Evolutionary Reversals During Viral Adaptation to Alternating Hosts

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ABSTRACT

Experimental adaptation of the bacteriophage ϕX174 to a Salmonella host depressed its ability to grow on the traditional Escherichia host, whereas adaptation to Escherichia did not appreciably affect growth on Salmonella. Continued host switching consistently exhibited this pattern. Growth inhibition on Escherichia resulted from two to three substitutions in the major capsid gene. When these phages were forced to grow again on Escherichia, fitness recovery occurred predominantly by reversions at these same sites, rather than by second-site compensatory changes, the more frequently observed mechanism in most microbial systems. The affected residues lie on the virion surface and they alter attachment efficiency, yet they occur in a region distinct from a putative binding region previously identified from X-ray crystallography. These residues not only experienced high rates of evolution in our experiments, but also exhibited high levels of radical amino acid variation among ϕX174 and its known relatives, consistent with a history of adaptation involving these sites.

VIRUSES differ in the numbers of host species they infect. Some, such as tobacco mosaic virus, infect a range of species. Others, such as poliovirus, infect a single host species. However, phylogenetic evidence suggests that viral host range is historically dynamic, even when each viral strain has a single host. Thus, a group of closely related viruses infects a range of species, yet individual strains are presently confined largely to one or a few host species (Gibbs *et al.* 1995). Such a pattern reflects occasional host shifts intermingled with periods of evolution on single hosts.

Host-range shifts in viruses and the evolutionary consequences of those shifts have implications to human health and agriculture. Recent natural shifts in host range have been the cause of some major epidemics in humans and other animals (Morse 1993; Nathanson *et al.* 1995; Gao *et al.* 1999). In contrast, deliberate shifts in host range have been used in beneficial ways: the most common method of producing attenuated vaccines has been to adapt a virus to novel environments, such as by growing it on cells of a novel host. The resulting adaptation to the new environment often reduces its virulence on the original host, such that the virus no longer causes disease but can still be utilized as an effective vaccine (Fenner and Cairns 1959; Bull 1994; Ebert 1998).

Viral adaptation to novel hosts is an example of the more general evolutionary phenomenon of invasion of and adaptation to a new niche. The new host may present challenges at the level of viral entry into cells, viral replication, or transmission from the host. Only a small minority of the initial pool of viral genotypes may survive these hurdles, but if a population is established on the new host, subsequent adaptation will likely lead to further improvements in the virus. Viral adaptation to the new host may attenuate its ability to grow on the former host and lead to progressive divergence from the ancestral stock, or the new viral population may instead spread across both hosts and displace the ancestral virus.

Despite the long-standing health implications of viral host shifts and their relevance to basic questions in evolutionary biology, only recently have researchers begun to explore the molecular genetics bases and the long-term evolutionary consequences of host switches (*e.g.*, Truyen *et al.* 1996; Parker and Parrish 1997; Speck *et al.* 1997). Here we present a model system to study viral adaptation to multiple hosts. We explore the molecular and evolutionary basis of host-specific adaptation in the bacteriophage ϕX174 by alternately adapting the virus to its typical laboratory host, *Escherichia coli* C, and to a novel host, *Salmonella enterica.* The basis of viral adaptation is studied at a population level and at the molecular level to observe the actual evolutionary process during prolonged adaptation to each of these hosts.

MATERIALS AND METHODS

Phage and host lineages: ϕX174 is a small icosahedral, lytic bacteriophage. The capsid contains a circular, single-stranded DNA genome 5386 bases long, encoding nine essential and two nonessential genes (Sanger *et al.* 1977; reviewed in Tessman and Tessman 1978). A wild-type (wt) isolate of ϕX174 was used as the ancestor for this study, whose sequence differed from the published sequence at five positions [GenBank acces-

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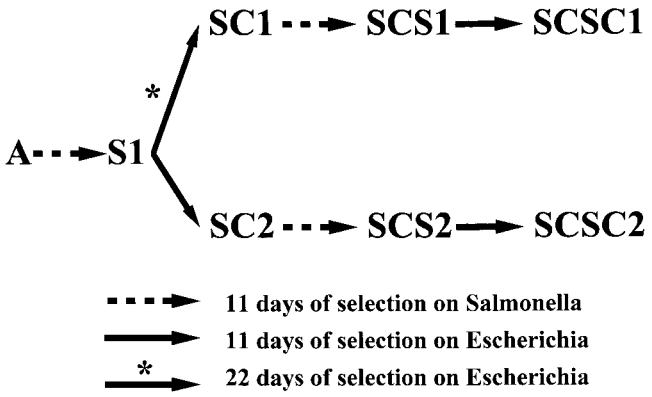

