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An experimental study of the population and evolutionary dynamics of *Vibrio cholerae* O1 and the bacteriophage JSF4

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Studies of *Vibrio cholerae* in the environment and infected patients suggest that the waning of cholera outbreaks is associated with rise in the density of lytic bacteriophage. In accordance with mathematical models, there are seemingly realistic conditions where phage predation could be responsible for declines in the incidence of cholera. Here, we present the results of experiments with the El Tor strain of *V. cholerae* (N16961) and a naturally occurring lytic phage (JSF4), exploring the validity of the main premise of this model: that phage predation limits the density of *V. cholerae* populations. At one level, the results of our experiments are inconsistent with this hypothesis. JSF4-resistant *V. cholerae* evolve within a short time following their confrontation with these viruses and their populations become limited by resources rather than phage predation. At a larger scale, however, the results of our experiments are not inconsistent with the hypothesis that bacteriophage modulate outbreaks of cholera. We postulate that the resistant bacteria that evolved play an insignificant role in the ecology or pathogenicity of *V. cholerae*. Relative to the phage-sensitive cells from whence they are derived, the evolved JSF4-resistant *V. cholerae* have fitness costs and other characters that are likely to impair their ability to compete with the sensitive cells in their natural habitat and may be avirulent in human hosts. The results of this *in vitro* study make predictions that can be tested in natural populations of *V. cholerae* and cholera-infected patients.

Keywords: cholera; bacteriophage; population dynamics; evolution; *Vibrio cholerae*

1. INTRODUCTION

Not only was the lysis of *Vibrio cholerae* by a heat-labile substance in river water the first evidence for the existence of bacteriophage (Hankin 1896), there is compelling field and patient evidence that suggests lytic bacterial viruses are responsible for the decline in the incidence of cholera during seasonal outbreaks (Pasricha *et al.* 1931; Faruque *et al.* 2005a,b). The hypothesis that lytic phage are responsible for the waning of cholera outbreaks is also supported by the results of a theoretical study, which employed a mathematical model that combined the epidemiology of cholera with the population dynamics of *V. cholerae* and a lytic phage (Jensen *et al.* 2006). In this model, for phage to modulate outbreaks of cholera, the bacterial virus, rather than growth-limiting resources, must limit the densities of *V. cholerae* in the environment.

Unfortunately, the proposition that predation by bacteriophage rather than resources limit the density of bacterial populations is inconsistent with the results of all the *in vitro* studies of the population and evolutionary dynamics of bacteria and lytic phage of which we are aware. Although phage may limit the densities of bacteria for extended periods (e.g. Chao *et al.* 1977), eventually bacterial mutants resistant to the phage evolve and the bacterial population becomes limited by resources

(Chao *et al.* 1977; Levin *et al.* 1977; Lenski & Levin 1985; Bohannan & Lenski 2000; Mizoguchi *et al.* 2003). This occurs even when the bacteria are confronted with three phage 'species' with different adsorption sites and when all three phage are sensitive to the restriction-modification system borne by these bacteria (Korona & Levin 1993). There may be a coevolutionary arms race, with mutations for bacterial resistance being countered by host-range phage mutations enabling them to replicate on the resistant cells. However, if the lytic phage of *Escherichia coli* are at all typical, these arms races end within a couple of cycles. Resistant bacterial mutants are generated to which the phage cannot produce host-range mutants capable of replicating on them (Lenski & Levin 1985). For a possible exception to this limited arms race see Buckling & Rainey (2002), but even in that study no evidence was presented for the bacteria being limited by phage rather than resources.

To test the hypothesis that populations of *V. cholerae* are limited by phage rather than resources in batch, chemostat and serial transfer culture, we performed population dynamic, evolutionary and parameter estimation experiments with the El Tor strain of *V. cholerae* O1 (N16961) and a lytic phage from a natural population (JSF4). Our results indicate that, when cultured together, *V. cholerae* resistant to JSF4 rapidly emerge and continue to dominate the bacterial populations, which are maintained at levels similar to that observed for phage-free, resource-limited cultures of bacteria.

While these observations are inconsistent with the hypothesis that predation by lytic phage, rather than

Table 1. *Vibrio cholerae* strains used in this study. Sm^r, streptomycin-resistant; JSF4^r, JSF4-resistant; Rif^r, rifampin-resistant. Ten or more JSF4-resistant mutants isolated from three additional chemostats were included in the fitness assay. Their source is noted in the legend of figure 2. A comprehensive version of this table is in the electronic supplementary material, table S2.

