.

The role of RpoS in *V. cholerae* pathogenesis has been previously studied [29,33]. However, prior studies did not focus on the later stages of the infectious process, nor did they employ the rabbit ileal loop model of cholera. In the work presented here, we have used the rabbit ileal loop model and confocal microscopy to obtain images that capture the pathogenic sequence of events that characterize the *V. cholerae* infectious process. These images show that the final step in this sequence, which we designate the "mucosal escape response," is marked by the detachment of bacteria from the epithelial surface. Analysis of this phenomenon by microarray expression profiling and the study of mutants show that it requires RpoS and entails the activation of motility and chemotaxis functions. Evidence is also presented

that the mucosal escape response may coincide with down-regulation of virulence gene expression.

Results

RpoS Is Required for the Mucosal Escape Response

To determine in our rabbit ligated ileal loop model if growth slows between 8 and 12 h post-inoculation as previously observed [32], we measured the number of colony forming units of *V. cholerae* in the luminal fluid during the 12 h post-inoculation time course (Figure 1E). The bacterial counts were expressed as colony forming units per centimeter of loop along the longitudinal axis of the intestine, to take differences in fluid accumulation into account. The kinetics of growth in vivo late in the time course showed

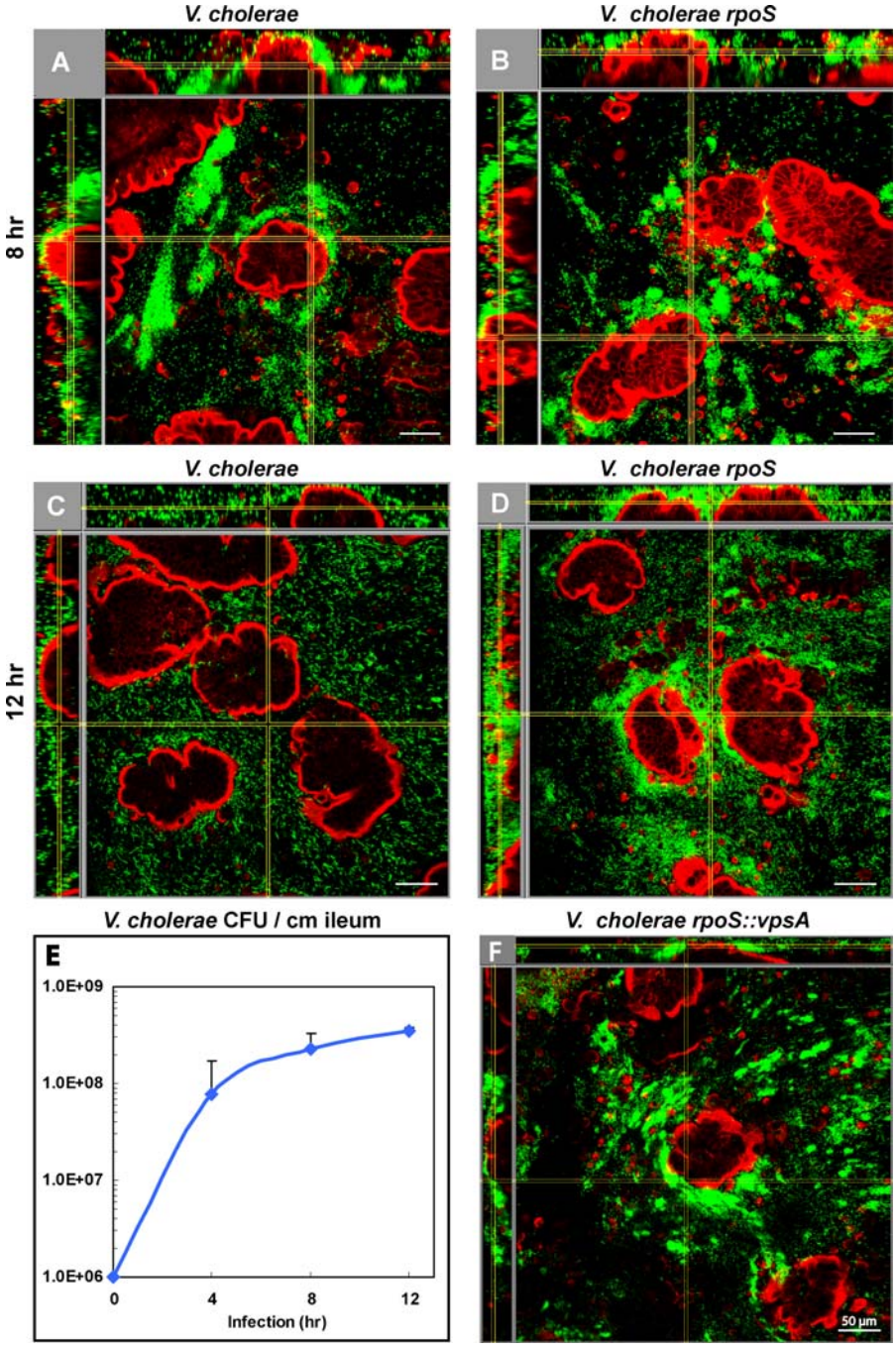

Figure 1. Growth of *V. cholerae* O1 El Tor, Strain A1552, in Ligated Ileal Loops and the Role of RpoS on Bacterial Localization during Infection

Bacteria were visualized using confocal microscopy 8 h (A and B) and 12 h (C, D, and F) post-inoculation of ligated rabbit ileal loops. The actin-rich epithelial surface was stained with phalloidin and is shown with red color, while GFP-tagged *V. cholerae* are shown with green color. Images are reconstructed Z-projections and show horizontal sections of the villi infected with wild-type *V. cholerae* (A and C), *V. cholerae rpoS* (B and D), and *V. cholerae rpoS/vpsA* (F). Scale bar represents 50 μm. (E) Growth of *V. cholerae* A1552 in rabbit ligated ileal loops was followed 4, 8, and 12 h post-inoculation based on multiple loops in at least three individual rabbit experiments per time point. Bacterial growth in the luminal fluid was determined by viable plate counts as colony forming units divided by the length of the loop to account for differences in fluid accumulation between loops.

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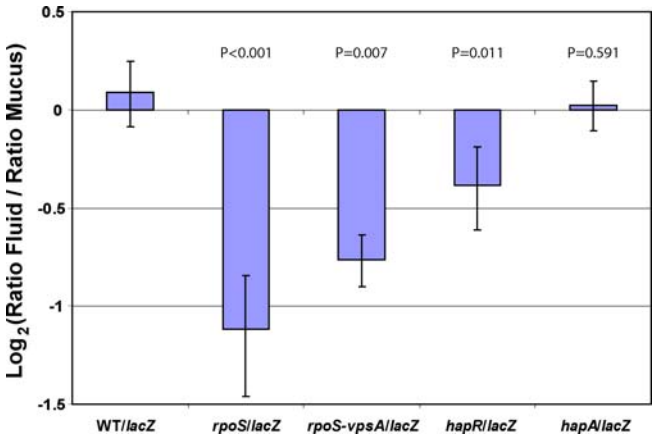

Figure 2. Distribution of Bacteria between Mucous and Luminal Fluid

Quantitative localization assays in rabbit ligated ileal loops were used to compare the distribution of wild-type bacteria and different deletion mutants between the mucus and the luminal fluid. A wild-type strain with a *lacZ* deletion was mixed in equal numbers with the wild-type parent or the *rpoS, rpoS/vpsA, hapR,* or *hapA* deletion mutants and injected into rabbit ligated ileal loops. After 12 h of infection, the bacteria in the two compartments were isolated and the ratio between the strains in each compartment determined by plating. Log_2 to the ratio between these two values is a measure of the distribution of the mutant between the two compartments when compared with the distribution of the wild-type parent. A localization ratio less than zero indicates that the mutant strain is more numerous in the mucus fraction than in the luminal fluid fraction. Two-sample two-tailed Students *t*-test with a significance level of 0.05 was used to indicate a significant difference between the wild-type and the different mutants. The actual *p*-values are shown above the bars.

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markedly slowed rates of replication, thus resembling entry into stationary phase in vitro, a state of growth characterized by the accumulation of RpoS.

To assess the role of RpoS during infection, an inoculum of green fluorescent protein (GFP)-labeled *V. cholerae* O1 El Tor (strain A1552) or the GFP-labeled *rpoS* deletion mutant of this strain, was injected into rabbit ligated ileal loops and the course of infection followed by confocal microscopy. This strain was selected for study because it was isolated from a patient with cholera, had not been subjected to multiple in vitro passages, and had an intact copy of *hapR* [34], unlike the sequenced *V. cholerae* strain N16961 [14,35]. Fluid was drained from infected loops 4, 8, and 12 h post-inoculation and the loops gently opened, mounted, trimmed, and fixed in formaldehyde in a way that preserved the mucus layer. Samples of the tissue were stained with phalloidin to visualize the actin-rich epithelial surfaces and scanning confocal laser microscopy was employed to visualize GFP-tagged bacteria attached to the epithelial surface or suspended in the layer of mucus covering the epithelial surface. Confocal images at 4 h post-inoculation showed beginning colonization of the epithelial surfaces for both the wild-type parent and the *rpoS* mutant (unpublished data). Images taken 8 h post-inoculation showed significant colonization of the epithelial surfaces and disclosed no differences in the distribution of the wild-type parent and the *rpoS* mutant between the epithelial surface and overlying mucus layer (Figure 1A and 1B). At 12 h post-inoculation, the wild-type parent was found mainly as single cells in the mucus layer (Figure 1C), consistent with its emigration from this compartment and its movement into the fluid-filled lumen as previously observed [2,5]. The *rpoS* mutant, however, continued to form dense bacterial aggregates on the epithelial surface (Figure 1D). The in vivo appearance of the *V. cholerae rpoS* mutant on villous epithelial cells was strikingly similar to the in vitro behavior of the same mutant in laminar flow cells studied by confocal microscopy, showing that it forms a thicker biofilm on a glass surface than the wild-type parent [36]. Increased biofilm formation by the *rpoS* mutant in laminar flow cells has been attributed to over-production of extracellular polysaccharide, owing to increased expression of the *vps* gene cluster [36]. To address this possible explanation for the *rpoS* in vivo phenotype depicted in Figure 1D, we deleted *vpsA,* a gene required for the biosynthesis of the extracellular polysaccharide [37]. The resulting *rpoS/vpsA* double mutant also failed to detach from the epithelial surface (Figure 1F), indicating that over-production of extracellular polysaccharide is not responsible for the *rpoS* in vivo phenotype. Moreover, a *vpsA* deletion mutant was found to colonize the epithelial surface in a way indistinguishable from the wild-type parent (unpublished data), demonstrating that extracellular polysaccharide is not required for intestinal colonization in the rabbit ileal loop model. The wild-type strain was mainly found as single cells 12 h post-inoculation as shown in Figure 1. However, some confocal images and microscopy of the luminal fluid also showed the presence of dense clumps of bacteria often associated with extruded epithelial cells and other membrane fractions at this time point. These observations are highly congruent with the recent findings by Faruque et al. showing that in vivo-formed biofilms may be important for the transmission of *V. cholera* between patients [38].