Figure 1.—Experimental design. The temporal and historical relationships of the isolates in the chemostat selection experiment are shown. The ancestral ϕX174 isolate (A) seeded the initial period of chemostat selection on Salmonella. After this initial 11-day selection, a single plaque was isolated from the S-adapted chemostat population (S1). This isolate was then used to seed two replicate lineages grown on Escherichia, resulting in the SC1 and SC2 isolates. Each of these isolates then seeded their own 11-day growths on Salmonella, producing the SCS1 and SCS2 isolates. Finally, the SCS1 and SCS2 isolates seeded their own 11-day growths on Escherichia, producing the SCSC1 and SCSC2 isolates. All selections lasted 11 days except for the S1-SC1 selection on Escherichia (22 days).

sion no. V01128 for the published sequence of Sanger *et al.* (1977); GenBank accession no. AF176027 for the sequence of our ancestor]. Two ϕX174-sensitive hosts were used: *E. coli* C and type I restrictionless (hsd), ϕX174^s *S. enterica* serovar Typhimurium, LT2 strain IJ750 [*xyl-404 metA22 metE551 galE719 trpD2 ilv-452 hsdLT6 hsdSA29 hsdSB121 fla-66 rpsL120 H1-b H2-e nix*] provided by M. M. Susskind to I. J. Molineux as MS3849. Below, we use the abbreviation C for the Escherichia host and S for the Salmonella host.

Chemostat: Phage populations were evolved in a continuous culture, two-chambered chemostat (Bull *et al.* 1997). Media (LB broth: 10 g NaCl, 10 g Bacto Tryptone, and 5 g yeast extract/liter, + 0.1% antifoam B, Sigma A5757) was pumped continuously into the first chamber, which contained cells only. Excess suspension from this cell chamber was drained continuously and unidirectionally into the second chamber, which contained phage. Thus, the phage were presented with a continuous supply of sensitive hosts that had not been exposed previously to phage.

The chemostat apparatus was sterilized and reinoculated with cells from a frozen stock every 2–3 days. The most recent phage sample, treated with chloroform (which kills cells but not phage), was used to inoculate the phage tube. Thus, any bacteria adapted to the chemostat or to the phage were discarded, while phage adaptation accumulated. A flow rate of 6–10 ml/hr resulted in total turnover of the cells and phage in the phage tube of ~100 times/day. Phage samples were collected from the chemostat daily, mixed with chloroform, and saved for future analyses and/or used to reinoculate the chemostat after sterilization.

Experimental design: Phage populations, usually 10^7–10^8, were grown in the chemostat at high temperature (43.5°) on one of the two hosts (Figure 1). After a set period of growth (selection) on a given host, a single plaque isolate was chosen to inoculate the chemostat for the next period of selection on the alternate host. Therefore, we imposed a severe population bottleneck of $N = 1$ at each host switching event (Figure 1).

The experimental design created two replicate lineages in which hosts were related in the following fashion (Figure 1). The wt ϕX174 isolate (A) was initially adapted to S over 11 days of gradually increasing temperature from 38° to 43.5°. A single isolate (S1) of this heat-adapted, S-adapted population was then used as the ancestor for both replicate lineages. In each of the replicate lineages, phage were subjected to three consecutive periods of selection, yielding three serial isolates for each replicate: SC1 → SCS1 → SCSC1 for replicate 1 and SC2 → SCS2 → SCSC2 for replicate 2. The letters in this nomenclature thus match the history of changes in selection. All lineages were grown for 11 days before transfer, except the one leading to SC1 (22 days).

