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A tale of tails: Sialidase is key to success in a model of phage therapy against K1-capsulated *Escherichia coli*

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ABSTRACT

Prior studies treating mice infected with *Escherichia coli* O18:K1:H7 observed that phages requiring the K1 capsule for infection (K1-dep) were superior to capsule-independent (K1-ind) phages. We show that three K1-ind phages all have low fitness when grown on cells in serum whereas fitnesses of four K1-dep phages were high. The difference is serum-specific, as fitnesses in broth overlapped. Sialidase activity was associated with all K1-dep virions tested but no K1-ind virions, a phenotype supported by sequence analyses. Adding endosialidase to cells infected with K1-ind phage increased fitness in serum by enhancing productive infection after adsorption. We propose that virion sialidase activity is the primary determinant of high fitness on cells grown in serum, and thus in a mammalian host. Although the benefit of sialidase is specific to K1-capsulated bacteria, this study may provide a scientific rationale for selecting phages for therapeutic use in many systemic infections.

Introduction

The use of phages to treat bacterial infections, phage therapy, is an enigma of Western medicine. Proposed and practiced before the development of antibiotics and before phage genetics was even understood, phage therapy was disgraced and displaced in the West by antibiotics during the 1940s, although it survives even today in Eastern Europe. Only now, amid an ever-growing threat from antibiotic resistant bacterial pathogens, is it being entertained again in the West. Yet after more than a decade of promulgation in the U.S., it has been approved only for treatment of processed food and plants and has seen little interest from Big Pharma (Brussow, 2005; Carlton et al., 2005; Coates and Hu, 2007; Hanlon, 2007; Leverentz et al., 2003; Mattey and Spencer, 2008; Projan, 2004; Schoolnik et al., 2004; Soothill et al., 2004; Sulakvelidze, 2005).

In concept, phage therapy seems invincible: a self-replicating organism selectively killing only a pathogen, amplifying itself only where it is most needed—sites where the pathogen is lurking and abundant. Yet successful treatment with phages is necessarily founded on dynamic principles just as pharmacodynamic properties determine whether a drug that kills a bacterium *in vitro* will actually be effective *in vivo* (Bull and Regoes, 2006; Levin and Bull, 1996, 2004; Payne and Jansen, 2001, 2003). A phage that does not amplify fast enough or cannot access its host in some tissues may fail clinically, even if it works well in the lab. Most lab practitioners are aware that phages do not sterilize cultures of cells even under ideal conditions and may not even kill cells under poor growth conditions, but this and other empirical realities have not helped balance the promise with the prospects of a viable phage therapy enterprise.

Much experimental and empirical research on phage therapy has omitted consideration of dynamics, relying on qualitative outcomes. However, a systematic study using *Escherichia coli* and laboratory phages in a mouse model has been initiated (Denou et al., 2009; Weiss et al., 2009). Nevertheless, there is yet little work to provide mechanistic insight on how to improve treatment, short of haphazardly testing multiple phages. The use of genetically engineered phages has shown that rational design of phage properties can improve efficacy (Hagens and Blasi, 2003; Hagens et al., 2004; Lu and Collins, 2007; Westwater et al., 2003), and artificial selection of a phage *in vivo* led to a reduced clearance rate of phage in mice (Merril et al., 1996). So some progress is being made toward understanding how to improve phage therapy success.

One promising system for discovering the bases of phage therapy success is a mouse infection model developed over 25 years ago (Smith and Huggins, 1982). Inoculation of 5×10^7 bacteria (*E. coli* O18:K1:H7) into the leg of a mouse was nearly always fatal after 24 h unless treated early by phages or antibiotics. The phages were wild isolates from sewage and pig farms in England. However, in this

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Table 1
Notation and origins for the seven phages characterized in detail.

Name[a]	Family	Genome size (bp)	Origin/identity
K1-dep(1)	*Siphoviridae*	41,632	Atlanta, GA sewage (øLH in Bull et al., 2002). Formally named K1H
K1-dep(2)	*Podoviridae*	45,251	K1E (Gross et al., 1977; Stummeyer et al., 2006; Tomlinson and Taylor, 1985)
K1-dep(3)	*Podoviridae*	44,385	K1-5 (Scholl et al., 2001; 2004)
K1-dep(4)	*Siphoviridae*	43,587	Superior growth rate in competition of 2,000 K1-ind and K1-dep phages from Austin, TX sewage, subjected to serial passage in serum on CAB1. Formally named K1G
K1-ind(1)	*Siphoviridae*	42,292	Atlanta, GA sewage (øLW in Bull et al., 2002) Formally named K1ind1
K1-ind(2)	*Siphoviridae*	42,765	Superior growth rate of 54 K1-ind phages; from Austin, TX and Moscow, ID, grown in serum on CAB1. Formally named K1ind2
K1-ind(3)	*Siphoviridae*	43,461	Superior growth rate in competition of 450 K1-ind phages from Austin, TX sewage, subjected to serial passage in broth on a K1E-resistant CAB1. Formally named K1ind3

[a] K1-dep indicates that the phage requires the K1 capsule, K1-ind that the phage does not require K1.

heterogeneous collection of phages, some performed much better than others, achieving near 100% survival when administered simultaneously with, but separately from, the bacteria. "Good" phages were of a type that required the bacterial K1 antigen for infection (K1-dependent, or K1-dep phages); of 9 isolates, 8 rescued 10 of 10 mice, and the 9th rescued 9 of 10. Six apparently independent phage isolates that did not require the K1 antigen were tested (K1-independent, or K1-ind phages), rescuing on average 33% of mice, the best rescuing 60%.