strain	relevant phenotype	comments	references
N16961	Sm^r smooth wild-type	El Tor O1 *V. cholerae*	John J. Mekalanos
R-N16961	Sm^r rugose wild-type	isolated from a batch culture	this study
Rif.R	Rif^r smooth N16961	spontaneous Rif^r mutant of smooth N16961	this study
RF	JSF4^r smooth N16961	isolated from a batch culture	this study
R1.1	JSF4^r smooth N16961 ⎫	isolated from chemostat 1 (figure 1*c*)—25 h after introduction	this study
R1.2	JSF4^r smooth N16961 ⎭	of phage	this study
R2.1	JSF4^r smooth N16961 ⎫	isolated from chemostat 2 (figure 1*a*)—75 h after introduction	this study
R2.2	JSF4^r smooth N16961 ⎭	of phage	this study
RR	JSF4^r rugose N16961	isolated from a batch culture	this study

resources, limits the densities of *V. cholerae* populations, the results of our other experiments suggest an alternative mechanism, whereby lytic bacteriophage can contribute to the waning of outbreaks of cholera. The phage-resistant *V. cholerae* mutants obtained in these experiments are less fit than wild-type in laboratory culture, possess a number of characteristics that are likely to further reduce capacity to be maintained in natural populations and may be avirulent in human hosts. We postulate that because of the impaired ecological fitness of phage-resistant *V. cholerae* mutants and their relative avirulence, lytic phage like JSF4 can modulate outbreaks of cholera. We discuss these hypotheses and the extent to which they are supported by current field, experimental animal and clinical studies, and suggest how they can be tested in natural populations of *V. cholerae* and human hosts.

2. MATERIAL AND METHODS

(a) *Organisms: bacteria, phage, nematodes and crustaceans*

The *V. cholerae* used in this study were derived from El Tor O1 N16961, a spontaneous streptomycin-resistant (Sm^r) mutant of a clinical isolate from Bangladesh. JSF4 is a naturally associated bacteriophage of *V. cholerae*. Table 1 lists the variants of *V. cholerae* employed in these experiments and their sources.

Two species of *Daphnia*—*D. pulex* and *D. magna*—were used to explore the relative capacity of *V. cholerae* isolates to adhere to the surfaces of zooplankton. These crustaceans were purchased from Carolina Biological Supply Inc.

The *Caenorhabditis elegans* used for the virulence assays is the wild-type reference strain var. Bristol (N2). *Escherichia coli* OP50 was used as a food source for *C. elegans*.

(b) *Media, culturing and sampling procedures*

Vibrio cholerae were grown in either Luria-Bertani Broth (LB) or a medium composed of 5 per cent LB and 95 per cent tap water. The density of *V. cholerae* N16961 was estimated from colony count data on LB agar or LB agar with rifampin (25 μg ml^{-1}). The density of JSF4 was estimated from plaque counts on LB agar overlaid with lawns of *V. cholerae* N16961. Unless otherwise noted, the experimental populations were maintained at 30°C with aeration via shaking or (in chemostats) bubbling.

The chemostats used in these experiments were of a 'homemade' design similar to that in the appendix of Chao *et al.* 1977 (see www.eclf.net for a description of the latest

incarnation of these chemostats). *Vibrio cholerae* N16961 and JSF4 were co-cultured in chemostats with 5 per cent LB and 95 per cent tap water at dilution rates ranging from 0.07 to 0.24 per hour for 120–340 h (in a volume of 20–25 ml). Unless otherwise noted, estimates of bacterial density and phage titres from chemostats were made from samples taken directly from the chemostat vessels.

(c) *Assays for phage-resistance*

Resistance of *V. cholerae* N16961 to JSF4 was first determined by spotting 10–20 μl of a $\sim 10^8$ ml^{-1} lysate of JSF4 on lawns derived from single colonies of the sampled culture. Bacterial clones for which there was no evidence for phage lysis on lawns were further tested for the ability to support the replication of JSF4 in liquid cultures. Those unable to support phage replication were deemed resistant.

(d) *Ecological fitness and virulence experiments*

(i) *Pairwise competition in liquid culture*

The fitness cost associated with resistance to JSF4 was estimated by competing JSF4-sensitive wild-type *V. cholerae* N16961 with JSF4-resistant isolates. To facilitate these assays, a spontaneous rifampin-resistant mutant of N16961 (designated Rif.R) was used. The relative frequencies of JSF4-sensitive and -resistant strains were estimated by plating on LB agar and LB agar with rifampin. For each competing pair, at least three replicate experiments were done. The Malthusian selection coefficient, s, was calculated using the formula

$$s = \frac{(\ln(W_t) - \ln(W_0))}{\ln(N_t/N_0)},$$

where W_t and W_0 are, respectively, the ratio of the densities of JSF4-resistant and -sensitive N16961 at time t and initial time 0, and N_t and N_0 the total densities of bacteria at those two times (Travisano & Lenski 1996). The relative fitness of each JSF4-resistant mutant to Rif.R was calculated as $1/(1 - s)$. As a control for the fitness cost of the rifampin resistance marker, pairwise competition experiments were performed with wild-type N16961 and Rif.R. *t*-test was used to analyse the fitness data and false discovery rate (FDR) was controlled at 0.05 to correct for multiple comparisons.