Growth rate assays: In assessing the nature of adaptations, we measured a fitness component, growth rate. Our measure of growth rate is analogous to the intrinsic rate of increase of a population, commonly utilized in population genetic studies. Growth rate assays (GR) were performed over 1 hr, assayed in small (2.5-ml) volumes in 15-ml glass tubes. Host cells were grown in LB batch cultures at 43° with shaking, to a density of $\sim 2 \times 10^8$ cells/ml (measured as Klett = 40). A volume of 0.2 ml of the suspension was added to individual tubes containing 2.5 ml LB. Phage were added to an initial concentration of $\sim 5 \times 10^2$/ml (moi $\approx 10^{-5}$), thereby limiting multiple infection of individual cells throughout most of the assay period. A volume of 0.5 ml of the phage-infected inoculum was removed immediately from the tube, vortexed with chloroform, and titered for a $t = 0$ value. The culture was then shaken (160 rpm) for 60 min in a 43° water bath, when a second ($t = 60$) sample was taken, treated with chloroform, and titered.

Growth rate was quantified as $\log_2$ of the ratio of phage concentration at $t = 60$ divided by the phage concentration at $t = 0$, as in Bull *et al.* (1997). Since ϕX174 attachment rates are enhanced by the presence of calcium, some GR assays were performed with LB media at 0.5 mm $CaCl_2$ to buffer against small fluctuations in calcium concentration in the media. This media supplement increased GR somewhat, so GR assays with supplemental calcium are compared only among themselves, not to assays lacking calcium.

Attachment assays: The rate at which phage attached to cells was measured in LB media with 0.1% antifoam and 0.5 mm $CaCl_2$. Phage and log phase cells were mixed (moi $\approx 10^{-5}$) and a $t = 0$ sample was immediately removed and titered. The culture was then placed in a shaking water bath at 43° (160 rpm) for 5 or 8 min, after which a second sample was centrifuged to remove cells and attached phage. The concentration of unattached phage was titered from the supernatant. Attachment rate was quantified as the coefficient α in the exponential decay process $p(t) = p(0)\, e^{-\alpha t}$, where $p(t)$ is the concentration of unadsorbed phage remaining in the supernatant at t minutes (5 or 8 min), and $p(0)$ is the initial concentration.

Sequence analysis: Complete genome nucleotide sequences were determined for the seven evolved isolates, for the ancestor, and for various site-directed mutations (GenBank accession nos. AF176027–AF176034 for the seven evolved isolates and ancestor sequences). Sequences were obtained from PCR products of viral DNA as template, using ABI 377 automated sequencers either from a core facility at the University of Texas or in the lab of H.A.W. at the University of Idaho. Sequences of the A, S1, SC1, and SC2 isolates have been reported previously (Bull *et al.* 1997); three errors in the reported changes in SC1 and one in SC2 are corrected.

Substitution frequencies: Frequencies of substitutions were analyzed from samples of viral populations by hybridization with radiolabeled oligonucleotides to nylon-immobilized phage DNA, using the protocol in Wichman *et al.* (1999). In the present study, 16 isolates were screened from each daily popu-

lation to estimate frequencies of particular changes. These 16 plaques were taken from plates of daily chemostat samples and stored in individual wells of microtiter plates. Phage from the microtiter plates were replica-plated onto lawns of Salmonella suspended in soft agar on large LB agar plates, grown for 6–8 hr at 43°, yielding a large plaque centered on each well. The agar slabs from these plates were then blotted onto a nylon membrane (Hybond-N^+ membrane) in 0.4 m NaOH, without pretreatment of the membrane or agar. After blotting, phage DNA was UV crosslinked and baked onto the membrane. For each site of interest, a 17-mer oligonucleotide was designed complementary to the viral strand of the ancestral genome sequence, and a second 17-mer was designed complementary to the evolved sequence. Oligos were end-labeled with [γ-^{32}P]ATP (Sambrook *et al.* 1989). Blots were incubated for 30 min at 48° in prehybridization buffer (6 m SSC, 5× Denhardt's, 5% salmon sperm DNA, and 0.3% SDS), which was replaced with hybridization buffer for a 2–8-hr incubation at 48° (3 m tetramethyl ammonium chloride (TMAC), 0.1 m $NaPO_4$, 1 mm EDTA, 5× Denhardt's, 0.6% SDS). TMAC buffer offers the advantage that hybridization properties are determined by oligonucleotide length and are independent of base composition, so that single-base mismatches are easily detected under a standard set of conditions (Ausubel *et al.* 1997). For each hybridization, both oligos were used but only one was radiolabeled (10^6 cpm/ml), and the unlabeled oligo was used in 100-fold excess. After hybridization, blots were washed in 2× SSC solution, wrapped in plastic wrap, and exposed to film. All isolates were screened separately for both evolved and ancestral states of a substitution.