No further investigation was undertaken by Smith and Huggins of the mechanisms underlying the difference between the two types of phages, except for a casual note that K1-ind phages were poor at lysing cultures in broth and formed smaller plaques than K1-dep phages. The authors further argued that the success of K1-dep phages lay in their ability to rapidly destroy the bacteria, a perspective that inspired, and was supported by, several theoretical studies (Levin and Bull, 1996, 2004; Payne and Jansen, 2001, 2003). These observations raised the specter that K1-ind phages grew poorly under many conditions and might have a genomic property incompatible with fast growth (cf. Bull et al., 2004), and in turn, that poor growth rate was inimical to treatment success. Bull et al. (2002) compared single K1-dep and K1-ind phage isolates using a different *E. coli* O18:K1:H7 strain, and obtained quantitatively similar results to those reported by Smith and Huggins (1982). In addition, growth rates (absolute fitness) of both phages were formally quantified on cells grown in broth or serum. The K1-dep and K1-ind phage grew equally well in broth but the K1-ind phage performed far more poorly in serum although there was considerable variance between replicate assays.

If the different *in vivo* performance of K1-ind and K1-dep phages is general, not merely an accident of the phages sampled, the system could offer insight to the determinants of phage therapy success. Understanding the cause of the difference could lead to a rational a priori choice of phages that perform well in treatment. The goal of the present study is to resolve the basis of the difference in fitness of K1-ind and K1-dep phages, extending the sample to a larger set of K1-ind phages in search of some with high *in vivo* fitness. Following the precedent of Bull et al. (2002), the *in vivo* environment is represented by using serum as growth medium.

Results

Phage samples

The phages subjected to detailed assays are described in Table 1, each capable of growing on the host CAB1 (*E. coli* O18:K1:H7). For ease of presentation, they will be denoted K1-ind(i) and K1-dep(j), to facilitate distinguishing whether they require K1 for infection, with subscripts distinguishing their identities within a type. Four phages were chosen without a priori knowledge of or bias toward fitness in broth or serum, only that they formed plaques on LB agar plates with CAB1 as host. The others were chosen for high growth rates from moderately large samples of otherwise uncharacterized phages.

K1-dep phage fitness is superior, but only in serum

Prior work raised the possibility that K1-dep phages have higher fitness than K1-ind phages in the environment of a mammalian host but sampling was limited and assays were variable. Here we consider the generality of that model. As before, the *growth rate* of a phage (measured as doublings per hour) describes its ability to amplify its numbers when hosts are not limiting, and it is used here as a measure of phage fitness. For example, a growth rate of 10 doublings/h indicates a 1024-fold (2^{10}) increase in numbers each hour, whereas a fitness of 15 indicates a 32,768-fold increase (under the assay conditions). A phage's growth rate reflects its ability to rapidly expand during treatment and kill bacteria to the extent that growth rate measured *in vitro* mirrors growth rate *in vivo*. The assays here used either broth or bovine serum, the latter interpreted as more closely approximating a within-host environment.

Growth rates of the seven phages were measured on CAB1 (Fig. 1). In broth, there was considerable overlap between the K1-ind and K1-dep groups. There was a clear separation between the two groups in serum: all K1-dep phages grew faster than all K1-ind phages. Yet there was substantial variance within each group; the best K1-dep phage exceeded the next-best K1-dep phage by the same amount as the difference between the worst K1-dep phage and the best K1-ind phage (4.2 doublings/h). The K1-dep phage isolated from competition in serum had the highest growth of all other phages in serum but not in broth. Likewise, the K1-ind phages isolated from competitions had higher growth rates than other K1-ind phages in the competition environment but not in the other environment. Thus, while there may be a correlation between fitnesses in different environments, within each phage type, the correlation is not perfect.

Common to most phages is that fitness is higher on cells grown in broth than in serum. The suppressive effect of serum is merely far greater for the K1-ind phages. The general effect of serum in reducing

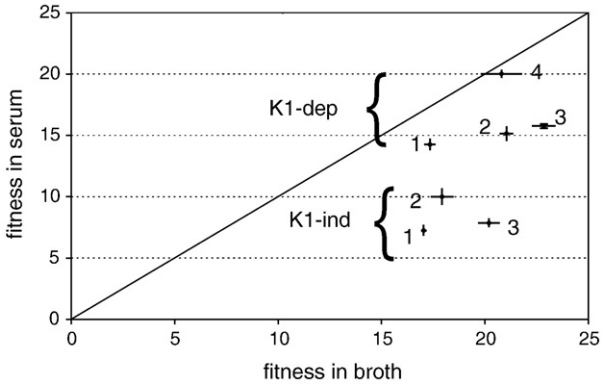

Fig. 1. Fitnesses (doublings/h) of four K1-dep phages and of three K1-ind phages measured in LB (broth) and in bovine serum. Points give means, bars are ±1 standard error. The diagonal line indicates equal fitness in broth and serum.

fitness of both phage types may have a different cause than its differential effect on K1-ind phages. Bacterial physiology may simply be less conducive to supporting phage growth in serum than in broth.