(ii) *Fitness in surface culture*

A membrane filter method similar to that employed by Fujikawa & Morozumi (2005) to study surface growth of *E. coli* was used to ascertain the relative fitness of the resistant and sensitive *V. cholerae* when they grew as colonies. Ten
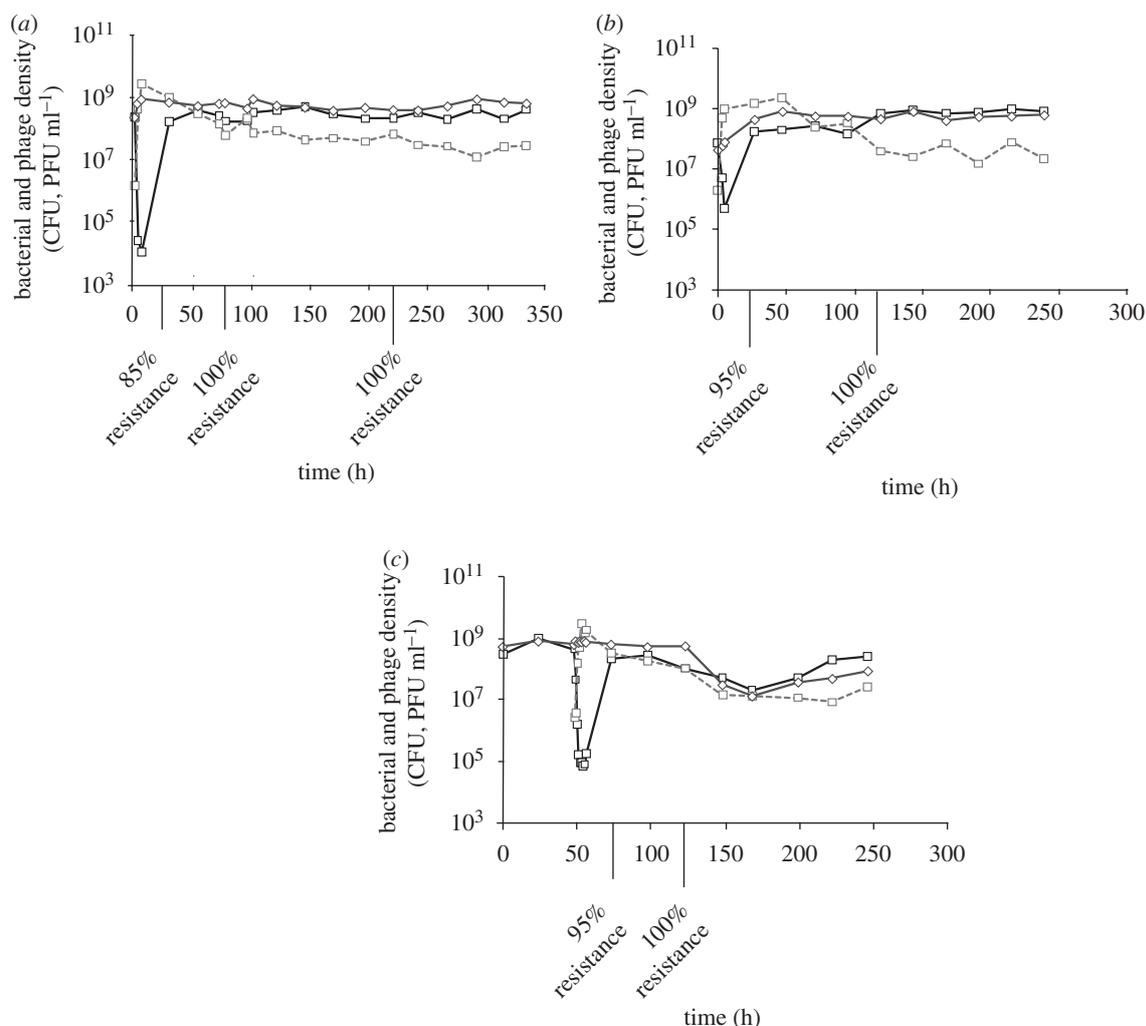
Figure 1. Change in the density of bacteria and phage in continuous culture, 5% LB, where the solid lines with squares and broken lines with squares are, respectively, the densities of the bacteria and phage in mixed culture, and solid lines with diamonds are the density of bacteria in the phage-free control. All chemostats were inoculated with bacteria from an overnight LB culture for an initial density of approximately 10^8 CFU ml^{-1} and approximately 10^6 PFU ml^{-1} of phage. The dilution rates, w, are the fraction of the total volume of the chemostat change per hour. The lines with '% resistance' denote the percentage of JSF4-resistant mutants out of 20 randomly chosen colonies at specific time points. (*a*) Chemostat inoculated with wild-type *V. cholerae* N16961, $w = 0.07–0.12$. (*b*) Chemostat inoculated with rugose variant of *V. cholerae* N16961, $w = 0.24$. (*c*) Chemostat inoculated with wild-type *V. cholerae* N16961; the phage JSF4 was introduced after the rugose variants in this chemostat reached a level of 9.4% of total bacteria, $w = 0.21$.

microlitres of culture containing approximately equal densities (approx. 3×10^5 cells ml^{-1}) of the competing bacteria were spotted onto 25 mm diameter of 0.45 μm Tuffryn membrane filters (Pall, 66221). These filters were placed on Petri dishes containing 5 per cent LB agar and incubated at 30°C for 24 h. To estimate the relative frequencies of the competing pair, the filters were removed from the agar, placed in 1 ml of 0.85 per cent saline and vigorously vortexed for 30 s to release the bacteria. The fitness effect of rifampin marker on the competing wild-type bacteria was also estimated in these surface cultures.