Quantification of the spatial distribution of bacteria in the ileal loop using image analysis is difficult due to the irregularity of the intestinal surface. Therefore, quantitative in vivo localization experiments were performed to test if emigration of the *rpoS* mutant from the epithelial surface was impaired. The wild-type *V. cholerae* strain, bearing a deleted *lacZ* gene, was mixed in equal numbers with either the wild-type parent or with the *V. cholerae* strain carrying a deletion of *rpoS* and the mixture of the two strains then inoculated into ligated ileal loops. After 12 h of infection, the fluid that had accumulated in the loops was collected, the loops incised to expose the mucous membranes, and the membrane surface gently washed in PBS buffer to remove any remaining luminal fluid. The tissue then was flattened and the mucus gel overlying the epithelium was gently scraped off and collected. Luria Bertani (LB) plates containing X-gal were used to enumerate *lacZ*-positive and *lacZ*-negative colonies from the luminal fluid and mucus gel samples. These counts were then used to compute the ratio of each pair of tested strains in the luminal fluid and the mucus gel compartments using values derived from a total of four loops from three different rabbits. The resulting localization ratios, for example, $Log_2[(rpoS/WT_{lacZ})_{lumen}/(rpoS/WT_{lacZ})_{mucus}]$, were taken as a measure of the relative distribution of the two strains between the two compartments. This approach largely eliminates the effect of possible differences in inoculum size between the tested strains and also possible differences in isolation efficiency between the mucus gel and luminal fluid samples. Ratios less than zero indicate that the mutant was less numerous than the wild-type parent in the luminal fluid compartment than the mucus gel compartment. Figure 2 shows that the *lacZ* deletion mutant derived from the wild-

type parent is neutral when compared to the *lacZ*-positive wild-type parent. By contrast, the *rpoS* mutant showed a very strong and statistically significant tendency to remain in the mucus gel layer compared to the wild-type parent, thus supporting the confocal imaging results shown in Figure 1. Of crucial importance to the interpretation of this result is the following observation: the total number of *rpoS* mutant bacteria in the mucus gel plus luminal fluid fractions was found to equal the total number of wild-type parent bacteria in both fractions combined. This indicates that the localization phenotype of the *rpoS* mutant was not due to growth rate differences between the mutant and wild-type parent in the intestine. Taken together, the results shown in Figure 1 and Figure 2 indicate that *rpoS* is required for the efficient emigration of bacteria from the epithelial surface and mucus gel compartments into the luminal fluid compartment during later stages of the infectious process. Although the effect on the localization ratio of the *rpoS/vpsA* double mutant was somewhat less than that of the *rpoS* single mutant, the double mutant also showed a statistically significant tendency to remain in the mucus gel layer compared to the wild-type parent (Figure 2). This supports the confocal images showing that overproduction of the extracellular polysaccharide does not cause the localization phenotype of the *rpoS* mutant that is depicted in Figure 1.

The images shown in Figure 1 suggest that the RpoS localization phenotype is stronger than is reflected in the quantitative localization data in Figure 2. This difference is possibly caused by technical difficulties in separating the luminal fluid from the mucus layer, which is loosely associated with the epithelial surface. In addition, because the *rpoS* mutant autoaggregates, it may be under-counted during the plating assay. Finally, it is possible that the wild-type parent, which is present in equal numbers, trans-complements the localization defect of the *rpoS* mutant by secreting a detachase or some other extracellular factor favoring the mucosal escape response.

Increasing RpoS abundance and cell density positively controls the expression of *hapR* [12,13]. In turn, HapR coordinately downregulates the expression of virulence determinants and biofilm formation (including production of an extracellular polysaccharide) and upregulates the production of HapA, a proteinase hypothesized to promote the detachment of *V. cholerae* from epithelial surfaces [18]. The regulatory effects of HapR and its induction by RpoS and increasing cell density prompted us to determine if HapR is also involved in the mucosal escape response. To test this idea, quantitative localization experiments were performed in the ileal loop model between the wild-type parent and a *hapR* deletion mutant, and the localization ratios computed as described above. The *hapR* deletion strain was found to localize mainly in the mucus compartment. Although still statistically significant, the magnitude of this phenotype was less pronounced than the effect of the *rpoS* mutation (Figure 2). This indicates that the effect of RpoS on the mucosal escape response cannot be ascribed entirely to a defect in *hapR* expression. To test if the phenotype of the *hapR* mutant was due to a polar effect, we complemented the *hapR* mutant with a single functional copy of *hapR* inserted onto the chromosome. The smooth colony morphology and the production of protease activity were restored to wild-type levels (unpublished data). Disruption of *hapR,* carried out in the smooth colony type genetic background, caused conversion to the rugose morphotype, as noted by others [13,36]. To determine if the rugose colony switch was responsible for the localization phenotype of the *hapR* mutant shown in Figure 2, we deleted *vpsA,* thus producing a *hapR/vpsA* double mutant. Deletion of the *vpsA* gene caused reversion of the strain to the smooth morphotype, but microscopy studies with the double mutant yielded the same localization phenotype as the *hapR* single mutant (unpublished data). Taken together, these results indicate that HapR likely contributes to the mucosal escape response, and that this role is not affected by changes in the production of the extracellular polysaccharide.

The hemagglutinin/protease encoded by *hapA,* which is positively regulated by RpoS and HapR, has long been believed to play a role in mucus penetration and detachment of *V. cholerae* from epithelial surfaces [18,19,30]. However, it was not found to be a virulence determinant when tested in an infant rabbit model, and it did not affect colonization in a suckling mouse model of cholera [18,39]. To determine if HapA is required for the mucosal escape response, a *hapA* deletion mutant was constructed, demonstrated to lack protease activity (unpublished data), and studied in ileal loop localization experiments with the wild-type parent, as described above. No localization phenotype was detected (Figure 2). To ensure that this result was not due to cross complementation by HapA protease secreted by the wild-type strain used in the quantitative localization experiments, we used confocal microscopy to study the GFP-labeled *hapA* mutant alone. Its phenotype in the intestine, including its localization behavior, was not distinguishable from the phenotype of the wild-type parent (unpublished data). Thus, the confocal microscopy and quantitative localization experiments show that RpoS and HapR are required for the mucosal escape response. However, the phenotypes depicted in Figure 1 and Figure 2 cannot be attributed to the effect of HapR on the production of HapA.

Chemotaxis and Motility Genes Are Upregulated during Stationary Phase and in the Ileal Loop during the Mucosal Escape Response

The data depicted in Figure 1 and Figure 2 suggest that *V. cholerae* moves from the epithelium into luminal fluid by hour 12 of the infectious process. We hypothesize that this involves activation of motility and chemotaxis functions. To investigate this issue, we compared expression profiles from *V. cholerae* collected from the luminal fluid of the ligated ileal loop 12 h post-inoculation and from stationary phase bacteria grown for 11 h in LB broth.

Bacteria in stationary phase were studied because viable plate counts of bacteria obtained from *V. cholerae*-infected ileal loops showed that growth rate declined between 8 and 12 h post-infection (Figure 1E). RpoS, the stationary phase alternative sigma factor, was in addition found to be induced in bacteria isolated from patient rice water stools when compared to an exponentially grown reference (original microarray data from [10], summarized in Table S6). Finally, as reported below, transcriptional profiles of bacteria isolated from rabbit ileal loops and from patient stool were similar with respect to the expression of chemotaxis and motility genes to in vitro-grown bacteria transitioning into stationary phase (summarized in Table S6). Together, these observations

