Cholera: **Nice bacteria and bad viruses**

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The genes coding for cholera toxin are borne on, and can be infectiously transmitted by, a filamentous bacteriophage, raising intriguing questions about the mechanisms and evolution of bacterial pathogenesis, and the taxonomy, epidemiology and control of cholera and other bacterial diseases.

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Otherwise nice bacteria can go wrong when they associate with a bad crowd of accessory genetic elements. Another case of this has now been discovered, and this time it is a really big one. In a recent article, Waldor and Mekalanos [1] have presented compelling evidence that the genes coding for the enterotoxin responsible for the full virulence of the cholera pathogen, *Vibrio cholerae*, can be borne on, and transmitted by, a bacteriophage, and one of those wimpy filamentous phages at that.

Using a combination of elegant classical and neoclassical (molecular, as we all must be [2]) procedures, Waldor and Mekalanos [1] demonstrated that the *ctxAB* loci that encode the A and B subunits of cholera toxin, along with other cholera-associated loci, can be carried and infectiously transmitted, in mice as well as *in vitro*, by a filamentous phage they designate CTXΦ. This single-stranded DNA phage, related to coliphage M13, infects host bacteria by adsorbing to a 'toxin-coregulated pilus' (TCP), another cholera virulence factor. The gene for the pilus protein is expressed under control of the ToxR regulatory system that also regulates the transcription of the cholera toxin genes. The CTXΦ genome is a 7–9.7 kilobase compound transposon, previously found to be associated specifically with toxigenic strains of *V. cholerae*. The CTXΦ element can become incorporated into the *V. cholerae* chromosome, often as an array of tandemly repeated copies, but can also replicate and be vertically transmitted as a plasmid.

These observations raise and remind us of a number of delicious, yet-to-be-answered questions about the mechanisms and evolution of virulence in *V. cholerae* and of bacterial pathogenesis in general, and about the taxonomy, epidemiology and control of cholera and other bacterial diseases. Why are so many bacterial virulence determinants encoded by accessory-element-borne, rather than chromosomal, genes? What are the ecological conditions and genetic processes responsible for the evolution and persistence of phage-, transposon- and plasmid-encoded virulence? What does the horizontal transfer of virulence genes mean for the identification and taxonomy of cholera-causing *Vibrio cholerae*? How much of the emergence of seemingly new bacterial diseases, and the waning of old ones, can be attributed to the acquisition and loss, respectively, of virulence-encoding accessory elements? What does the observation that virulence is encoded by an accessory element tell us about the design of procedures to limit the dissemination of cholera and other bacterial diseases, and to reduce their pathogenic effects following infection?

Ever since the discovery of the β prophage of *Cornybacterium diptheriae* [3,4], it has become increasingly clear that much of bacterial virulence is encoded, at least in part, by genes that are either carried on phage, plasmids and transposons, or packaged together as 'chromosomal islands'. Without these elements, pathogenic strains of *Shigella*, *Salmonella*, *Yersinia*, *Staphylococcus*, *Clostridium* and *Escherichia coli* would be less virulent and maybe even benign [5–10]. On the other hand, these genetic elements alone are usually not sufficient to make an otherwise nonpathogenic bacterium virulent — other loci are generally required.

This is certainly the case for *V. cholerae*, where the expression of the chromosomal genes that encode the proteins that make the TCP, which is required for both virulence and absorption of the CTXΦ phage, and the cholera toxin genes carried by the CTXΦ phage genome, are regulated by another chromosomal gene (*toxR*). How did this evolve? It would seem that, even without the CTXΦ-encoded cholera toxin, the production of toxin-coregulated pili must confer a fitness advantage on *V. cholerae*. Toxin production must further augment fitness, in either a complementary or very different way, possibly even acting in a different bacterial habitat. Just how expression of these pili and toxins augments the fitness of *Vibrio cholerae* remain no more than untested hypotheses. Do the pili facilitate host colonization, or contribute to *V. cholerae* replication and maintenance in the human gastrointestinal tract? By causing rampant diarrhea, do cholera toxins really increase the rate of infectious transmission of *V. cholerae*?

This new study of *Vibrio cholerae* may be the first report of a filamentous phage serving as vehicle for the carriage and transmission of virulence-determining genes. We would be surprised, however, if cholera toxin turns out to be the unique example of this. As Waldor and Mekalanos [1] point

out, the morphology of a filamentous phage is not hampered by the amount of heterologous DNA it carries, and these bacterial viruses are not very deleterious to the bacteria they infect. While not officially temperate, these phage ooze out of their bacterial hosts without killing them and, most importantly, they can be transmitted vertically as well as horizontally.

In a study of the population dynamics of the filamentous coli phage *f1*, Bull and colleagues [11,12] found that, if phage carry genes homologous to those of the host, so that their genomes can integrate into the host chromosome, they will do so, particularly when maintained under culture conditions favoring vertical, rather than horizontal, transmission. One effect of this incorporation is to reduce the fitness burden the phage genomes impose on their host, as one would expect for a vertically transmitted, parasitic genetic element [13]. The carriage by these elements of genes that augment their host bacteria's fitness is just a good example of enlightened self-interest, and a molecular one at that. Moreover, in situations where the host densities are too low to maintain the phage as parasites by horizontal transmission alone, the phage could be maintained if they had the capacity for vertical transmission and carried genes that augment their host's fitness [14].

Why bacteria rely on sometimes fickle accessory-element-borne genes for their virulence, or other characters, rather than know-their-place chromosomal genes, is not clear. Presumably — though it has rarely been demonstrated — the expression of virulence determinants augments the bacteria's fitness in at least some ecological conditions [15,16]. Could it be that the encoding of virulence — or other characters, such as antibiotic resistance — by accessory elements represents a primitive evolutionary state and that the genes determining those characters will eventually become permanent parts of the host chromosome. Or could it be that, under most conditions, virulence is not an adaptive character for a bacterium, and the only way virulence-determining genes can be maintained is by being carried on horizontally transmitted genetic elements that can infect a variety of different strains or species of bacteria in specific habitats?