(iii) Colonization of V. cholerae N16961 on Daphnia

As a measure of the relative capacity of the JSF4-sensitive and -resistant *V. cholerae* to colonize the chitinous surfaces of crustaceans, we used *D. pulex* and *D. magna*. *Vibrio cholerae* were grown on stationary phase in LB and washed three times with autoclaved pond water obtained from Lullwater Park (Emory University). After gently washing with sterile pond water, two to three *Daphnia* were incubated with

$10^6–10^7$ CFU of bacteria in 1.5 ml Eppendorf tubes at room temperature overnight in the dark.

After overnight co-incubation, the tubes were rinsed with autoclaved pond water three to five times to remove the unattached bacteria. The density of free bacteria after washing was determined by diluting and plating. The *Daphnia* in the tubes were then homogenized using a pellet pestle (Kontes, K749510). The difference between the post-homogenizing and pre-homogenizing estimates was used as our estimate of the density of *V. cholerae* adhering to the *Daphnia*. To control for contribution of aerobic bacterial flora of the *Daphnia*, we used LB agar with streptomycin; N16961 is resistant to this antibiotic. *t*-test was used to analyse the colonization data and FDR was controlled at 0.05 to correct for multiple comparisons.

(iv) C. elegans mortality assay

Nematode growth medium (0.3% NaCl, 1.7% agar, 0.25% peptone, 0.001 M MgSO$_4$, 0.001 M CaCl$_2$, 0.005 mg ml^{-1} cholesterol in ethanol, 0.025 M pH 6.0 K$_3$PO$_4$ buffer) agar

plates were prepared and inoculated with 100 µl of overnight LB cultures of *V. cholerae*. These plates were incubated for 8 h at 37°C, at which time distilled-water-washed *C. elegans* L4 stage (adults) were added to agar plates bearing *E. coli* OP50 or JSF4-sensitive or -resistant *V. cholerae* and incubated at room temperature (approx. 23°C). Each day, the number of viable worms was estimated and every second day all of the viable worms were transferred to fresh plates containing the same strain of bacteria.

3. RESULTS

(a) *Population dynamics of V. cholerae N16961 and lytic bacteriophage JSF4 in chemostat culture*

We open this section with the results of experiments addressing the question of whether populations of *V. cholerae* are limited by phage rather than resources. The changes in the density of bacteria and phage in three of the eight chemostat experiments of this type are presented in figure 1.

Two to four hours after the introduction of phage into chemostats bearing wild-type N16961, the density of bacteria in the chemostats dropped by approximately four orders of magnitude, while that of the phage rose to between 10^9 and 10^{10} PFU ml^{-1} (figure 1a). No substantial change in the density of bacteria was observed in the phage-free control chemostats. Similar dynamics were observed with the introduction of JSF4 to chemostats with rugose colony variants of N16961 and mixtures of rugose and wild-type smooth colony N16961, albeit with less of a decline in the bacterial density than observed, when the phage were added to chemostats bearing solely wild-type N16961 (figure 1b,c). Rugose variants were examined this way because it had been proposed that these variants, which produce copious quantities of exopolysaccharide, have higher rates of survival in the environment (Ali *et al.* 2002) and are presumed to be more refractory to phage than smooth colony types (Beyhan & Yildiz 2007).

Most importantly, in all three cultures depicted in figure 1 (and in five similar chemostat experiments; electronic supplementary material, S1), the drop in bacterial density following the addition of phage was transient. The bacterial population recovered, and by 24 h the densities of the bacteria in these cultures with JSF4 were similar to those in the corresponding phage-free controls. The same results of resource- rather than phage-limited cultures were obtained in analogous experiments performed in 1/100 dilution 10 ml serial transfer cultures (data not shown).

While the density of JSF4 decreased after its initial bloom, the phage continued to persist at approximately 10^6–10^7 PFU ml^{-1}. We interpret this as evidence for phage replication. Were that not the case, with the dilution rates of the chemostats in figure 1a–c, following a peak density of 10^9 PFU ml^{-1}, the density of phage would be less than 10 PFU ml^{-1} by 184, 77 and 88 h in the chemostats, figure 1a, 1b and 1c, respectively.

Resistant bacteria and host-range phage: the majority of bacteria recovered from these cultures were resistant to the original phage by approximately 25 h after the phage were introduced. This was also the case for all the colonies tested in later samples (figure 1). We interpret this as evidence that the bacterial populations in the cultures

with phage were dominated by phage-resistant cells. No JSF4-resistant colonies were observed in the samples taken from the phage-free control cultures. Presumably, the phage are maintained in these chemostats by replicating on a minority population of planktonic sensitive bacteria (Levin *et al.* 1977) or on sensitive cells adhering to the walls of the chemostats (Schrag & Mittler 1996). To ascertain whether the JSF4 phage produced host-range mutants capable of replicating on the resistant N16961, the lysates from final samples of the chemostat were mixed with lawns of resistant mutants from the corresponding chemostat; no plaques were observed.