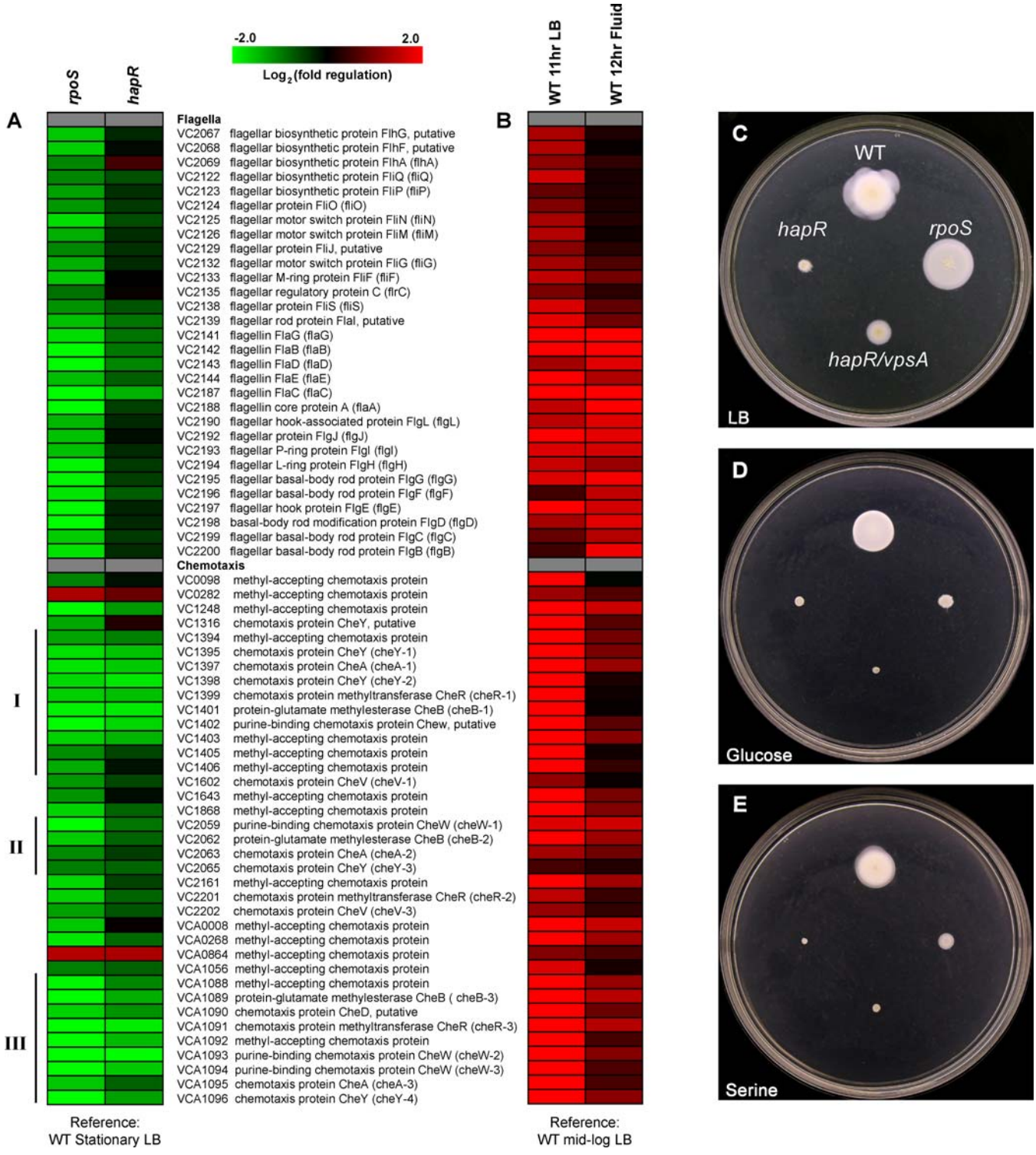

Figure 3. RpoS and HapR Are Required for the Regulation of Chemotaxis and Motility Genes in Stationary Phase

(A) Gene expression of *V. cholerae, rpoS,* and *hapR* deletion mutants in stationary phase LB cultures was analyzed and compared to the wild-type parent under identical conditions. Only chemotaxis and motility-annotated genes with at least a 2-fold statistically significant change in the *rpoS* mutant are shown (except for *flrC,* which was induced 1.9-fold with statistical significance). Genes requiring RpoS or HapR for their induction are identified with green colors. The three genomic chemotaxis clusters are marked and numbered to the left of the cluster.

(B) Gene expression of the wild-type parent during stationary phase after 11 h growth in LB was analyzed using RNA from an exponentially growing culture as a reference. The expression of these genes is compared with the expression of genes from bacteria isolated from luminal fluid after 12 h of infection of rabbit ileal loops using the same mid-exponential phase reference. Genes induced under these conditions are identified with a red color.

(C–E) The motility of the wild-type parent was compared with the mutants by spotting wild-type *V. cholerae* (top), *hapR* (left), *rpoS* (right), and *hapR/vpsA* (bottom) on low-percentage agar plates formulated with either LB (C), glucose (D), or serine (E) as the only carbon source. The plates were incubated at 37 °C for 24–48 h.

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support the idea that some aspects of *V. cholerae* physiology during the last stages of the infectious process in vivo resemble adaptations that occur during entry into stationary phase in vitro. In both the in vivo ileal loop and in vitro stationary phase expression profiling studies described below, we used RNA from exponential phase, LB-grown bacteria as the reference condition.

The expression of 531 genes was found to be more than 2-fold increased in LB-grown stationary phase bacteria when RNA from exponential phase bacteria was used as the reference condition (Table S1). Here we focus on genes upregulated in stationary phase and within that group on the large subset of genes annotated by the *V. cholerae* genome project [40] to specify chemotaxis or motility functions. Of the 114 genes in this functional category, 72 (63 %) were more than 2-fold upregulated during stationary phase compared to their expression in mid-exponential phase cultures. Thus, adaptation to stationary phase coincides with a powerful and quite broad activation of chemotaxis and motility functions. Among these are 24 of the 43 genes encoding methyl-accepting chemotaxis proteins (Figure 3B and Table S5). Also upregulated during stationary phase are 19 genes specifying other components of the chemotaxis signal transduction system. The latter mostly reside in three separate genomic clusters [41] (two clusters on Chromosome I and one on Chromosome II). Genes in each of these clusters were found to be induced during stationary phase (Figure 3B), including the majority of genes in cluster II that were previously shown to be important for chemotaxis [41]. Remarkably, 30 of the 44 annotated flagellar biosynthesis genes were strongly upregulated during stationary phase.

Expression profiling was also used to identify genes whose expression during stationary phase requires RpoS or HapR. For this purpose, RNA from stationary phase wild-type bacteria was used as the reference condition. RpoS was found to be required for the upregulation of 60 of the 72 chemotaxis and motility genes whose expression was increased in wild-type bacteria during stationary phase (Figure 3A). Amongst the RpoS-dependent genes in this functional class are 19 encoding methyl-accepting chemotaxis proteins, most of the chemotaxis signal transduction genes in the three genomic clusters, and 29 of the 44 genes encoding proteins involved with the biosynthesis and function of flagella. Thus, while the alternative sigma factor RpoN (σ^{54}) is known to be required for the expression of flagellar biosynthesis genes during both exponential growth and stationary phase ([42,43] and unpublished data), most of these genes also require RpoS for their induced expression during stationary phase. In contrast, only one chemotaxis gene was found to require RpoS for a more than 2-fold induction during exponential growth [36].

HapR, shown in Figure 2 to be required for the mucosal escape response, was found to control the expression of 16 of the 72 (22%) chemotaxis and motility genes upregulated during stationary phase (Figure 3A and Table S3). All the HapR-dependent genes also require RpoS for their expression and therefore are a subset of the RpoS-dependent set of genes. The HapR subset includes five genes coding for methyl-accepting chemotaxis proteins, ten genes coding for chemotaxis signal transduction proteins in clusters I and III (Figure 3A), and two genes coding for flagellar biosynthesis proteins. The two HapR-dependent flagellar genes encode the "alternate" flagellins FlaD and C. The genes in chemotaxis cluster II, which have been shown to be important for *V. cholerae* chemotaxis [41,44], are strongly regulated by RpoS, whereas they seem to be relatively independent of HapR as shown in Figure 3A. Thus, unlike RpoS, which controls the expression of both flagellar biosynthesis genes and chemotaxis genes (including those in cluster II) during stationary phase, HapR shows a strong bias toward the control of chemotaxis genes, particularly those belonging to clusters I and III. During exponential growth, Yildiz et al. found that HapR is required for the induction of a number of flagellar biosynthesis genes including the flagellins, *flaACD* [36]. Promoter motif analysis further revealed that a wide range of both chemotaxis and flagellar biosynthesis genes have the HapR signature [36]. Included amongst these were the chemotaxis clusters I and II and the flagellar biosynthesis genes VC2066–69, VC2120–37, *flaC,* and *flgG*. Thus, a substantial overlap exists between the computationally predicted HapR regulon and the experimentally determined genes that require HapR for their induction. Since the experimentally determined HapR regulon was identified under narrow physiological conditions, it is likely that additional HapR dependencies may be observed under other conditions.

Microarray expression profiling was also used to compare the regulation of chemotaxis and motility genes in stationary phase with their regulation in ileal loop fluid 12 h post-inoculation. For ileal loop expression experiments, RNA from exponential phase bacteria grown in LB was used as the reference condition. 36 of the 114 annotated chemotaxis and motility genes (32%) were found to be upregulated in the ileal loop at this stage of the infection (Figure 3 and Table S4) compared to their expression in exponential phase cultures. Moreover, 29 of the 36 chemotaxis and motility genes that were upregulated in the ileal loop were also induced during stationary phase growth, thus demonstrating the similarity between the growth state of bacteria in the 12-h ileal loop and during stationary phase with respect to genes in this functional category. Of the genes upregulated in ileal loop fluid, ten code for methyl-accepting chemotaxis proteins; three of these (VC1248, VC1898, and VCA0008) were powerfully induced in the ileal loop fluid at this time point. Also induced were five genes coding for components of the chemotaxis signal transduction cascade *(cheA-1, cheW-1, cheW-2, cheY-4,* and *cheB-2).*

Of the 44 genes annotated to be involved with the biosynthesis or function of flagella, 20 were found to be induced in ileal loop fluid coincident with the mucosal escape response. When these genes were classified using the four-class flagellar transcriptional hierarchy scheme of Prouty et al. [43], nearly all of the flagella genes upregulated at this time point in the ileal loop were found to belong either to Class III (encoding the basal-body rod, hook, rings, and the "core" flagellin FlaA) or to Class IV (encoding the alternate flagellins FlaB, FlaD, FlaE, and FlaC). Class III genes require FlrC together with the RpoN holoenzyme for their expression, whereas Class IV genes require σ^{28} [43]. Interestingly, while most of the genes in transcriptional hierarchy Class II were upregulated in LB broth stationary phase cultures, they were not upregulated in the ileal loop 12 h post-inoculation. However, production of the Class II proteins precedes production of Class III and Class IV proteins [43]. Therefore,