Adaptation of a K1-ind phage fails to overcome the fitness constraint

Given that we could not isolate K1-ind phages with high fitness in serum, we attempted to evolve one. Both phages and bacteria commonly show fitness improvement when adapted to growth conditions (e.g., Bull and Molineux, 2008; Ebert, 1998; Fong et al., 2006). However, 50 h of serial passage of K1-ind(1) in serum failed to elicit an increase in its fitness (data not shown, the net population expansion was approximately 10^{300}). Thus the limitation of K1-ind phages to have low fitness on cells grown in serum appears to be strong.

K1-ind and K1-dep phages do not have fundamentally different genomes

A possible explanation for the different serum fitnesses of K1-dep and K1-ind phages is that the two phages represent fundamentally different genomic types, each with different limitations in their abilities to grow in some environments. For example, phages of the *Podoviridae* exhibited higher evolved fitnesses than phages of some other families given similar opportunities for adaptation (Bull et al., 2004). The two previously characterized phages in our sample of seven are *Podoviridae*, and both are K1-dep (Table 1).

To explore phage identities, genome sequences were determined for the 5 remaining phages, representing two K1-dep and three K1-ind isolates obtained from sewage (annotations provided in Table S1). Surprisingly, all five phages are extremely similar in genome structure, even though K1-dep(1) and K1-ind(1) were isolated ~10 years before the other three and from a distinct geographical area. (Note that phages isolated from Austin sewage were not amplified on CAB1 before isolation.) The five genomes range from 41632 bp to 43857 bp and with one major exception, contain the same complement of genes. Gene order is essentially syntenous, differing only by minor indels, and in the cases of K1-dep(4) and K1-ind(3), by the acquisition of, respectively, a putative homing endonuclease and an uncharacterized *Listonella* phage protein. A dotplot analysis of four genomes reveals stunning similarity, even between K1-dep and K1-ind types (Fig. 2; K1-ind(3) is not included as it is virtually identical to K1-ind(2), with 98% nucleotide identity over the entire genome). Their closest relatives in the database, as judged by BlastP searches of predicted orfs are the *Salmonella* typing phages SETP3 (De Lappe et al., 2009) and KS5, although nucleotide similarity to putative homologs is not high. It is noteworthy, if not surprising, that the five phages isolated for this and earlier work, although similar among themselves, differ profoundly from the two K1-dep phages that were included in our sample based on a priori knowledge of their ability to infect K1-capsulated bacteria.

Only K1-dep phages possess endosialidase activity

The major difference among the five genome sequences lies in the putative tailspike gene. Both K1-dep phage tailspike genes contain an endosialidase, with greater than 96% amino acid sequence identity to the enzyme characterized from the tail of phage 63D (Kataoka et al., 2006; Machida et al., 2000a; Machida et al., 2000b). Both K1-dep(2) and K1-dep(3) (K1E and K1-5, respectively) also have documented endosialidases (Leggate et al., 2002; Leiman et al., 2007; Muhlenhoff et al., 2003). In contrast, sequences of the three K1-ind phages indicate a glycosidase that is most closely related to the coliphage HK620 tailspike. The HK620 enzyme is an endo-N-acetylglucosaminidase that degrades the O-antigen of *E. coli* O-18A1 serotype (Barbirz et al., 2008).

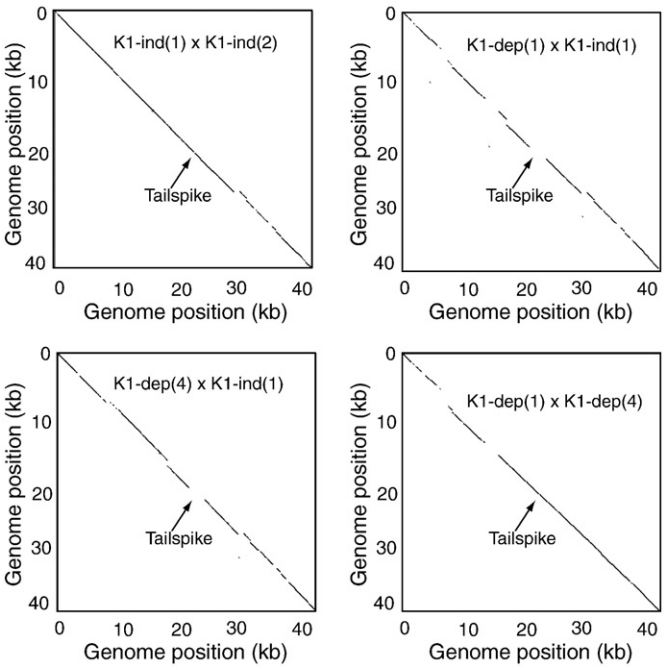

Fig. 2. Dot plots of the phages K1-ind(1), K1-ind(2), K1-dep(1) and K1-dep(4). The main difference between K1-dep and K1-ind types is in the tailspike gene (arrows). Window size is 19 bases, and each dot implies identity over all 19 bases. K1-ind(3) is not included because it is virtually identical to K1-ind(2).