(b) *Competitive performance and virulence of the JSF4-resistant mutants of V. cholerae N16961*

If phage rather than resource limitation is a necessary condition for these viruses to be responsible for the waning of outbreaks of cholera (Jensen *et al.* 2006), the results of these chemostat and serial transfer experiments are inconsistent with that hypothesis. However, as we now show, the results of our experiments characterizing the resistant mutants suggest that in natural habitats, JSF4-resistant mutants may not be significant players in the ecology of *V. cholerae* and epidemiology of cholera.

(1) *Most but not all JSF4-resistant mutants have a fitness disadvantage when competing with wild-type in liquid culture.* Using procedures similar to those used by Travisano & Lenski (1996), we estimated the Malthusian fitness of 12 smooth and 4 rugose JSF4-resistant strains relative to a phage-sensitive rifampin-resistant mutant, Rif.R. The results of these pair-wise competition are presented in figure 2a.

Relative to wild-type, the fitness of the JSF4-sensitive Rif.R, was 0.71 ± 0.03, indicating that the rifampin resistance marker engenders a substantial fitness cost. To facilitate our comparison of the relative fitness of the different resistant mutants and wild-type, in figure 2a, we assign a unit value (1.0), the horizontal line, for the fitness of the Rif.R common competitor, which relative to wild-type has a fitness of $1/0.71 = 1.41$. By this criterion, seven resistant mutants were significantly more fit than the Rif.R (*t*-test, $p < 0.05$) and 11 of the 12 resistant mutants were significantly less fit than wild-type (*t*-test, $p < 0.005$). While the JSF4-sensitive rugose variant was more fit than Rif.R, all four JSF4-resistant rugose variants were significantly less fit than Rif.R (*t*-test, $p < 0.005$).

(2) *The resistant strains of similar fitness to wild-type in liquid culture are less fit than wild-type in surface culture.* As observed in liquid, in surface culture the rifampin-resistant, JSF4-sensitive common competitor, Rif.R, is less fit than the wild-type N16961 (figure 2b). On the other hand, when competing as colonies in surface culture, the phage-resistant mutants R2.1 and R2.2 were less fit than the Rif.R common competitor, rather than substantially more fit as they were in liquid (figure 2b). We interpret this to mean that in surface culture, these resistant mutants are substantially less fit than wild-type.

(3) *JSF4-resistant V. cholerae N16961 are less motile than wild-type V. cholerae N16961.* The swarming motility of more than 200 smooth phage-resistant mutants separately isolated from the batch and chemostat with N16961 and JSF4 was examined. These resistant mutants were either
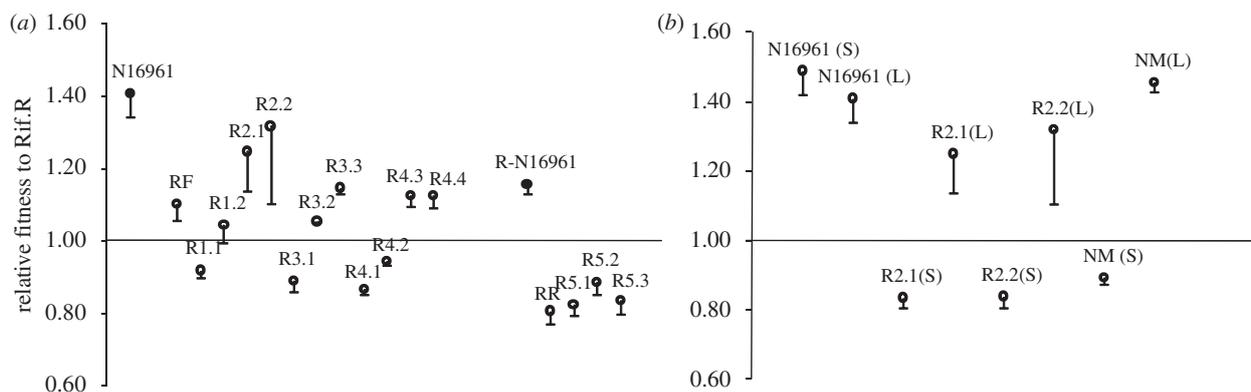
Figure 2. (*a*) Relative Malthusian fitness of a rifampin-resistant JSF4-sensitive common competitor in pair-wise competition with rifampin-sensitive JSF4-sensitive wild-type N16961, rugose variant of N16961 and 16 rifampin-sensitive JSF4-resistant mutants (12 are smooth colony and four are rugose colony types). The horizontal line denotes the fitness of the common competitor, the rifampin-resistant mutant. The error bars are the standard deviations of the mean fitness for at least three independent pair-wise competition experiments. For clarity and aesthetic reasons, we have plotted only the negative standard error. RF, R1.1, R1.2, R2.1, R2.2, RR: JSF4-resistant mutants (see table 1 for the source). R3.1, R3.2, R3.3, R4.1, R4.2, R4.3, R4.4: smooth JSF4-resistant mutants. R5.1, R5.2, R5.3: rugose type JSF4-resistant mutants. All of them were isolated from three different chemostats. The source of these mutants is listed in the electronic supplementary material, table S2. (*b*) Relative fitness of different *V. cholerae* N16961 in competition with rifampin-resistant JSF4-sensitive common competitor in liquid and in surface culture. (S): competition on surface; (L): competition in liquid; R2.1, R2.2: non-motile JSF4-resistant mutants; NM: a non-motile JSF4-sensitive isolate.