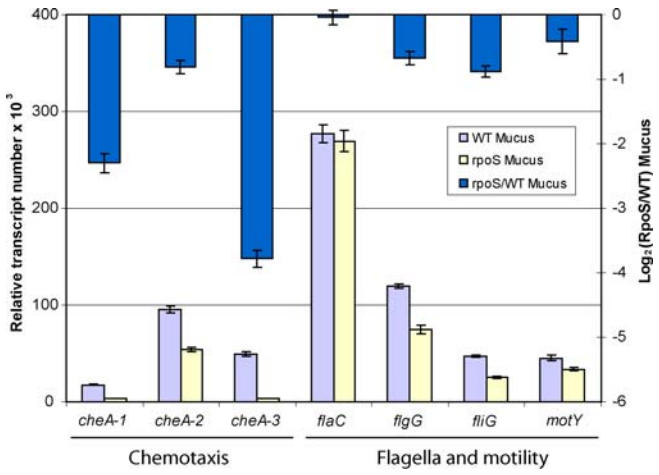

Figure 4. RpoS Is Required for the Expression of Chemotaxis and Motility Genes in Rabbit Ileal Loop Mucus

Real-time RT-PCR was used to assess relative gene expression of selected motility genes in the wild-type parent or the *rpoS* mutant isolated from rabbit ileal loop mucus 12 h post-inoculation for selected genes involved in chemotaxis and flagellar biosynthesis. The left vertical axis depicts the relative transcript number ($\times 10^3$) for the wild-type parent (light blue) and the *rpoS* mutant (yellow). The right vertical axis depicts the Log_2 to the ratio of transcript abundance for the *rpoS* mutant and the wild-type parent (dark blue). Data from one typical experiment are shown.

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it seems likely that some of the Class II structures (the membrane/supramembrane ring, switch, and export apparatus) were pre-assembled during an earlier phase of the infectious process. These results also suggest that the Class III and IV structures described above, which must have been produced at earlier stages in the infectious process in order for the bacteria to swim through mucus to reach the epithelia surface, may have been lost or otherwise degraded in some bacteria of the attached population. If so, then they would have to be re-synthesized prior to or during the mucosal escape phenomenon, thus explaining why they, but not Class II genes, were upregulated in the ileal loop 12 h post-inoculation. Indeed, one possible scenario entails loss of the flagellar filament, hook, rings, and basal-body rod by bacteria attached to epithelial surfaces (when flagellar-dependent motility would be superfluous) and then their re-synthesis as a prelude to the emigration of the bacteria from the surface and into luminal fluid. Unfortunately, we were not able to test this idea directly since the resolution of the confocal images precludes seeing if bacteria attached to the epithelial surface still retain flagellar filaments. However, a careful review of previously obtained SEM of *V. cholerae*-infected human small intestine and rabbit ileal loops at different time points showed that many of the bacteria attached to the epithelial surface lack flagella [5,45].

Of the 36 chemotaxis and motility genes upregulated more than 2-fold in the rabbit ileal loop 12 h post-inoculation, 29 (81%) were found to require RpoS for their induction during stationary phase. These include 16 of the 20 flagellar biosynthesis genes upregulated in the ileal loop, thus explaining why RpoS is required for the motility component of the mucosal escape response. In addition, two genes (VC1248 and VCA0008) encoding methyl-accepting chemotaxis proteins stand out because they are strongly expressed in the ileal loop and require RpoS for their expression during stationary phase. Their induction suggests that the chemoreceptors they encode may be responding to chemical gradients extant in the intestine at this stage of the infection. Of the 36 motility and chemotaxis genes upregulated in the ileal loop 12 h post-inoculation, ten were found to require HapR for their expression during stationary phase. Only two of these, *flaD* and *C* code for proteins involved with flagellar biosynthesis. Rather, most HapR-dependent genes upregulated in the ileal loop specify chemotaxis proteins, leading us to predict that the *hapR* mutant will produce near normal numbers of flagella, but exhibit a disproportionate loss of chemotactic function.

The mucosal escape response appears to be initiated at the interface between the epithelial surface and the overlaying layer of mucus. To corroborate the expression profile results of bacteria in luminal fluid and to focus more precisely on the bacteria at this interface, real-time RT-PCR was performed using a mucus-associated fraction of bacteria in 12-h ileal loop samples to monitor the expression of seven genes involved in chemotaxis and motility *(cheA-1, cheA-2, cheA-3, flaC, flgG, fliG,* and *motY).* Gene-specific transcripts were detected for each of the seven tested genes for wild- type bacteria in the mucus layer 12 h post-inoculation, indicating that these chemotaxis and flagellar biosynthesis genes were expressed in this compartment at this time point. Particularly abundant were transcripts corresponding to *cheA-2, flaC,* and *flgG* (Figure 4). To determine if the expression of these genes was stronger in bacteria in the mucus fraction than bacteria in the luminal fluid, we also performed real-time RT-PCR on luminal fluid from the same loops. On average this showed that the seven chemotaxis and motility genes were expressed 1.9-fold more strongly in the mucus than in the luminal fluid (unpublished data). Real-time RT-PCR assays were also performed to determine the transcript abundance of the same seven genes for the *rpoS* mutant in the mucus compartment at the same time point. Compared to wild-type bacteria, six of the seven genes were found to be more strongly expressed in the wild-type parent than in the *rpoS* mutant (Figure 4). Of these, the fold-difference was most striking for *cheA-1* (~4-fold) and *cheA-3* (~16-fold). In contrast, the measured *flaC* transcript abundance for the wild-type and the *rpoS* mutant was found to be the same, even though *flaC* requires *rpoS* for its expression during stationary phase (Figure 3A). This discordance points to the possibility that the regulation of some genes may be different in vivo and in vitro.

RpoS and HapR Control Motility and Flagellar Biosynthesis during Stationary Phase

The data presented above demonstrated that more than 80% of the chemotaxis and motility genes which were upregulated in the ileal loop 12 h post-inoculation also required RpoS for their induction during stationary phase. To further investigate this observation, we studied motility under contrasting conditions of nutrient availability. The wild-type parent and the *rpoS, hapR,* and *hapR/vpsA* mutants were inoculated onto either nutrient-rich swarm plates containing LB broth or onto nutrient-limited swarm plates formulated with a minimal medium containing ammonium as the principal nitrogen source and either 0.5% glucose or 0.5% serine as sole carbon source. In contrast to growth on swarm plates containing LB broth, these nutrient-limited conditions

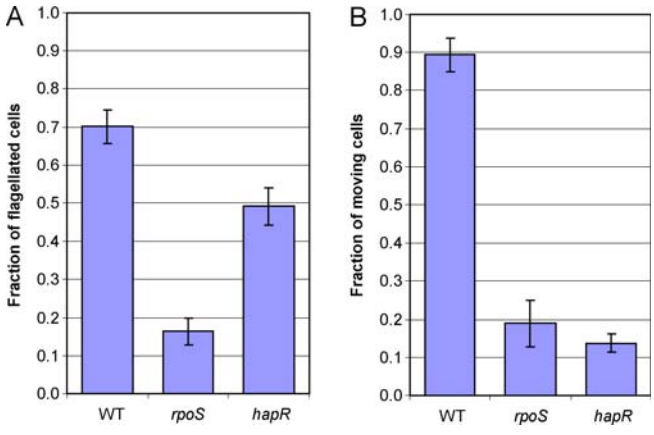

Figure 5. Fraction of Flagellated and Motile Cells in Stationary Phase

Wild-type *V. cholerae* and the *rpoS* and *hapR* deletion mutants were analyzed for the fraction of flagellated cells using SEM after 11 h growth in LB broth (A), and for motility using video tracing microscopy after 11 h growth in a minimal medium supplemented with lactate as the only carbon source (B).

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likely result in the accumulation of RpoS owing to decreased replication rates and induction of the stringent response [22]. On LB swarm plates, the *rpoS* mutant exhibited a wild-type motility phenotype, while the motility of the *hapR* mutant was significantly affected. This motility defect is not solely due to conversion from the smooth to the rugose colony morphotype, as the smooth *hapR/vpsA* double mutant also showed diminished motility (Figure 3C). The motility phenotype of the *hapR* mutant on LB swarm plates corroborates previous data by Yildiz et al., who demonstrated an 80% reduction of motility of the *hapR* mutant under the same conditions of growth [36]. The motility phenotype depicted in Figure 3C and the observation of Yildiz et al. are commensurate with microarray data showing that HapR is required for the expression of several flagellar biosynthesis genes during exponential and stationary phase ([36] and Figure 3A). When the *rpoS, hapR,* and *hapR/vpsA* mutants were grown on nutrient-limited swarm plates, all were found to be significantly less motile than the wild-type parent (Figure 3D and 3E). Similar motility phenotypes were obtained when the *rpoS* and *hapR* mutants were tested on swarm plates with nutrient-limiting concentrations of lactate, maltose, and glycerol phosphate (unpublished data).

The motility phenotype of the *hapR, hapR/vpsA,* and *rpoS* mutants during growth under nutrient-limiting conditions could be due to the reduced production of flagella, to impaired flagellar function, or to defects in chemotaxis. SEM of stationary phase bacteria obtained from culture conditions identical to those used for the expression profiling studies shown in Figure 3 revealed that only 20% of the *rpoS* mutant bacteria had intact flagella after entry into stationary phase, whereas intact flagella were detected on approximately 50% of the *hapR* mutant bacteria and on 70% of the wild-type parent after entry into stationary phase (Figure 5A). Video tracing microscopy was used to quantify the effect of the *rpoS* and *hapR* deletions on motility. Fewer than 20% of the *rpoS* or *hapR* mutants were motile, whereas almost all of the parental bacteria swam rapidly after 11 h growth in a minimal medium supplemented with lactate. Taken together, these data show that *rpoS* and *hapR* are required for the production of flagella and for motility during stationary phase.