Site-directed mutagenesis: Site-directed mutants were generated by hybridizing mismatched mutagenic oligos (25-mers) to single-stranded ϕX174 DNA and extending this duplex with T4 polymerase [modified from Sambrook *et al.* (1989)]. Resultant DNA was electroporated, and transfectants were screened with radiolabeled oligonucleotides as above. Mutant plaques were identified, replated, and verified by a second round of probing; the entire genome was then sequenced to verify the existence of the targeted change and the absence of any additional changes.

Classification and structural location of amino acid replacements: Location of host-specific substitutions in the ϕX174 F protein were visualized using RasMol v2.6 software and the published coordinates for the ϕX174 pentameric unit containing the F, G, and J proteins [McKenna *et al.* (1994); Protein Data Bank entry 2BPA]. Classification of amino acid replacements was based on the criteria of Miyata *et al.* (1979), who divided amino acids into six groups based on their physicochemical properties of size and hydrophobicity (FYZ, VLIM, HKR, QEND, PAGST, and C). Amino acid replacements within a group were designated "conservative"; replacements between groups were designated "radical."

RESULTS

The phage ϕX174 was adapted alternately between a Salmonella host and an Escherichia host (Figure 1). With one exception, all periods of adaptation to a host lasted 11 days (the exception was a 22-day period). At the end of each period of adaptation on one host, an isolate was obtained for sequencing and growth-rate analysis, and this isolate was also used to initiate the viral population for the next round of adaptation, on the other host. Seven evolved isolates were obtained. In addition, daily samples were retained to study changes in substitution frequencies and in growth properties.

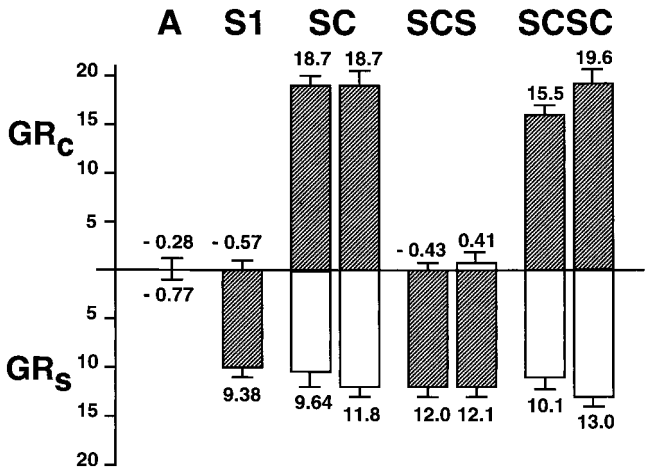

Figure 2.—Asymmetric pattern of host-specific adaptation. Growth rates of the ancestor A and the seven evolved isolates were measured under defined conditions at 43° and expressed as $\log_2$ of phage increase per hour (see Bull *et al.* 1997). A growth rate of 10 is a 1,000-fold phage increase per hour, and a value of 20 a 1,000,000-fold increase. Hatched bars show growth rate on the host of most recent selection, and clear bars show growth rate on the alternative host. Vertical lines represent half of 95% confidence intervals of the mean. Where two bars are present together, replicates 1 and 2 are the left and right bars, respectively. The low growth rate of A predominately reflects its inability to grow at high temperature.

Growth rates of evolved isolates: Adaptation to Salmonella resulted in phages that grew poorly on Escherichia and that were difficult to propagate initially on that host (*e.g.*, in some cases they did not form visible plaques). Yet, adaptation to Escherichia did not measurably affect growth on Salmonella. The fitness component of phage growth rate was measured to quantify this phenomenon. Growth rate was measured as doublings of phage concentration per hour at low phage density, offering a general measure of the phage's ability to grow on a host. The growth rate of the ancestral isolate A was near zero on each host, reflecting the known inhibitory effect of high temperature on ϕX174 (Figure 2; Dowell 1980; Bull *et al.* 1997). After the initial period of selection on Salmonella (S), growth rate on S improved dramatically ($GR_S = 9.38$ for isolate S1, representing a 666-fold expansion of the phage population in 1 hr). All descendants in both lineages maintained at least this magnitude of GR_S, regardless of the host of most recent selection (Figure 2).