non-motile or somewhat motile, but all were less motile than wild-type. The average swarming distance of the 209 phage-resistant mutants examined was 6.1 ± 4.6 mm (mean $\pm$ s.d.). Of these 209, 77 were non-motile, with an average swarming distance of 1.4 ± 0.16 mm. The remaining 132 resistant mutants were at least somewhat motile, with an average swarming distance of 9.0 ± 3.2 mm. All of these JSF4-resistant mutants are significantly less motile than wild-type N16961, which have an average swarming distance of 23.2 ± 4.74 mm (t-test, $p < 10^{-7}$).

To exclude the possibility that the reduced motility observed among JSF4-resistant mutants is associated with the adaptation of *V. cholerae* to chemostat culture, rather than phage resistance, 252 isolates from two JSF4-free control chemostats were tested for motility. Of these 252 isolates, 20 appeared to be less motile than the wild-type. All 20 of the reduced-motility colonies were isolated from the later samples of the chemostat cultures. None of the clones isolated at similar time points to those of the JSF4-resistant mutants described above had a reduced motility phenotype. Since the JSF4-sensitive rugose variants were substantially less motile than wild-type (swarming distance of about 4 mm rather than 23 mm), we did not assay for the difference between JSF4-sensitive and -resistant rugose variants.

(4) *JSF4-resistant mutants are more likely to form clumps and switch to rugose at lower frequencies than wild-type*. The methods and results of the experiments demonstrating this are in the electronic supplementary material, S2 and S3.

(5) *JSF4-resistant mutants have a disadvantage relative to wild-type in colonizing crustaceans*. In their natural aquatic habitat, *V. cholerae* are associated with zooplankton such as copepods, where they colonize the carapace and other parts of the chitin exoskeleton (Huq *et al.* 1983; Cottingham *et al.* 2003; Levy 2005). The relative ability of JSF4-sensitive and -resistant *V. cholerae* to colonize zooplankton was examined by culturing single clones of

JSF4-resistant and -sensitive *V. cholerae* N16961 with *D. magna* and *D. pulex* (Chiavelli *et al.* 2001; Kirn *et al.* 2005). In the course of these experiments, we noted that, for any given strain of bacteria, the more intensely red *Daphnia* supported higher densities of colonizing *V. cholerae* than those that were more colourless (i.e. had less haemoglobin; Pirow *et al.* 2001). To control for this haemoglobin effect, we used the same batch of *Daphnia* for each colonization experiment.

In figure 3*a*, we present the results of these colonization experiments with *D. pulex* and in figure 3*b* those with *D. magna*. For both JSF4-sensitive and JSF4-resistant *V. cholerae*, the number of bacteria recovered from *D. magna* was substantially greater than that from *D. pulex*, presumably because of the greater size and thus greater surface area of *D. pulex*. In all cases, significantly fewer JSF4-resistant cells were recovered from both species of *Daphnia* than the wild-type (t-test, $p < 0.05$).

Although it is beyond the scope of this report to ascertain the reasons that *V. cholerae* are more successful in colonizing dead crustaceans than live ones (Mueller *et al.* 2007), for this study it seemed reasonable to ascertain whether the differences in the relative ability of JSF4-sensitive and -resistant *V. cholerae* to colonize viable *Daphnia* can be attributed to their relative capacity to adhere to chitin. To determine if this is the case, we examined the ability of JSF4-sensitive and -resistant strains to colonize chitin in an aqueous suspension (electronic supplementary material, S4). Among five smooth JSF4-resistant mutants, only two mutants, which are non-motile, were significantly less capable of colonizing chitin than wild-type (t-test, $p < 5 \times 10^{-7}$). This result is consistent with the observation that motility is positively associated with the ability of these bacteria to colonize dead crustaceans (Mueller *et al.* 2007). As noted above and contrary to this observation, there seemed to be no association between motility and the colonization of viable *Daphnia*. There was also no significant difference
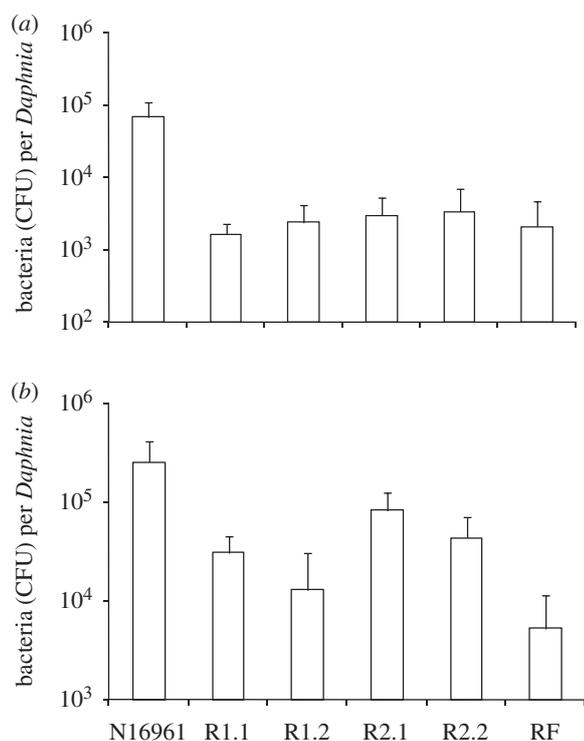
Figure 3. Colonization of wild-type *V. cholerae* N16961 and JSF4-resistant mutants on (a) *D. pulex* and (b) *D. magna*. RF, R1.1, R1.2, R2.1, R2.2: JSF4-resistant mutants. Error bar represents standard deviation of bacteria (CFU) attached to single *Daphnia*.