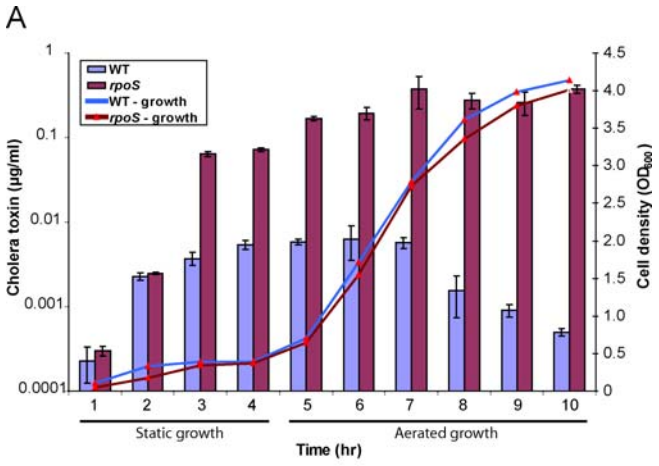
These data also suggest that the flagella seen by SEM in stationary phase cultures of the *rpoS* mutant had been formed during early and mid-exponential phase growth. Based on cell density measurements and the fraction of *rpoS* mutant bacteria with flagella (as determined by SEM during stationary phase), flagella production by this mutant appears to occur at cell densities below $OD_{600} = 0.9$ in LB media. At higher cell densities a decrease in growth rate is observed, and this is associated with a period of RpoS-dependent flagella production.

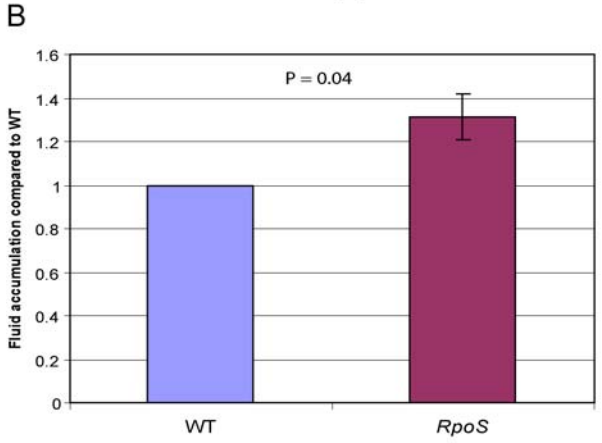
Differences were observed between the effect of the *hapR* deletion on flagella production (~29% reduction from wild-type levels, Figure 5A) and the effect of the deletion on motility (~83% reduction from wild-type levels, Figure 5B). This suggests that HapR may affect flagella function and/or chemotaxis in a manner that is independent of its control of flagellar biosynthesis. This supposition is supported by expression profiles of the *hapR* mutant (Figure 3A), which show that chemotaxis genes located in clusters I and III require HapR for their induction during stationary phase growth, and that these are more powerfully affected than the effect of HapR on flagellar biosynthesis genes.

Taken together, these results show the following: many of the genes that specify chemotaxis and motility functions are upregulated by wild-type bacteria in stationary phase cultures and in the luminal fluid of *V. cholerae*-infected ileal loops 12 h post-inoculation. The induction of these genes is synchronous with the RpoS-dependent mucosal escape phenomenon shown in Figure 1. These microarray expression results also show that during stationary phase RpoS is required for the upregulation of nearly all the chemotaxis and motility genes that were found to be induced in the rabbit ileal loop 12 h post-inoculation (Figure 4A and 4B). The in vitro data on the role of RpoS in the upregulation of chemotaxis and flagellar genes were found to be congruent with results obtained by real-time RT-PCR assays for the mucus-associated fraction of ileal loop bacteria 12 h post-inoculation (Figure 4). We therefore propose that the RpoS-dependent induction of motility and chemotaxis functions is an essential component of the mucosal escape program and that this response enables bacteria to leave the mucosal surface of the intestine and enter the luminal fluid.

The Effects of RpoS on Virulence Gene Expression

In addition to the activation of motility and chemotaxis functions, we reasoned that the mucosal escape response would logically entail coordinate downregulation of virulence gene expression late in the infectious process when continued production of virulence determinants would be ineffective. We also hypothesized that this too might require RpoS, owing to its capacity to increase the production of HapR [31], which negatively regulates genes specifying CT and TCP [14]. To test this idea we measured the concentration of CT in cultures containing either the *rpoS* mutant or the wild-type parent during growth in an vitro condition previously shown to strongly induce the genes encoding CT and TCP [46]. Bacteria were cultured statically for 4 h in AKI medium and then shifted to aerobic growth for 6 h using shaken culture flasks. Growth rate, monitored by optical density, showed that replication ceased after a few hours of static growth, then





C

Relative gene expression - Mucus 12hr

Gene	Gene product	RpoS / WT
VC0583	Transcriptional regulator (*hapR*)	0.11 (±0.02)
VCA0865	Hemagglutinin/protease (*hapA*)	0.66 (±0.10)
VC2647	Regulatory protein (*aphA*)	2.56 (±0.19)
VC0838	Virulence regulatory protein (*toxT*)	4.21 (±0.41)
VC0828	Toxin co-regulated pilin (*tcpA*)	1.33 (±0.21)
VC1457	Cholera enterotoxin (*ctxA*)	1.30 (±0.14)
VC0917	Polysaccharide biosynthesis (*vpsA*)	4.61 (±1.06)
VC0934	Capsular polysaccharide biosynthesis gene	10.4 (±3.85)

Figure 6. Role of RpoS in the Expression of Virulence-Associated Genes In Vitro and In Vivo

(A) CT production during AKI growth conditions. Bacterial growth and CT concentration were measured for wild-type *V. cholerae* (blue) bacteria and for the *V. cholerae rpoS* deletion mutant (red) during growth in AKI medium [46]. The cultures were kept static during the first 4 h of the experiment followed by aerobic growth with shaking during the rest of the experiment. Cell density is shown with solid lines, and CT concentrations are shown with bars plotted on a logarithmic scale.

(B) Fluid accumulation in the rabbit ileal loop model was compared between neighboring loops inoculated with either wild-type *V. cholerae* or the *rpoS* deletion mutant. The graph shows the average fluid accumulation of the *rpoS* mutant when compared with the neighboring wild-type strain in four pairs of loops from three different rabbits. The variance of the individual ratios is indicated on the graph. Paired one-tailed Students *t*-test with a significance level of 0.05 was used to ascertain the level of significance of the increased fluid accumulation of the *rpoS* mutant in the neighboring pairs of loops.

(C) Real-time RT-PCR assessment of relative expression levels of selected virulence-associated genes, regulators of virulence gene expression, and genes involved in production of the extracellular polysaccharide in bacteria isolated from rabbit ileal mucus 12 h post-inoculation. The ratio of transcript abundance between RNA isolated from the *rpoS* deletion strain and the wild-type parent is shown together with standard deviations indicated in brackets.

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resumed during aerobic growth and until the cultures entered stationary phase during the last 2 h of the assay (Figure 6A). CT production by the wild-type strain (monitored by ELISA) was evident soon after inoculation of the culture, but its rate of accumulation in the medium then declined coincident with the onset of growth arrest in the static cultures. In contrast, the *rpoS* mutant produced comparatively more CT throughout the static growth period, eventually achieving more than 10-fold higher concentrations than the wild-type parent. This coincided with increased *ctxA* and *tcpA* transcript levels in the *rpoS* mutant during the third and fourth hour of incubation in the static cultures (unpublished data) indicating that the accumulation of CT in this medium and at these time points is likely due to increased transcription of the corresponding genes rather than increased secretion or decreased degradation of CT. During the aerobic phase of the assay period, CT concentrations declined in the wild-type culture, whereas the concentration of CT remained high in the *rpoS* mutant culture (Figure 6A). Thus, in this in vitro assay, widely used to assess the production of virulence determinants by *V. cholerae,* RpoS was found to downregulate CT production, particularly during periods of growth deceleration or stasis.

To further test if RpoS is required to terminate the *V. cholerae* virulence program during the last stages of the infectious process, the accumulation of ileal loop fluid after 12 h of infection was investigated. Neighboring loops infected with the wild-type parent or *rpoS* mutant were compared directly due to differences in fluid yield in the different sections of the rabbit ileum and between different rabbits. The *rpoS* deletion mutant was found to stimulate the production of approximately 30% more fluid than the wild-type parent (Figure 6B). A paired one-tailed Student *t*-test of the four pairs of loops in three separate rabbits resulted in a *p*-value of 0.04 demonstrating significantly increased fluid accumulation by the *rpoS* mutant.

To further study the nature of RpoS-dependent regulation of the virulence program in the later stages of infection, real-time RT-PCR was used to compare the expression of genes coding for virulence determinants in the wild-type parent and *rpoS* mutant using RNA isolated from the mucus-associated fraction of bacteria 12 h post-inoculation. Compared to the wild-type parent, *hapR* expression was found to be profoundly reduced in the *rpoS* mutant, a result that is consistent with the proven role of RpoS as a positive regulator of *hapR* expression. As expected, this was associated with reduced expression of *hapA,* which codes for HA protease (Figure 6C). By contrast, *aphA* and *toxT,* which code for transcription factors that upregulate *ctxAB* and *tcpA,* were found to be expressed at significantly higher levels in the *rpoS* mutant than the wild-type parent. However, the effect of the *rpoS* mutation on the expression of *aphA* and *toxT* did not result in a strong overexpression of *ctxAB* or *tcpA* by the *rpoS* mutant. The discrepancy between the powerful effect of the *rpoS* mutation on the expression of *ctxAB* in vitro and the modest effect of the same mutation on *ctxAB* and *tcpA* expression in vivo is currently unexplained and is the focus of ongoing studies.