Although sequence similarity between K1-dep and K1-ind phages is low across these tailspike enzymatic domains, it is high on both flanks. Between the K1-dep and K1-ind phages, the N-terminal 35–46 amino acids of the tailspike are 100% identical, and the N-terminal 115, likely the tail-binding domain, are 78% identical. (The three K1-ind phages contain identical 5′-terminal nucleotide sequences of the tailspike gene.) A region of >100 bp identity lies upstream of the tailspike initiation codon in all five phages, and this region is extended upstream at ~95% similarity for ~3.5 kb. Downstream of the tailspike gene, high similarity between the K1-ind and K1-dep phages again becomes apparent, also persisting for several kb. These closely related K1-ind and K1-dep phages thus present a clear example where an enzyme domain has been exchanged by recombination while maintaining a common tail-binding domain of the tailspike (Barbirz et al., 2008; Leiman and Molineux, 2008; Scholl et al., 2004; Stummeyer et al., 2006).

Inference of sialidase activity in all K1-dep phages and its absence in all K1-ind phages was confirmed by direct enzyme assay of virions. All K1-dep phage virions contain sialidase, whereas the enzyme is absent in all K1-ind virions (data not shown). From the combined sequence comparisons and enzyme assays, the essential difference between K1-dep and K1-ind phages lies in their tailspike, with K1-dep phages having an enzyme that degrades the bacterial K1 capsule and K1-ind phages having an enzyme that degrades O-antigen.

Exogenous endo-N restores most of a K1-ind phage's fitness in serum

The invariant absence of sialidase activity with K1-ind virions raises the possibility that the K1 capsule of serum-grown cells is responsible for the major fitness deficit of K1-ind phages. One test of this model is provided by the addition of free endo-N-acylneuraminidase (endo-N) to the culture. If the lack of sialidase in K1-ind phages is responsible for low fitness in serum, and if the capsule is more of a barrier in serum than in broth, addition of exogenous endo-N should greatly increase fitness in serum but have little effect in broth. The consequence of endo-N addition in serum was a fitness increase of

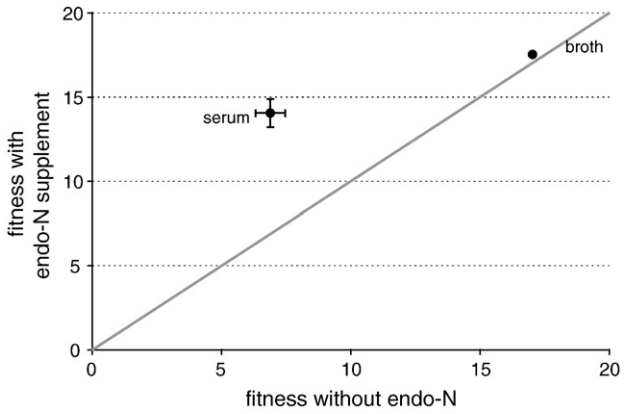

Fig. 3. Fitnesses of phage K1-ind(1) in serum and broth with and without endo-N supplementation. The diagonal line, at which fitness is equal under the two treatments, is provided only to facilitate visualizing the difference between the two treatments. A point above the line indicates higher fitness with endo-N supplementation than without. Standard error bars are shown in both dimensions, but may be obscured by the data point.

over 7 doublings/h (Fig. 3). Furthermore, the effect was specific to growth in serum, with no appreciable benefit in broth.

Phage tails are thought to affect the early part of the phage life cycle, host recognition and delivery of the genome into the host. Thus if the tailspike accounts for the consistently lower serum fitness of K1-ind phages relative to K1-dep phages, the effect should be manifest in either adsorption or genome entry rates. The effect should be striking if adsorption was the only trait affected: at a density of 10^8 cells/mL, a fitness drop from 15 to 10 dbl/h requires a 15-fold drop in adsorption rate (see Bull, 2006). Although all three fitness parameters (adsorption rate, lysis time and burst size) likely change between cells grown and infected in broth versus serum, there should be a profound reduction in adsorption or genome entry rate that is common to K1-ind phages that is abolished by addition of free endo-N.

Although adsorption rate is the obvious a priori candidate for the effects of tailspike, it was not obviously affected by endo-N addition. The adsorption rate in the absence of endo-N ($\sim 1\times10^{-9}$ mL min^{-1}) is already close to the proposed theoretical upper limit (approximately 10^{-8}, Adams, 1959) and could not improve enough to account for the observed fitness gain. Indeed, adsorption rates, measured as the ratio of unadsorbed to total phages after dilution into 37 °C medium, were not significantly higher with addition of endo-N [$t(6) = 0.9$, $P \approx 0.2$, one-tailed t-test].