The horizontal transfer of cholera toxin genes may well explain some of the curious epidemiology of cholera. Of three waves of cholera that swept the world since the aetiological agent was discovered, the first two were caused by the 'classical' biotype of *V. cholerae* serogroup O1, while the current pandemic, ongoing since 1961, is caused by the 'El Tor' biotype. The two biotypes are easily separable by phenotypic assays and by electrophoretic enzyme typing, indicating that there is substantial genetic distance between them [17]. However, the sequences of toxin subunit B genes are the same in classical and some El Tor strains [18], whereas other El Tor strains have different sequences, indicating that the phylogeny of the toxin is to some degree independent of the phylogeny of the host bacterium. This suggests that new pathogenic strains may occasionally be produced following phage infection of a susceptible, nontoxigenic strain of *V. cholerae* O1, and that pandemics may be caused by strains other than those we are familiar with now.

The transfer of cholera toxin genes by a phage that attaches to a specific pilus on the host bacterium may also account, at least in part, for the extraordinarily restricted host range of toxigenic *Vibrio cholerae* O1. Like most *Vibrio* species, these strains appear to be adapted to the estuarine environment and to invertebrate marine hosts. On the other hand, they also have the perverse capacity for rapid and sustained multiplication, causing severe diarrheal illness in humans but in essentially no other terrestrial animal. The roles of the toxin and coregulated pilus in this host specificity are unknown, but it is tempting to speculate that a new niche — humans — was opened up to this parasite of marine invertebrates by the chance acquisition, first of a human-adapted intestinal attachment factor and then of a toxiniferous phage that adsorbs to this pilus. If so, the evolutionary event(s) that led to cholera may have occurred only after humans colonized the seacoast and became sophisticated enough to partake in the gastronomic delights of shellfish.

By and large, however, Waldor and Mekalanos' observations [1] raise more questions than they answer. If the high rates of cholera toxin transfer observed in the mouse experiments obtain in general, why are there not a greater variety of toxigenic strains? Among the large number of *V. cholerae* non-O1 serotypes, the presence of cholera toxin genes is extremely rare. Even within serogroup O1, a broad variety of nontoxigenic strains exist [19,20]. At a time when cholera cases are being reported in greater numbers and more countries than ever before, it is difficult to believe the nontoxigenic strains of *V. cholerae* remain free of cholera toxin simply because of lack of exposure to toxiniferous phage. Do these strains lack the pilus receptor, or are the toxiniferous phage restricted in host range for other reasons? Are there circumstances under which the pilus itself can be acquired by otherwise innocuous *V. cholerae*, which would make them susceptible to CTXΦ? Could the ready loss of CTXΦ be one of the reasons for the end of previous pandemics? It would certainly be interesting to know more about the conditions under which CTXΦ prophage and free phage are maintained and their rates of loss from individual and populations of *Vibrio*. Does the host range of CTXΦ include the various live nontoxigenic vaccine strains of *V. cholerae* O1 and O139?

Waldor and Mekalanos' observations [1] may also have practical implications. Public health immunization strategies may target a variety of antigens in ways that interfere

with the organisms themselves — as the pneumococcus or meningococcus vaccines do — or block the action of specific toxins — as tetanus and diphtheria vaccines do. If many bacterial toxins or other virulence factors can be transferred horizontally by phage, or other mobile elements, the latter immunization strategy actually targets the accessory element rather than the host bacterium. We may speculate that high levels of antibacterial immunity would make it advantageous for a phage to move from its original host bacterium to another, and advantageous for those bacteria to change their surface antigens and thus escape the limiting effect of the immunity.

Could naturally acquired and vaccine-induced immunities also promote the emergence of new pathogens, as well as new stereotypes of established pathogens? High levels of immunity directed against phage-encoded products might make it advantageous to any host bacterium to lose or inactivate the phage responsible for the synthesis of the target antigen, and thus ultimately to become nonpathogenic. This type of immunity in the vertebrate host population would also make it advantageous to the phage to change the antigenicity of its products. This ultimately may lead to the disappearance of old pathogens, and to the appearance of new toxin types. Before wide-scale vaccination programs are established, some consideration should perhaps be given to the evolutionary as well as the clinical and epidemiological consequences of these programs [21].

These observations further highlight the possibility that phage — and other infectiously acquired accessory elements — may play an important role in the emergence of seemingly new pathogens. As noted earlier, other bacterial pathogens carry important virulence factors on accessory elements, including lysogenized phage. How many additional pathogens have phage-encoded virulence determinants? It would seem that phage can contribute to the appearance of new bacterial pathogens in other ways than by transferring exotoxin genes. In 1992, a new epidemigenic serogroup of *V. cholerae* (serogroup O139) appeared in India, and subsequently spread to much of Asia. These strains are very closely related to the 'El Tor' biotype strains of serogroup O1, but produce an entirely different surface lipopolysaccharide, possibly as the result of gene transfer by an as yet unidentified phage [22]. The new surface antigen may have permitted these O139 strains to spread through a population that already had some immunity to infection by the previously dominant O1 strains, in a manor analogous to the antigenic shifts exhibited regularly by the influenza virus. Changes in ecological conditions, rather than genetic changes, are probably the major factor responsible for the emergence of new pathogens [23]. On the other hand, once these ecological conditions are met, the receipt of an infectiously transmitted accessory element by that population of bacteria could result in a seemingly precipitous origin of a new bacterial disease.

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