Discussion

V. cholerae has previously been shown to move through the mucus gel and attach to the epithelial surface during the first 8 h of infection in the rabbit ileal loop model (Figure 1 and [1,2]). This period coincides with the accumulation of luminal fluid, indicating that the genes encoding CT are induced early in the infectious process. However, 12 h post-inoculation, mass emigration of the population is evident as bacteria move away from the epithelial surface and into the fluid-filled lumen of the intestine (Figures 1 and 2), a phenomenon we have termed the mucosal escape response. During this process, the bacteria undergo a switch from rapid replication to bacteriostasis (Figure 1E) and from attachment to the epithelial surface to detachment (Figure 1). This process is associated with upregulation of chemotaxis and motility genes (Figures 3 and 4). Although RpoS was also shown to be required for reduced production of CT in an in vitro condition that induces expression of genes coding for CT and TCP (Figure 6A), evidence is suggestive, but not as compelling, that this occurs in vivo coincidental with the mucosal escape response (Figure 6B and 6C). We believe that this transition prepares the bacteria to leave the intestine, for survival in the environment, and for eventual transmission to a new host.

The results we describe in this report come from the combined use of confocal microscopy, expression profiling, and the study of the *rpoS* and the *hapR* mutants in the rabbit ligated ileal loop model of *V. cholerae*. This model was chosen because it entails a controlled and synchronized infection of the intestine. Although this model appears to simulate infection in humans in many respects, it employs a closed loop system, and thus the timing of the observed mucosal escape response may not reflect the progression of infection in a human host.

Flagella production and motility are also required early in the infection as the organism moves through the mucus gel toward the epithelial surface [32]. However, unlike the physiological state of the organism late in the infection, earlier stages are characterized by rapid growth and nutrient availability (Figure 1E). Moreover, at this time period, confocal images of the infectious process show that the in vivo phenotypes of the wild-type parent and *rpoS* mutant strain are indistinguishable (Figure 1). Together, these observations show that RpoS is not required for flagella production at early time points. Thus, although the production of flagella is required at both the early and later stages of the infectious process, RpoS is only required for this function late in the infection.

A possible explanation for this apparent paradox comes from the well-established central role of RpoN as a positive regulator of flagella production [42,43] (Figure 7). Studies of *E. coli* show that the concentration of RpoN does not change upon entry into stationary phase [24]. Rather, its activity as a regulator of a subset of RpoN-dependent genes is determined by the abundance of different RpoN-dependent transcriptional activators [47]. Our microarray expression studies of the *V. cholerae rpoS* mutant show that RpoS is required, during stationary phase, for the downregulation of four genes coding for RpoN-dependent transcriptional activators (*luxO*, VC1817, VCA0182, and VC0706) and for the 1.9-fold upregulation of one *(flrC)* (Table S2 and Figure 3). The

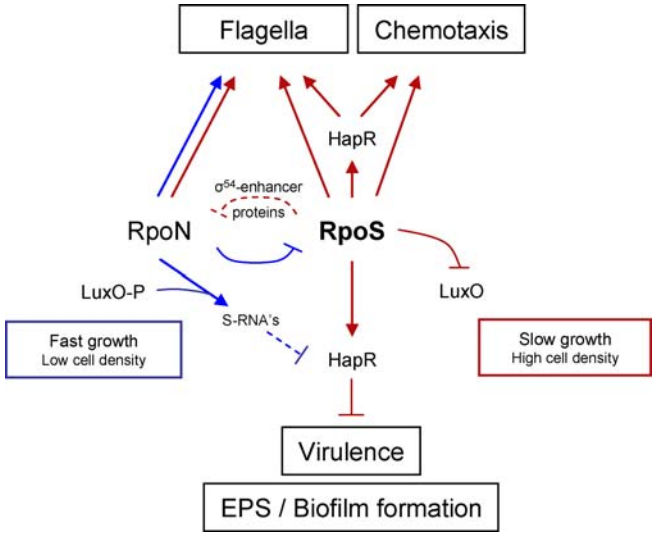

Figure 7. Model Depicting the Role of RpoS in the Regulation of CT (Virulence), Extracellular Polysaccharide, and Chemotaxis/Motility Genes during Different Growth States (Fast and Slow)

The regulation of functions during the initial stages of infection, characterized by fast growth and low-cell density, is shown in blue. Regulation late in the infection, characterized by slow growth and high-cell density, is shown in red. Solid arrows indicate direct transcriptional regulation whereas dotted arrows indicate indirect transcriptional or posttranslational regulation. During low-cell density and fast growth, RpoS is largely silent. However, RpoN acts as an inducer of flagellar biosynthesis. LuxO is phosphorylated and acts as an RpoN enhancer protein causing the production of small RNAs that degrade the virulence repressor HapR [51]. This in turn leads to induction of virulence functions and EPS production. By contrast, during conditions of slow growth and high-cell density, RpoS together with RpoN is required for the induction of flagellar biosynthesis genes in both a *hapR*-dependent and a *hapR*-independent manner. RpoS also represses the expression of *luxO,* which together with quorum sensing mediated dephosphorylation of LuxO-P, relieves repression of *hapR* expression. Together these cause a strong induction *hapR*. Finally, RpoS indirectly affects the activity of RpoN by repressing the expression of three other RpoN enhancer proteins (VC0706, VC1817, and VCA0182) in addition to *luxO*. EPS, extracellular polysaccharide.

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RpoN-dependent transcriptional activator FlrC together with RpoN, upregulates the expression of flagellar biosynthesis genes in class III of the proposed flagellar gene expression hierarchy [42,43]. Accordingly, the fact that RpoS is required for the induction of *flrC* during stationary phase might explain, in part, the observed effect of RpoS on flagella production that is depicted in Figure 5. In *Salmonella* it was recently shown that integration host factor is involved in RpoS-dependent activation of flagellar gene expression [48]. However, we did not find that the expression of genes coding for the α or β subunits of integration host factor are regulated by RpoS under the growth conditions used in this study. Quorum sensing also has previously been shown to be associated with induced flagella production and motility in *E. coli* [49]. Because HapR is a key regulator of the quorum-sensing response, the mucosal escape phenotype of the *hapR* mutant suggests that quorum sensing may be involved in regulating chemotaxis and motility in *V. cholerae*. However, as we were unable to show changed motility in swarm plate assays for either a *luxO* or a *luxS* deletion mutant (unpublished data), quorum sensing does not seem to be implicated in the RpoS-dependent induction of motility and chemotaxis.

The mechanism by which RpoS controls flagellar biosynthesis during stationary phase will require further study.

The in vivo and in vitro results presented above suggest the following scenario. Late in the infectious process, bacteria adjacent to the epithelial cell surface experience conditions that cause RpoS to accumulate. Evidence that RpoS accumulates at this time point comes from the following observations. First, measured growth rates in the rabbit ileal loop show that late in the infection replication slows significantly (Figure 1E). This growth deceleration in vivo resembles entry of the organism into stationary phase during in vitro growth in batch cultures, a period that is known to be associated with the accumulation of RpoS. Second, in vivo microarray expression data show that RpoS is upregulated 12 h post-inoculation in bacteria obtained from ileal loop luminal fluid and from rice water stools from patients with cholera [10] (summarized in Table S6). Finally, the deletion of *rpoS* causes a dramatic localization phenotype evident by confocal microscopy (Figure 1D). The regulator *hapR* is expected to be induced at the same time and site by the quorum-sensing circuit owing to increasing cell density [12], and because its expression is directly upregulated by RpoS [36] (Figure 7). These regulatory events coincide with upregulation of flagella production and activation of motility and chemotaxis functions late in the infectious process allowing the organism to swim away from the epithelial surface and into the luminal fluid.

The results presented above weigh into an area of unresolved controversy in the field. Our study provides evidence that chemotaxis and motility genes are upregulated in ileal loop bacteria 12 h post-inoculation (Figure 3B), a time period coinciding with the mucosal escape response as determined by confocal microscopy. By contrast, Merrell et al., using a microarray expression profiling method that employed stationary phase reference RNA, found that three *cheW* and *cheR* genes and 17 of 43 genes coding for methyl-accepting chemotaxis proteins were downregulated in cholera patient rice water stools compared to their level of expression in stationary phase cultures [10]. More recently, these findings were extended by the observation that the CheW-1 protein, measured by Western blot analysis, was less abundant in rice water stool bacteria than in stationary phase bacteria [44]. Further, they showed that this was associated with a chemotactic defect, i.e., the bacteria were motile, but exhibited smooth, but not "tumbly" swimming. From these observations and studies using an infant mouse model of cholera, they concluded that the altered chemotactic state of bacteria in rice water stools renders them hyperinfectious and thus contributes to the rapid spread of the organism through human populations [44]. Differences in the results of our study and those of Merrell et al. and Butler et al. might come from their use of stationary phase cultures as the source of reference RNA and bacteria for microarray expression studies and Western blot analysis, respectively. The potential magnitude of this confounding effect is evident from Figure 3B, which shows that genes in this functional category, including *cheW-1,* are powerfully upregulated in stationary phase compared to exponential phase cultures. Consequently, their use of a stationary phase reference for their microarray expression and Western studies may have led to an underestimation of the level of expression of chemotaxis and motility genes in clinical samples.