In contrast, genome internalization and phage development appeared to be greatly enhanced by the addition of endo-N. Following adsorption of K1-ind(1) to CAB1 in serum, addition of endo-N resulted in a 15-fold increase in burst size (Fig. 4). It is unlikely that degradation of the capsule would profoundly improve the intracellular host environment in such a short time, and we interpret the burst size increase as an increase in the number of adsorbed phages that go on to infect cells productively. With this interpretation, the K1 capsule of cells growing in serum normally blocks efficient genome entry into the cell for over 90% of adsorbed K1-ind phages, but those phages remain functional (at least in the short term), and the block can be reversed by capsule reduction either enzymatically or upon

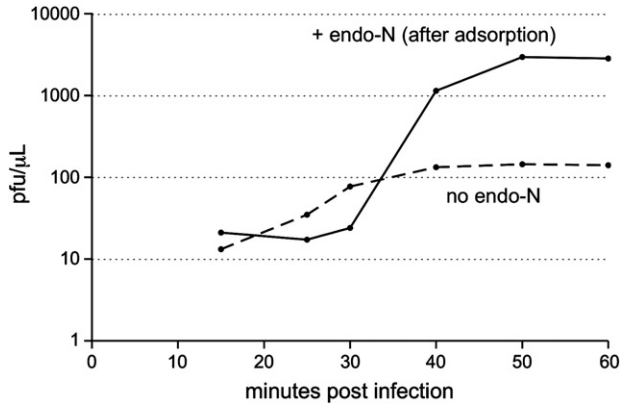

Fig. 4. Representative one-step growth curve of K1-ind(1) on CAB1. Exponentially growing cells in serum were allowed to adsorb phage (multiplicity of ~0.1) for 5 min, and then diluted 1000× into either serum (no endo-N, dashed curve) or serum with endosialidase (+endo-N after adsorption, solid curve). At 60 min, the estimated burst size for the endosialidase treatment was 500, versus 30 for no-sialidase. Infective centers were a high enough proportion of total added phage (36% for the endosialidase treatment, 47% for the pure serum treatment) that the difference in burst size cannot be attributed to an artifact of sampling error.

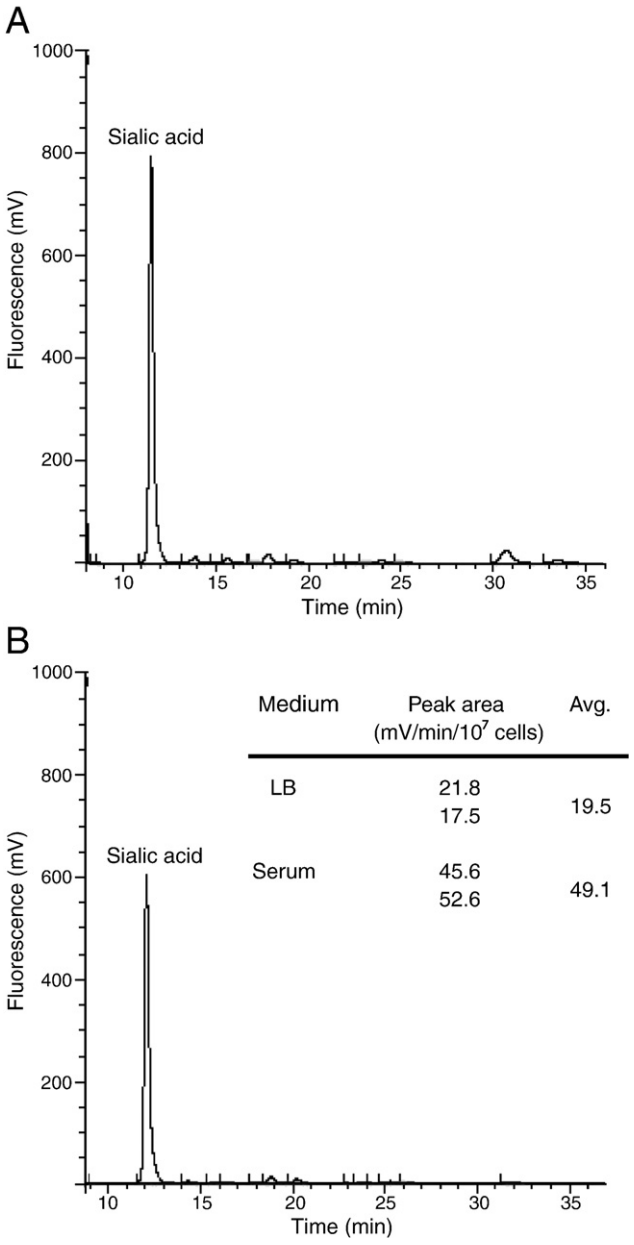

Fig. 5. Relative amount of capsular sialic acid in strain CAB1 grown in LB or bovine serum. (A) Representative chromatographic profile of sialic acid from cells grown in LB medium. (B) Representative profile of cells grown in serum; the sample size was one-half that used in panel A. The insert gives the peak area in millivolts min^{-1} normalized for cell number for two independent experiments under each growth condition. Data for the two conditions are different ($p = 0.01$) by a one-tailed Student's t-test where a probability <0.05 was considered significant.

growth in broth. Presumably, the addition of endo-N allows adsorbed K1-ind phages to complete an infection cycle rather than releasing them into the medium.