in the colonization on chitin between JSF4-sensitive and -resistant rugose mutants (*t*-test, $p > 0.1$).

(6) *JSF4-resistant mutants are less virulent than wild-type when ingested by* C. elegans. Using a procedure similar to that in Vaitkevicius *et al.* (2006), we estimated the relative rate of mortality of *C. elegans* feeding on six independent JSF4-resistant mutants, a wild-type N16961 and *E. coli* OP50. At a qualitative level, our survival results are consistent with those of Vaitkevicius and colleagues; the rate of decline in viable *C. elegans* ingesting wild-type N16961 was less than those fed *E. coli* OP50 (figure 4). Most importantly for this study, the decline in viable *C. elegans* feeding on JSF4-resistant *V. cholerae* was significantly less than those ingesting phage-sensitive, wild-type *V. cholerae* (log-rank test, $p < 0.01$).

4. DISCUSSION
If, as postulated in Jensen *et al.* (2006), the necessary condition for phage to modulate outbreaks of cholera is that these viruses, rather than resources, limit the densities of *V. cholerae* populations, the results of our chemostat and serial transfer experiments are inconsistent with this hypothesis. When the lytic phage JSF4 are introduced into chemostat and serial transfer cultures of the El Tor O1 *V. cholerae* N16961, JSF4-resistant cells rapidly ascend to dominance. The densities of these experimental populations of *V. cholerae* and the phage JSF4 are no different from those of phage-free, resource-limited, control cultures. Presumably, by replicating on minority populations of phage-sensitive *V. cholerae* (Levin *et al.* 1977), the phage persist in these mixed experimental

cultures. These results are what would be anticipated from studies of the population dynamics of *E. coli* and its phage (Bohannan & Lenski 2000; Mizoguchi *et al.* 2003), as well as those of *Pseudomonas syringae* and its phage Phi6 (Lythgoe & Chao 2003) and *Pseudomonas aeruginosa* with phage PP7 (Brockhurst *et al.* 2005).

Although our *in vitro* population dynamic experiments with *V. cholerae* N16961 and the phage JSF4 reject the hypothesis that this bacterial virus limits the density of *V. cholerae*, the other experiments we performed are not inconsistent with the epidemiological/clinical hypothesis that lytic phage contribute to the waning of outbreaks of cholera. We postulate that because of their lower fitness in their natural habitat and possible avirulence in humans, phage-resistant *V. cholerae* play a relatively insignificant role in the ecology of these bacteria and the epidemiology of cholera. In support of this interpretation are the following observations.

— In pair-wise competition between JSF4-resistant mutants and the JSF4-sensitive *V. cholerae* in liquid culture, the majority of the resistant mutants are significantly less fit than the sensitive cells from whence they were derived. Although one of the phage-resistant clones we tested was not less fit than wild-type in liquid, it had a profound fitness disadvantage relative to wild-type in surface culture.

— All the JSF4-resistant mutants we examined were less motile than wild-type. We believe that this reduced motility may account for why some phage-resistant mutants were less fit than wild-type in surface but not in liquid culture (figure 2b). We postulate that in surface and semi-solid culture, motility enables bacterial colonies to spread and, by diffusion, sequester more resources than non-motile colonies. As a result, colonies of motile bacteria produce more cells than non-motile colonies and thereby have a competitive advantage in a manner analogous to the way the diffusion of bacteriocins provides a selective advantage to producing colonies in competition with bacteriocin-sensitive, non-producing populations (Chao & Levin 1981). We are currently testing this resource sequestration hypothesis for the advantages of motility in physically structured habitats.

— In their natural habitat, *V. cholerae* colonize copepods and other crustaceans (Mueller *et al.* 2007). *In vitro*, the JSF4-resistant cells appear less able to colonize viable crustaceans than the sensitive cells. The number of JSF4-resistant *V. cholerae* recovered from *D. pulex* and *D. magna* was significantly lower than the corresponding number of phage-sensitive wild-type.