Differences in the results presented here and in the studies of Merrell et al. and Butler et al. prompted us to formally compare expression data for four separate conditions (wild-type in stationary phase, wild-type from rabbit ileal loop 12 h fluid, *rpoS* in stationary phase, and patient rice water stools from [10]) using a common exponentially grown reference. The results of this analysis are depicted in Table S6. Indeed, re-analysis of the original microarray expression data using exponential grown bacteria as the reference condition showed a 1.3-fold average induction of all annotated chemotaxis and motility genes and a 1.4-fold upregulation of chemotaxis genes in cluster I and III in bacteria in rice water stools (Table S6). The study of gene expression in rice water stools by Bina et al. used a genomic DNA-based reference, and we were therefore unable to include it in our comparison. However, they also found that chemotaxis genes were strongly expressed in rice water stools from cholera patients [9].

Another possible explanation for the dissimilarities in the data presented here and the results of Merrell et al. and Butler et al. comes from possible differences in the stage of the infection studied. Our study focuses on an event that transpires in the small bowel and on the process by which bacteria disassociate from the epithelial surface. By contrast, bacteria in rice water stools from cholera patients may undergo additional adaptations that occur at later time points and during passage through the colon. Finally, it is possible that the rabbit model fails to reproduce this stage of the infectious process in humans.

RpoS was found to be involved in the downregulation of virulence gene expression in vitro and also in vivo, although the in vivo results were less conclusive (Figure 6C). The effect of RpoS on virulence is likely due to the RpoS-dependent induction of HapR [31], a negative regulator of virulence gene expression. However, in addition, the induction of *hapR* could also occur indirectly owing to RpoS-dependent down-regulation of *luxO* (Table S2 and Figure 7), another RpoN-dependent transcriptional activator and a central regulator of the quorum-sensing circuit [50,51]. At low-cell densities, phospho-LuxO represses *hapR* expression. At high-cell densities, LuxO becomes dephosphorylated, the repression is relieved, and HapR is produced [12,14,50,51]. Thus, the repression of *luxO* expression by RpoS during stationary phase would accentuate the quorum-sensing effects on LuxO activity that occur in high-density populations. The reduced expression of *luxO* together with its dephosphorylation would further relieve repression of *hapR* expression. In turn, the production of HapR would be increased, leading to the repression of *ctxAB* and *tcpA* expression (Figure 7).

The work presented here used confocal microscopy to identify a distinctive stage in the *V. cholerae* infectious process, the mucosal escape response, which we believe simultaneously causes the organism to detach from the mucosal surface and activate motility and chemotaxis functions. We suspect that these effects may be associated with other changes which prepare the organism to leave the host, enter environmental reservoirs, or be transmitted to another individual. Such a complex transformation of the organism's physiological state and niche strongly suggests that it is controlled by a genetic program. Here we have dissected a part of this genetic program by demonstrating that RpoS is required for the

mucosal escape response and for the control of chemotaxis and motility functions during stationary phase.

Materials and Methods

Bacterial strains and media. The *V. cholerae* strains used were smooth phase variants of A1552 (wild-type, El Tor, Inaba, and RifR). An RpoS mutant of the parental strain has previously been described [29]. The *E. coli* strain DH5 was used for standard DNA manipulation experiments, and the *E. coli* strain S17–1 pir was used for conjugation with *V. cholerae*. Bacteria were grown in standard LB broth with 0.5% NaCl at 37 °C. When appropriate, 100 μg/ml ampicillin, 100 μg/ml rifampicin, or 50 μg/ml gentamycin was added to the media. For motility assays, bacteria were spotted onto the surface of 0.3% agar plates with either LB or M9 minimal media [52], supplemented with the carbon sources L-serine, glucose, lactate, maltose, and glycerol phosphate to a final concentration of 0.5% and incubated at 37 °C for 24–48 h. Virulence gene expression was studied during bacterial growth in AKI conditions [46], and CT concentrations were determined using a CT ELISA assay as previously described [53]. Bacteria used for confocal microscopy studies of rabbit ileal loop infections were labeled with GFP carried on pJB664 based on the broad-host-range low-copy-number plasmid pME6031 conferring gentamycin resistance (kindly provided by J. B. Andersen).

Generation of deletion mutants. Nonpolar deletions were generated essentially as described [54]. Crossover PCR was performed to amplify a fragment (with primers 1 and 4) that brings an upstream gene fragment (produced by PCR with primers 1 and 2) to a downstream gene fragment (produced by PCR with primers 3 and 4) thereby creating an in-frame deletion. The fragment was ligated into the sucrose-based counter selectable plasmid pGP704-Sac28 (CYW, unpublished data). The plasmid was introduced into *V. cholerae* A1552 by biparental mating. Sucrose-based counter selection was done essentially as described [54]. Deletions were confirmed by PCR. In addition, the *hapR* deletion was confirmed by a rugose phenotype and by the complete lack of HA hemagglutinin/protease production in stationary phase cultures as tested by the azocasein assay [30]. Primers used for construction of mutants are listed in Table 1.

Complementation of *hapR*. The BglI fragment from pBK-mini-Tn7-eyfp-a [55,56] containing a mini-Tn7 was cloned into pGP704 [57] between the EcoRI and SalI sites. A NotI fragment internal to the Tn7 was removed, and a SacI site outside of the transposon was destroyed by blunting and religation. The region from 290 bp upstream of *hapR* to 154 bp downstream of *hapR* was amplified and cloned into the Tn7 NotI site of the Tn7 vector. The transposon was introduced into *V. cholerae* A1552 by triparental mating using helper plasmid pUX-BF13 (carrying the transposase genes) followed by selection on TCBS/gentamycin. Transposition into the chromosome was confirmed by PCR, and the complementation of HapR was tested by restoration of the smooth phenotype, restoration of the motility phenotype, and by complete restoration of the production of the HA hemagglutinin/protease in stationary phase cultures using the azocasein assay described above.

Rabbit ileal loop model. The animal experimental protocol was reviewed and approved by the institutional animal care and use committee of Stanford University. Methods for ileal loop preparation and inoculation were slightly modified from previous descriptions [58,59]. New Zealand White rabbits weighing 2 kg were fasted for 36 h prior to surgery. After premedication with an intramuscular (IM) injection of glycopyrrolate (0.02 mg/kg), anesthesia was induced with ketamine (40 mg/kg IM) and xylazine (5 mg/kg IM). An intravenous catheter was placed to allow fluid administration during the surgery (10–20 ml/kg/h lactated Ringer's solution), an intratracheal tube was placed, and anesthesia was maintained using isoflurane gas. A midline celiotomy was performed to expose the bowel. The ileocecal junction was identified, and the ileum was double-ligated with silk ties just proximal to the sacculus rotundus. A series of ligated loops was then prepared starting with the terminal ileum and working retrograde along the small intestine, with particular care taken to preserve the vasculature and keep the gut moist. Loops ranged in length from 2–3 cm in length (for histology studies) to 4–5 cm (for secretion or gene expression studies). A non-inoculated "spacer" loop of 2 cm was left between all inoculated loops and, to prevent potential cross-contamination, a double spacer was placed on both sides of those loops that were anticipated to experience significant distention based on the administration of different pathogenic strains. Loops were carefully stretched and measured after preparation and then inoculated with bacterial suspensions or control media using a 25-gauge needle at a rate of 0.1 ml per cm of loop to provide a uniform initial distension. After inoculation, the small intestine was returned to its normal anatomic position, and the abdominal and skin incisions were sutured closed. Isoflurane administration was discontinued and the animals were moved to a recovery area and placed on a warm-water heating pad. The intratracheal tubes were removed when the animals regained consciousness, and pain control was provided by the administration of oxymorphone post-operatively at a dose of 0.15 mg/kg which was repeated every 2–4 h as needed. Additional intravenous or subcutaneous injections of lactated Ringer's solution were given as needed to maintain hydration. At end points of 4–12 h post-inoculation, the animals were euthanized with an intravenous overdose of pentobarbital.

Rabbit ileal loop quantitative localization assays. Bacteria were diluted 100-fold from overnight culture and grown to OD_{600} of 0.3. Each of the mutant stains and the wild-type parent were mixed in equal numbers with a *lacZ* deletion mutant and diluted to OD_{600} of 0.03. Mixtures were kept at room temperature until injected into rabbit ileal loops as described above. After 12 h of infection rabbits were sacrificed and the loops isolated. Fluid was collected from the loops and the loop cut open and washed gently in PBS. The remaining tissue was then vortexted forcefully in PBS buffer with glass beads to release any bacteria from the epithelial surface and associated adherent mucus. Samples were diluted and plated on LB plates containing 20 μg/ml X-gal (Qbiogene, Irvine, California, United States).

Scanning confocal laser microscopic analysis of *V. cholerae*-infected rabbit ileal loops. Bacteria were grown with 50 μg/ml gentamycin overnight, diluted 100-fold in fresh LB, and grown to an OD_{600} of approximately 0.3 without antibiotic selection. Bacteria were diluted

Table 1. Primers Used for Genetic Manipulations

Gene	Primer	Primer Sequence 5′–3′
hapR	VC0583 1	TTTGAGCTCCCTTTGGCTGAGTTGATTAC
	VC0583 2	TAGTTTCTTGGGCAGCAC
	VC0583 3	GTGCTGCCCAAGAAACTATCGAGGGCGTTTTTC
	VC0583 4	TTTCCATGGTCGACTTGATGAGTCAGATG
vpsA	VC0917 1	TTTGAGCTCCAACACCGTGAGATGTTAGA
	VC0917 2	CGCATCAAGCATTGG
	VC0917 3	CCAATGCTTGATGCGCAGGCACACAACCCTTAT
	VC0917 4	TTTCCATGGCAGTACGCAATCTTCACATC
hapA	VCA0865 1	TTTTCTAGAGGGCAATATCGAACAATCAC
	VCA0865 2	GTTGTTTTTGAAGGGCAGTT
	VCA0865 3	AACTGCCCTTCAAAAACAACTTCTCAATCCTAGAGATGTTGAATG
	VCA0865 4	TTTGAGCTCCCTTGGGTATTTATGATCGC

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to OD_{600} of 0.03 in PBS buffer and kept at room temperature until injected into the ileal loops. Plasmid loss during the relative short infection of the ileal loops was not observed. Ileal loops used for confocal microscopy were cut open and stretched gently onto cardboard discs. The tissue was allowed to adhere to the cardboard before it was gently submersed into 2% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) and allowed to fixate for 2 h. The fixative was washed away in three subsequent washes with PBS buffer before 0.3-cm^2 blocks were excised and transferred to a 96-well microtiter plate. For experiments with GFP-tagged bacteria, samples were stained overnight with Alexa Fluor 594 phalloidin (A-12381, Molecular Probes, Eugene, Oregon) diluted 100-fold in staining buffer (PBS with 1% saponin and 3% bovine albumin serum, BSA) to visualize the actin-rich epithelial surface. After staining, samples were gently washed twice in PBS buffer and mounted for microscopy in Slowfade Light Antifade Kit (Molecular Probes). Samples were imaged with BioRad (Hercules, California, United States) MRC1000 confocal microscope and the z-stacks were reconstructed into z-projections using the Imaris-software (Bitplane, Zurich, Switzerland) and figures were assembled with Photoshop CS (Adobe, San Jose, California, United States).