These data suggest that cells grown in the presence of serum possess more capsular material than the same cells grown in broth. We therefore determined the sialic acid content of CAB1 cells growing in each medium. A 2.5× increase in the amount of sialic acid was observed in serum-grown cells (Fig. 5). Note that the absence of material eluting later than sialic acid means that any phase variation involving O-acetylation of the sialic acid is not affected by growth in LB or in serum.

Discussion

Smith and Huggins (1982) concluded that phage therapy success in a mouse infection depended fundamentally on the type of phage used. For an *E. coli* bacterial host of serotype O18:K1:H7, the best phages required the bacterial capsular K1 antigen for infection (K1-dep phages), whereas phages that did not require K1 (K1-ind phages) were less effective. Smith and Huggins casually suggested that K1-ind phages were intrinsically poor at growth under all conditions. Subsequent work with a single phage of each type refuted that suggestion, showing that a K1-ind phage grew equally well as a K1-dep phage when cells were incubated in broth (Bull et al., 2002). K1-dep phage appeared to have superior fitness when cells were grown in serum, but the reproducibility and generality of those data required confirmation.

The goals here have been to determine if the apparent inferiority of K1-ind phages in therapeutics is general and if so, its basis. In broth, fitnesses of K1-dep and K1-ind phages on the K1-capsulated strain CAB1 overlapped, but the genome sequences do not even reveal clues for why fitnesses of closely related phages differ. However, a fitness pattern appears to be general in serum, fitnesses of K1-ind phages are lower than K1-dep phages. Thus, in serum medium, an environment more characteristic of a mammalian host than rich broth media, the specialist K1-dep phages grow better than phages that can plaque on both capsulated and non-capsulated strains.

Three phages were intentionally selected for high fitness, and these represent the high-fitness tails of the distributions. However, amplification of K1-ind(2), which was selected for high fitness in serum by serial passage of a mixture of K1-ind phages, was 16-fold lower than the least fit [K1-dep(1)], and 1000-fold lower than the fittest K1-dep phage [K1-dep(4)] in serum. This difference may have profound effects in therapeutic applications.

Several observations support the thesis that endosialidase activity of the tailspike is both the determinant of the K1-dep phenotype and is necessary for high fitness in serum. (1) Of three K1-ind phages and four K1-dep phages assayed for fitness, sialidase activity of the virion correlated perfectly with the K1-dep phenotype and thus with high fitness in serum. (2) The genome sequences of all four K1-dep phages reveal that the tailspike protein contains a sialidase domain whereas tailspike in the three K1-ind phages is predicted to contain a different glycosidase. Indeed, the sequence of the tailspike enzyme domain is the only major difference between some K1-ind and K1-dep phage genomes. (3) Addition of free endosialidase to a culture of the capsulated strain CAB1 infected by a K1-ind phage raised its fitness substantially, but only in serum. Serum-grown cells were shown to contain 2.5× more K1 capsule than when grown in rich broth. The extra capsular material could be manifest by longer chains of sialic acid, making a thicker capsule, or more chains that make the capsule more dense. We favor the latter possibility because longer chains would likely inhibit the initial adsorption of K1-ind phages to their O-antigen receptor, but this was not observed. Conversely, a more dense K1 capsule might be expected to inhibit or at least decrease the rate of O-antigen hydrolysis by K1-ind phages as the entire virion needs to penetrate the capsule in order to reach the cell surface when genome penetration of the cytoplasm could be initiated.

Merril et al. (1996) evolved two laboratory phages for long term retention in mice. Enhanced persistence of λ was due to a single amino acid change in the viral capsid protein (Vitiello et al., 2005). This result raises the prospect of using selection *in vivo* to improve phage performance. However, selection for faster serum growth of a single K1-ind phage was unsuccessful; too many simple mutations are presumably necessary to change the enzyme specificity of a K1-ind tailspike to a sialidase. Thus, success in selection of a more useful therapeutic agent by short-term adaptation of a single phage is not assured. If the original phage has already been carefully characterized, it should be possible to engineer the desired derivative. Alternatively, and perhaps more practical as a general approach, selection from a pool of phages is likely to allow isolation of a superior therapeutic phage. We have shown here that phages need not be tested individually to recover those with high growth rate; competition within a large pool may be used and conditions can easily be chosen where recombination can occur between different phages in the pool. Serial passage under selective conditions will then allow isolation of the "best" phage. This is, of course, the approach successfully followed for decades in Eastern Europe, but in the West may result in regulatory obstacles.

The ramifications of this study for use of phages in treating bacterial infections are threefold:

(1) Bacterial susceptibility to treatment with a particular phage depends fundamentally on the specific growth environment. In the context of this work, good phage performance in a host-like environment requires a specific tailspike enzyme to penetrate the bacterial capsule. This result underscores the need to test phage performance in an appropriate environment.
(2) The poor growth rate on a K1-capsulated strain of a phage possessing a tailspike that degrades O-antigen is not overcome by short-term adaptation, either as an individual or by allowing recombination with other phages with the same receptor type. However, given the genome similarity between some K1-dep and K1-ind phages, adaptations that allow recombination between different phage genomes could prove useful.
(3) A broad host range phage is an oft-cited desirable goal for therapeutic applications. However, the broad host range phages studied here, those growing on *E. coli* possessing an O18 antigen but blind to the presence of a K1 capsule (at least in broth cultures), are distinctly inferior to the narrower host range K1 capsule-dependent phages in the environment where they are likely to be used.

