Taken together, these studies indicate that a mature form of VirG, probably lacking a carboxy-terminal portion, can interact with F-actin. Recently, we have used antibody that recognizes the amino-terminal but not the carboxy-terminal portion of VirG to detect secreted VirG and F-actin-associated VirG in intracellular bacteria, and the results from these studies further support this view. If the cleavage of surface-exposed VirG that is seen in bacteria grown *in vitro* is also necessary for intracellular bacteria to spread, then the isolation of a mutant defective in *in vitro* cleavage of surface-exposed VirG and showing some change in spreading ability would confirm the scenario depicted by Goldberg.

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Multiple regulatory systems in *Vibrio cholerae* pathogenesis

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Vibrio cholerae has long been a nice model for studying a simple, noninvasive, human mucosal pathogen: There is a good animal model for its colonization and virulence. Many of its virulence factors have been identified. The study of the regulation, structure and function of these factors is currently under way[1,2]. Their importance in the construction of vaccine strains is being assessed[3]. The organism produces a potent exotoxin (cholera toxin; CT), which has been used to plumb various aspects of eukaryotic G-protein biology[4]. The crystal structure of a very closely related toxin (LT) from *Escherichia coli* has been solved[5]. Nevertheless, at practically every level, we are continuing to learn new features of *Vibrio cholerae*: In 1991, an epidemic of impressive proportions occurred in South America for the first time this century. A new strain of *V. cholerae* is currently overtaking the strains that were previously endemic to parts of Asia[6]. New pilus and toxin genes have been identified recently[7,8], genes required for getting CT out of the bacterium have been cloned only within the past year[9], and the crystal structure of the CT-like LT toxin from *E. coli* reveals a subunit arrangement that makes it difficult to understand how CT actually intoxicates eukaryotic cells[5]. Furthermore, recent work from the laboratory of Paul Manning in Australia has identified what may be a previously unrecognized regulatory system influencing virulence[10].

Strains of *V. cholerae* are traditionally classified as either classical or El Tor. For years, a feature attributed to El Tor strains of *V. cholerae* has been the production of a soluble hemolysin (Hly) (Ref. 11), although the phenotype is not absolutely restricted to El Tor strains[12], and it is debatable whether the phenotype is even critical to virulence[11]. The ability to produce hemolysin is not uniform among *V. cholerae* isolates, and even among Hly$^+$ strains, the phenotype is readily lost[13]. Now, Williams *et al.* suggest that hemolysin may be part of a newly identified coordinate regulatory system required for virulence[10].

So far, two other regulatory pathways critical for *V. cholerae* virulence have been characterized. In response to specific environmental conditions, the ToxR–ToxT system controls the expression of CT, as well as that of the genes encoding the toxin-coregulated pilus (TCP) and the accessory colonization factor. ToxR, a transmembrane, transcriptional activator protein, interacts with another membrane regulatory protein, ToxS, to control transcription of the CT genes. ToxR controls other virulence genes by activating the expression of ToxT, an AraC-like protein that can activate transcription of several genes[2]. Signal-dependent ToxR control occurs largely at the level of its activation of *toxT* transcription in response to environmental cues[2].

Another regulatory system that influences virulence in *V. cholerae* is controlled by iron levels through the Fur repressor. This system regulates the expression of at least one virulence factor, encoded by the *irgA* gene[14] (IrgA has sequence similarity with the TonB-dependent outer membrane proteins of *E. coli*). Although mutations in *irgA* attenuate virulence by at least 100-fold, its exact role in virulence remains unclear. A unique model for iron regulation of *irgA* expression was proposed by Calderwood and his colleagues, who suggested that Fur repression of a positive activator, *irgB*, which is encoded in the opposite orientation from *irgA* on the *V. cholerae* chromosome, maintains low levels of IrgA when the concentration of iron is high. Under

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limiting iron concentrations, derepression of *irgB* expression occurs, and the resulting IrgB activates *irgA* transcription to high levels[14]. Preliminary data suggest that IrgB controls the expression of other genes as well as *irgA* (S.B. Calderwood, pers. commun.).

Manning and his colleagues have shown that the HlyU protein, which regulates expression of the hemolysin gene (*hlyA*) in El Tor strain O17, may control other proteins necessary for full virulence that have not been identified so far[10]. HlyU belongs to a family of small regulatory proteins that share a common helix-turn-helix domain, a characteristic feature of many classes of DNA-binding proteins. The introduction of a mutation into either *hlyA* or *hlyU* results in an approx. 100-fold increase in the LD_{50} value in an animal model of *V. cholerae* virulence. Although the hemolytic activity of exponentially growing *hlyU* mutant cells is practically nil, the difference in measurable hemolytic activity of the *hlyU* mutant and wild-type cells is not as great when using overnight cultures. The authors suggest that HlyA can accumulate in *hlyU* mutants. This residual amount of hemolysin may be enough to supply whatever benefit it confers *in vivo*; if this is so, then the attenuation of the *hlyU* mutant could be attributed to a decreased production of some other determinant controlled by HlyU.

More compelling evidence for the existence of other HlyU-regulated virulence factors comes from *in vivo* competition experiments in infant mice, and from SDS–PAGE analysis of proteins from wild-type and mutant cells. In mixed infection experiments, the *hlyA* mutant can compete with the wild-type strain for survival *in vivo*, whereas the *hlyU* mutant consistently (although not dramatically) cannot maintain a 1:1 ratio when coinfected with wild-type cells[10]. One possible reason for the competitiveness of the *hlyA* mutant in mixed infection may be that the mutant cells derive a benefit from the hemolysin produced by the wild-type cells. However, the *hlyU* mutant, which would be exposed to hemolysin from wild-type cells as well, may be missing some other HlyU-dependent factor that cannot be supplied by wild-type cells. In SDS–PAGE analysis, a 28 kDa protein is detected in concentrated culture supernatants from *hlyU*$^+$ cells that is not present in similar fractions from the *hlyU* mutants. This protein is not immunologically related to HlyA and, while its level in culture supernatants depends on *hlyU*, it is independent of *hlyA* (Ref. 10). By the same reasoning used for mixed infections between wild-type and *hlyA* mutant cells, it might be expected that the 28 kDa protein could also be supplied by wild-type cells to *hlyU* mutants in mixed infections. Perhaps HlyU also controls a critical, nonsecreted colonization factor.

Despite the presence of a hemolysin determinant and a mechanism to control its expression, the role of this factor in the pathogenesis of *V. cholerae* infection has not been established. Typically, invasion of host cells is not part of its lifestyle; indeed, ToxR-regulated gene products, particularly TCP, are designed specifically to enhance mucosal colonization. Stoebner and Payne showed that iron levels influence hemolysin production and proposed that hemolysin may be an alternative to the vibriobactin siderophore system for acquiring iron while infection is becoming established[13]. Recent work from Payne's laboratory supports this model: a double mutant with lesions in the genes encoding vibriobactin and a heme-uptake protein is attenuated in the infant mouse model of infection (S.M. Payne, pers. commun.).

The interaction between these regulatory pathways, if any, needs to be determined. ToxR does not seem to affect the expression of hemolysin or of Fur–IrgB-regulated genes. Apparently, the level of available iron is a common signal for the synthesis of both hemolysin and IrgA. Given that Fur controls the positive activator IrgB, as proposed by Calderwood *et al.*, the obvious question is whether Fur also controls HlyU expression. Whatever the answers to these problems turn out to be, it is already clear that colonization by *V. cholerae* is the result of the ability of the organism to integrate different signals through multiple pathways.

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