Microarray experiments. For expression analysis of wild-type *V. cholerae*, *hapR*, and *rpoS* deletion mutants in mid-exponential or stationary phase, the strains were grown to either OD_{600} of 0.3 or for 11 h in LB media at 37 °C, and bacteria from 2-ml culture were quickly pelleted, resuspended in Trizol reagent (GIBCO/BRL, San Diego, California, United States), and frozen on dry ice. At least four microarray experiments were performed for each of two biological replicates for the tested strains. Transcriptional analysis of wild-type *V. cholerae* isolated from the rabbit ileal loop was performed by quickly pelleting bacteria from the fluid accumulated in the ileal loops after 12 h of infection. The bacteria were then resuspended in Trizol reagent and frozen on dry ice. The wild-type bacteria from three independent experiments in three different rabbits were analyzed. RNA was isolated from the Trizol agent, treated with DNaseI (Ambion, Austin, Texas, United States), and cleaned by using the RNeasy kit (Qiagen, Valencia, California, United States). Labeling of cDNA and microarray hybridizations were performed as described [36]. RNA from the wild-type strain was labeled with Cy3, whereas mutant RNA was labeled with Cy5. Microarrays were scanned with a GenePix 400A instrument (Axon Instruments), using the GENEPIX 5.0 software. To avoid fluctuations in intensity values from genes that are not expressed to a measurable level, we designated a minimum background level for each channel [57]. Statistically significant changes in gene expression were identified by conducting a one-class analysis using the Significance Analysis of Microarrays program [60] with a threshold of 2-fold change and a 0% false discovery rate for all samples. Hierarchical clustering was performed using the average relative intensity values for each experiment with the program Genesis [61]. Average relative expression values of each gene after adjustment of background values are shown in Tables S1–S5. Raw microarray data are available at http://genome-www5.stanford.edu.

Quantitative real-time RT-PCR Mucus- and cell-associated bacteria were isolated from the ileal loops 12 h post-inoculation by scraping the epithelial surface of the intestine with a disposable plastic cell scraper after the loops were cut open and gently rinsed in PBS buffer to wash away remaining luminal fluid. RNA from bacteria in the mucus was recovered, and an equivalent of 20 ng of total RNA was used in a real-time RT-PCR reaction essentially as previously described [57]. Validation experiments were performed for all TaqMan probe and primer sets, and these showed a linear relationship between the cycle threshold, and the logarithm of the template amount (genomic DNA) as expected. All probe-primer sets yielded a curve with approximately the same slope demonstrating that the amplification efficiency of the various targets was similar (unpublished data). To select an internal reference for normalization, we initially performed real-time RT-PCR with primer-probe sets for six putative target genes with the cDNA samples from the different experiments. We then used the program GENORM to identify the most stably expressed control gene in these samples as previously described [57]. Relative expression levels in the different samples were calculated by using the comparative cycle threshold method with VC1186 and VC2233 as internal control.

Video tracing microscopy. The fraction of swimming bacteria after 11 h growth in M9 medium supplemented with 0.5% lactose was determined from video sequences captured using the tracing software of the Hamamatzu image processor Argus in combination with an Olympus (Tokyo, Japan) CK2 microscope equipped with a XC-77 CCD video camera (Hamamatzu, Japan). Video was recorded on a Panasonic time-lapse AG-6010s VCR. The M9 minimal medium was used, since the fraction of actively swimming wild-type bacteria in this medium is very high, thereby facilitating more accurate quantification.

SEM. Bacteria were grown for 11 h in LB and allowed to attach to a poly-L-lysine coated glass cover slip for 10 min prior to fixation with 2% glutaraldehyde in 0.5 mM $CaCl_2$ in 0.1 M $NaPO_4$ buffer (pH 6.8) for 1 h at room temperature. Fixation solution was gently replaced with 1% OsO_4 in dH_2O and stained for 1 h. Samples were washed gently three times with dH_2O prior to staining with 1% uranyl acetate in dH_2O for 1 h. Samples were dehydrated in subsequent washes of 50%, 70%, 95%, and 100% ethanol before being critical point dried (Autosamdri-814, Tousimis, Rockville, Maryland, United States). The fraction of flagellated bacteria was determined on eight different fields of view each with approximately 50–100 bacteria using a scanning electron microscope (FEI XL30 Sirion SEM).

Supporting Information

Table S1. Complete List of Differentially Regulated Genes between Wild-Type *V. cholerae* in Stationary Phase after 11 h Growth in LB when Compared to RNA from an Exponentially Grown Reference

The gene expression data were analyzed using SAM with a 0% false-positive discovery rate and a 2-fold transcript abundance difference between samples in order to define significantly regulated genes. The genes are listed in gene order (Column 1), with Log_2(expression ratio) (Column 2), and SAM score (Column 3).

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Table S2. Complete List of Differentially Regulated Genes between the *V. cholerae rpoS* Deletion Mutant and the Wild-Type Parent at Stationary Phase after 11 h Growth in LB

The gene expression data were analyzed using SAM with a 0% false-positive discovery rate and a 2-fold transcript abundance difference between samples in order to define significantly regulated genes. The genes are listed in gene order (Column 1), with Log_2(expression ratio) (Column 2), and SAM score (Column 3).

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Table S3. Complete List of Differentially Regulated Genes between the *V. cholerae hapR* Deletion Mutant and the Wild-Type Parent at Stationary Phase after 11 h Growth in LB

The gene expression data were analyzed using SAM with a 0% false-positive discovery rate and a 2-fold transcript abundance difference between samples in order to define significantly regulated genes. The genes are listed in gene order (Column 1), with Log_2(expression ratio) (Column 2), and SAM score (Column 3).

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Table S4. Complete List of Differentially Regulated Genes between Wild-Type *V. cholerae* Isolated from Ileal Fluid 12 h Post-Inoculation when Compared to RNA from an Exponentially Grown Reference

The gene expression data were analyzed using SAM with a 0% false-positive discovery rate and a 2-fold transcript abundance difference between samples in order to define significantly regulated genes. The genes are listed in gene order (Column 1), with Log_2(expression ratio) (Column 2), and SAM score (Column 3).

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Table S5. Complete List of the Differential Regulation of Genes Annotated to Be Involved in Chemotaxis and Motility Functions

Log_2 to the differential expression values for all genes are shown for the *rpoS* and *hapR* mutants in stationary phase versus the wild-type parent also in stationary phase in Columns 3 and 4. Log_2 to the differential expression values for the wild-type parent in stationary phase after 11 h growth in LB versus exponential growing cells are shown in Column 5, while the wild-type parent isolated from rabbit ileal loop fluid after 12 h of infection versus exponential growing cells are shown in Column 6.

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Table S6. Regulation of Genes Involved in Chemotaxis and Motility Including *rpoS* and *hapR* in Stationary Phase, Ileal Loop Fluid, and Patient Stool

Differential expression values are shown for RNA isolated from

stationary phase LB culture versus exponentially growing wild-type parent (Column 3), *rpoS* versus the wild-type parent in stationary phase (Column 4), the wild-type parent isolated from rabbit ileal loop fluid after 12 h of infection versus exponentially growing cells (Column 5), and patient stool versus exponentially growing cells based on data obtained from the original microarray data provided at the SMD database (see "microarray analysis" in Merrell et al. [10]). Average expression values are shown for all annotated chemotaxis and motility genes, flagellar biosynthesis genes, and chemotaxis cluster 1–3.

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Accession Numbers

The TIGR-CMR genome database (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?database=gvc) accession numbers used in this paper are *cheA-1* (VC1397), *cheA-2* (VC2063), *cheA-3* (VCA1095), *cheB-2* (VC2061), *cheW-1* (VC2059), *cheW-2* (VCA1093), *cheY-4* (VCA1096), *ctxA* (VC1457), *flaA* (VC2188), *flaB* (VC2142), *flaC* (VC2187), *flaD* (VC2143), *flaE* (VC2144), *flaG* (VC2141), *flgG* (VC2195), *fliG* (VC2132), *flrC* (VC2135), *hapA* (VCA0865), *hapR* (VC0583), *lacZ* (VC2338), *luxO* (VC1021), *motY* (VC1008), *rpoN* (VC2529), *rpoS* (VC0534), *tcpA* (VC0828), and *vpsA* (VC0917).

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Author contributions. ATN, NAD, and GKS conceived and designed the experiments. ATN, NAD, and GO performed the experiments. ATN and NAD analyzed the data. GO performed all rabbit operations. ATN, NAD, MCM, and CYW contributed reagents/materials/analysis tools. ATN and GKS wrote the paper.

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