It has commonly been assumed that the success of phage therapy to treat systemic infections is tied, at least qualitatively, to a phage's ability to grow within the mammalian host. Assumptions of this nature can be traced to early reviews of (and doubts about) phage therapy, for which it was believed that phages could not cure infections unless they were replicating *in vivo* (Eaton and Bayne-Jones, 1934; Krueger and Scribner, 1941; Morton and Engley, 1945). Smith and Huggins (1982) explicitly considered that treatment success in their model was a function of phage growth *in vivo*, and several mathematical models of phage therapy have assumed a direct relationship between phage growth rate and infection clearance (Levin and Bull, 1996, 2004; Payne and Jansen, 2001, 2003).

Yet there are several alternatives to phage fitness as the main determinant of treatment success. First, some experimental evidence supports the view that, when applied in sufficient doses, non-replicating phage have superior treatment properties over phages that grow by reducing the release of endotoxins (Hagens et al., 2004; Matsuda et al., 2005; Westwater et al., 2003). That is, high phage growth rate can harm the host by releasing toxins too quickly. Second, and of greater relevance to the present study, is the demonstration

that treatment of *E. coli* O18:K1:H7-infected mice with endosialidase alone can cure an infection (Mushtaq et al., 2004). Thus, the *in vivo* treatment superiority of K1-dep phages may be partly or largely due to free enzyme released at lysis rather than growth rate per se. Phage assembly is typically imperfect in that not all components are exhausted, so that potentially large numbers of structural components are released as free proteins at lysis. The importance of phage growth rate versus endosialidase to treatment success could be tested by either (i) the comparison of treatment success of a K1-ind and a K1-dep phage with the same growth rate, or (ii) the comparison of treatment success of two phages differing in growth rate but with the same type of tail fiber. For the collection of phages tested here, only (ii) is possible; fitnesses in serum of K1-dep(4) and K1-dep(1) differ by over 5 doublings/h (a 50-fold difference) yet their genomes, and presumably then their mode of infection and development in host cells, are highly similar.

The question remains of how best to identify, a priori, phages useful for therapy. If serum proves to be a suitable alternative to an *in vivo* environment for systemic infections, and if phage growth rate *in vitro* reflects *in vivo* success, then the task is simple. For non-systemic infections, phage growth in, for example, laboratory-generated biofilms may prove useful. At least for human bacterial infections, the success of phage therapy may depend on developing appropriate *in vitro* environments to select for those phages most likely to function well in the environment where the infection is focused.

Methods

Media

LB (10 g NaCl, 10 g Bacto tryptone, 5 g Bacto yeast extract per liter) and 100% bovine serum (Bioreclamation Inc., Nicksville, NY) were used for bacterial growth. The serum was subjected to 0.1 μ filtration and stored in bulk at −80 °C; aliquots were heat treated (55–58 °C for 10–30 min) within 3 h of use.

Strains

Bacteria CAB1 (*E. coli* O18:K1:H7) and CAB281 (a K1-chimera of CAB1 and *E. coli* K12) were used (Bull et al., 2002). Most phages that infect CAB281 also infect CAB1 but do not require the K1 capsule for infection. Phages K1E and K1-5, referred to here as K1-dep(2) and K1-dep(3), respectively, were from the collection of IJM; phages ϕLW and ϕLH were described in Bull et al. (2002), they are referred to here as K1-ind(1) and K1-dep(1), although they have been formally named K1ind1 and K1H, both respectively.

Most phage strains used or screened here were initially isolated by their ability to plate on CAB1 (sometimes on CAB281). Post-sedimentation sewage water was obtained either from Walnut Creek plant, Austin, TX or from the municipal plant in Moscow, ID, USA. Sewage water, with or without chloroform treatment, was brought to 1 M NaCl and 10% PEG, and allowed to precipitate overnight at 4 °C. After centrifugation at 3000 rpm for 15 min, the precipitate was resuspended in LB containing 20% glycerol and stored at −80 °C. Resuspensions were plated directly (without amplification) at 37 °C on CAB1 to obtain isolated phages, or were added to liquid cultures of CAB1 or CAB281 to initiate serial transfer. The only phages used from Idaho were 21 isolates included in the 54 K1-ind isolates tested for growth rate in serum on CAB1; the phage with highest growth rate in this sample, K1-ind(2), was from Austin.

Serial transfer and fitness assays

Serial transfer and fitness assays followed protocols used in previous studies (e.g., Bull et al., 2004; Heineman and Bull, 2007; Heineman et al., 2005). Briefly, cells from a frozen aliquot were added to 10 mL media (125 mL flask, 37 °C, 200 rpm in a water bath), grown for 60 min (LB) or 90 min (serum) to approximately 10^8 cells/mL. Serum as medium yields a more variable density at 90 min than LB at 60 min, and cell density at 90 min in serum was often as low as 5×10^7, but this variation did not greatly affect the outcome of fitness assays.