— The rate of mortality of the nematode *C. elegans* feeding on JSF4-resistant *V. cholerae* was significantly less than when these worms feed on susceptible *V. cholerae*. This too may be a reflection of the reduced motility of these bacteria. There is evidence for a direct relationship between motility and virulence of *V. cholerae*, but that appears to depend on the nature of the mutations responsible for the non-motile phenotype (Gardel & Mekalanos 1996). We have not yet ascertained whether the mutations responsible for the decreased motility of JSF4-resistant *V. cholerae* are of this virulence-reducing type.
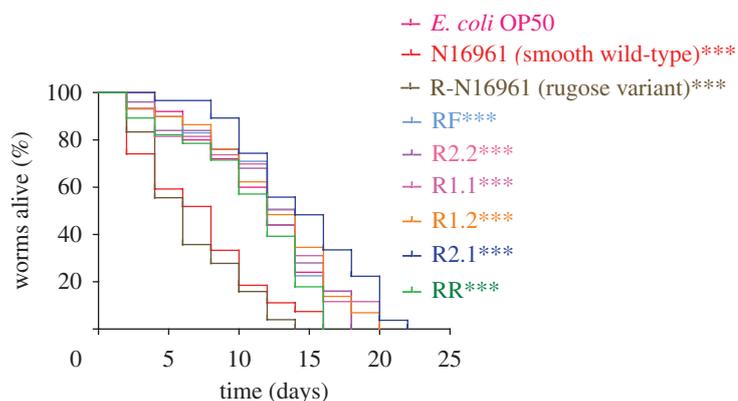
Figure 4. Rate of mortality of *C. elegans* feeding on phage-sensitive and -resistant *V. cholerae* and an *E. coli* OP50 control. RF, R1.1, R1.2, R2.1, R2.2: smooth phage-resistant mutants; RR: rugose phage-resistant mutant. Triple asterisks (***) indicate a significant *p*-value of log-rank test for comparison of survival curves of JSF4-resistant relative to JSF4-sensitive.

(a) *Predictions, current evidence from natural population and clinical isolates, and suggestions for testing hypotheses*

Model systems, like mathematical models, provide a way to generate hypotheses about natural populations, but cannot be used to test those hypotheses. One prediction of these experimental results is that if *V. cholerae* and its lytic phage are together and bacterial densities are high enough for the phage to replicate, the majority of the bacteria would be resistant to the phage. This prediction is at least somewhat consistent with the results reported by Faruque and colleagues (Faruque *et al.* 2005*b*). Of 15 samples from river and lake water, where both *V. cholerae* and its lytic phage were present, bacteria resistant to the co-isolated phage were found in 10 of the samples. Whether the *V. cholerae* were eliminated by the phage in the 87 samples, where only *V. cholerae*-specific phage but no bacteria were isolated, is not clear, although these phage must have replicated on *V. cholerae*. Whether the *V. cholerae* in their 49 phage-free samples were resistant to the phage in the same lake or river is not reported.

One problem in interpreting the results of the existing field studies is that we do not have good estimates of the densities of the bacteria and phage populations. Samples are commonly enriched and even then the densities of bacteria in those samples are relatively low (less than 10^3), which is too low to maintain a phage population that has even a modest rate of loss (see the electronic supplementary material, S5). Obviously, there are habitats or sub-habitats where the densities of bacteria are sufficiently high to support the replication of the phage. One goal of a field study testing the predictions generated from this experimental model would be to identify the habitats or sub-habitats, where the densities of bacteria are sufficiently high to support the replication of phage. In these habitats we would anticipate that (i) the bacterial population would be dominated by cells resistant to the co-existing phage, and (ii) if a novel phage that can replicate on the bacteria in that habitat is introduced, resistance will evolve and these resistant cells will ascend to dominance. Presumably because of the infective dose requirement (Cash *et al.* 1974), these sub-habitats with high densities of *V. cholerae* are those from whence humans obtain symptomatic cholera.

A second prediction is that *V. cholerae* resistant to lytic phage are ecologically less fit than wild-type. We are unaware of the evidence in support of or in opposition to this prediction, but believe that it can be tested in natural populations. Phage-sensitive and -resistant bacteria *V. cholerae* can be released in habitats where phage are not present, and their relative rates of recovery can be monitored in open water and colonizing on copepods and other crustaceans in these natural communities. Although there have been studies of the colonization of different strains of *V. cholerae* on viable and dead crustaceans in the field and *in vitro* (Huq *et al.* 1983; Mueller *et al.* 2007), to our knowledge there have not been field studies that have explored the relative abilities of otherwise isogenic phage-sensitive and -resistant *V. cholerae* to colonize viable and dead crustaceans.

A third prediction made from these *in vitro* experimental results is that phage-resistant *V. cholerae* are less virulent in human hosts than otherwise isogenic sensitive cells. There are at least two lines of evidence in support of this hypothesis. First, a recent report presented evidence that the minimum infective dose of *V. cholerae* O1 in infant mice was ten times greater when the bacteria were mixed with JSF4 than when they were not (Zahid *et al.* 2008). In that study, as in our own study, while JSF4-resistant *V. cholerae* C6706 emerged and dominated *in vitro* LB culture, in the mice only JSF4-sensitive *V. cholerae* were recovered from the stools. Second, the *V. cholerae* recovered from stools of cholera patients infected with both bacteria and phage were susceptible to the phage isolated from these stools (Zahid *et al.* 2008). If these results are general, we would expect that if mixtures of *V. cholerae* and JSF4 are introduced into human volunteers (i) the phage would replicate and (ii) the bacteria recovered from the stools would be primarily, if not exclusively, sensitive to these phage.

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