Fitness assays measure phage growth rate under ideal conditions of unlimited hosts. Phage were added and grown for 30 min or 60 min (with phage density remaining well below cell density), at which time a small aliquot was transferred directly to the next culture, already grown for the requisite time. The transfer volume included phage and live (infected and uninfected) cells to preserve the 'age' distribution of infections. A sample from the completed culture was treated immediately with chloroform and stored. The recipient flask was grown for the same length as the first, and the process repeated as needed. Fitness was estimated from $P_t = P_0\, 2^{Dt}$, where t is measured in hours and D represents doublings/h. P_0 was estimated from the sample of the first culture, P_t was typically estimated at least an hour later.

Essentially the same protocol was used for serial transfer as for fitness assays, except that large populations ($>10^5$ phages) were maintained with serial transfer to enhance mutation input and to avoid stochastic loss of rare types. Phage densities were sometimes allowed to exceed cell densities near the end of a culture. Serial transfer was used to (i) adapt phages to specific growth conditions, and (ii) select the fastest growing phage from a mixture of many phages.

Adsorption assays and one-step growth curves

For most assays, cells were grown in serum for 90 min as for fitness assays, and phage added. Phage and cells were plated on LB agar. Cell density (C) was estimated by plating 1–2 min before phage addition.

Assays to estimate adsorption rates with and without exogenous endo-N were performed in parallel. Cells were grown in 10 mL serum for 75 min, then divided equally into two flasks, one of which received crude endo-N (equivalent to 10^{10} K1-dep(4) pfu/mL). At 90 min, an aliquot of the culture was plated to determine cell concentration, and phage was added to the remainder. Five minutes after phage addition, each culture was diluted 1000× into pre-warmed serum (with or without endo-N, according to the treatment) to halt further infection. Within ten minutes, an aliquot was plated to determine the total phage titer (free and attached phages, P_t), and the supernatant from the same aliquot (centrifuged at 10,000 rpm, 30–60 s) was plated to estimate the free, unadsorbed phage (P_u). The adsorption rate (k) was estimated from $P_u = P_t\, e^{-kCt}$, where t is the duration of adsorption (minutes), and C the cell density from the endo-N-free culture.

One-step growth curves were paired between serum-only and serum + endo-N treatments. Cells grown in serum in a common flask for 90 min were infected with phage (multiplicity of 0.1–0.2) for 5 min, then diluted 1000× or more into serum, with or without endo-N, to reduce further infection. A few minutes later (before any phage release), an aliquot from each dilution was plated to determine the total phage titer (free and attached phages); the supernatant from a centrifuged sample was also plated to estimate unadsorbed phage. Together, these two measures provided an estimate of the density of infective centers. At subsequent times, both before and after the burst, aliquots were plated to determine the titers of combined free phage and infected cells. Burst size is given per infective center.

Detection and overproduction of endo-N

The endo-sialidase activity of endo-N (endo-N-acylneuraminidase) was determined as described in Petter and Vimr (1993). Briefly, 10 μg colominic (polysialic) acid (Sigma) was incubated for 2 h at 37 °C with either phage or recombinant endo-N in PBS. The entire reaction contents (6 μL) were spotted on silica gel thin layer plates

and developed in solvent prior to visualization of degradation products by staining with orcinol reagent.

Recombinant endo-N was overproduced by IPTG-induction of plasmid pEndo-N, which harbors the cloned PK1E endo-sialidase gene, as previously described (Steenbergen and Vimr, 2008). Under these conditions, approximately 2% of the soluble protein is endo-N. Enzyme was released from cells by a combination of freeze-thaw and sonication. The supernatant was used directly at a final concentration equivalence of 10^{10} K1-dep(4) pfu/mL.

Quantification of capsular sialic acid

Overnight cultures of CAB1 started from single colonies were diluted 1:30 into fresh LB at 37 °C and grown to an A600 of about 0.6. Samples of these cultures were then diluted 1:30 into fresh LB or an equal volume of heat-inactivated bovine serum and allowed to grow with aeration for 90 or 120 min in LB and serum, respectively. Cells were then plated on LB medium to determine their concentration in colony forming units/mL.

The relative amount of sialic acid was determined by reverse-phase chromatography of residues derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) essentially as described in Steenbergen et al. (2006). Briefly, cells from 3-mL portions of LB or serum were collected by centrifugation and washed twice with equal volumes of cold PBS before a final wash in water. Pellets from the final wash were resuspended in 0.1 mL of water, and an equal volume of 4 M acetic acid was added prior to hydrolysis for 3 h at 80 °C to release total free sialic acid. Cell debris was removed by centrifugation and the supernatants concentrated 20-fold by vacuum centrifugation. Samples were derivatized with DMB and the modified sialic acid were detected by fluorescence after reverse-phase chromatography. Peak areas (millivolts min^{-1}) were calculated and normalized for cell number.

Sequencing and bioinformatics

Genomic DNA of phage isolates was subjected to 454 pyrosequencing and contig assembly by the University of South Carolina genomics core facility. No attempt was made to establish the physical ends of genomes and the left genome end was arbitrarily set to the first base of the initiation codon for the putative terminase. Orfs and significant BlastP similarities are shown in Table S1; the five genome sequences have been deposited in GenBank with Accession Numbers GU196277–GU196281.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.11.040.

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