Growth rates measured on Escherichia (GR_C) were highly variable among isolates (Figure 2). Isolates from lineages most recently selected on host C had very high growth rates ($GR_C \approx 16$–20; a value of 20 represents a 1,000,000-fold expansion of the phage population). In contrast, isolates from lineages most recently selected on S had growth rates near zero when measured on C. There is thus a marked asymmetry between the conse-

TABLE 1

Summary of nucleotide substitutions for evolved isolates

	S1	SC1	SCS1	SCSC1	SC2	SCS2	SCSC2
Days of adaptation	11	22	11	11	11	11	11
New substitutions	15	28	6	8	15	4	3
Substitutions per day	1.4	1.2	0.55	0.73	1.4	0.36	0.27
Accumulated substitutions	15	27	31	33	24	26	25

Isolates are listed as column headings. The first row is the number of days of adaptation for each lineage. The second row is the number of new substitutions for each isolate accumulated from its most recent ancestral isolate; these values include reversions back to previous states. The third row is the per day rate of new substitutions accumulated from the most recent isolate. The fourth row is the total number of accumulated substitutions per isolate that differ from the original starting ancestral isolate A (hence omits reversions back to the original ancestral state).

quences of adaptation to C and to S, one that persisted both between replicate lines and across multiple host shifts within a line.

Higher viral growth rates were achieved on C than on S. This difference may stem from little more than differences in the hosts' intrinsic abilities to support phage growth, although its cause was not investigated. For example, C grows more rapidly than S at 43°, and rapid host growth is generally supportive of rapid phage growth.

Molecular analysis of evolved isolates: Complete sequences of the ancestor and of all seven evolved isolates revealed 79 substitutions at 55 different nucleotide positions affecting ~1% of the nucleotide sites in the ϕX174 genome (summarized in Table 1). Substitutions occurred in all ϕX174 genes except the short, nonessential gene K. We also observed one 2-base insertion, one 27-base deletion, and relatively large magnitudes of parallelism and reversion. Isolates differed from their most recent ancestor by 3–28 substitutions, averaging 10 new changes per 11 days of selection (Table 1).

The rate of substitution was more rapid during the beginning of the experiment than later. The early isolates S1, SC1, and SC2 all had substitution rates of at least 1.2 nucleotide substitutions per day (S1 = 1.4, SC1 = 1.2, and SC2 = 1.3). The later isolates had rates <0.75 substitutions per day (Table 1; SCS1 = 0.55, SCS2 = 0.36, SCSC1 = 0.73, and SCSC2 = 0.27; *t*-test of equal rates: $P = 0.002$).

Nucleotide reversions associated with host switches: With the exception of the initial period of adaptation to high temperature and to the chemostat, the design manipulates host as the only experimental variable and thus allows identification of nucleotide substitutions responsible for host-specific adaptation. In particular, reversion of an earlier substitution provides an obvious and easily identified candidate, since it is a change that arose during adaptation to one host and subsequently reverted to the previous base during adaptation to the alternative host. Because each period of selection was initiated with a single, plaque-purified isolate, nucleotide reversions necessarily stemmed from *de novo* back-mutations, and were not merely changes in the frequencies of residual polymorphisms maintained over long periods in the chemostat. Reversions were observed at 11 of the 55 sites of substitution (Table 2). Furthermore, some sites showed multiple reversions, switching back and forth with host on several occasions. One nucleotide position (1305) showed a perfect correlation between base and host, evolving back and forth at every host-switching event, and two others (2009 and 2167) showed multiple reversions (Table 2). Interestingly, the substitutions at 2 of these 11 sites were silent with respect to coding regions (Table 2).

Over half of the reversion events occurred in gene F, encoding the major capsid protein. For this reason, and because the capsid protein is believed to have important interactions with the host cell membrane and the host lipopolysaccharide receptor (Hayashi *et al.* 1988; McKenna *et al.* 1994), our molecular analyses focused on these gene F reversion sites.

Population frequencies and growth rate changes during adaptation: Growth rates on C changed dramatically over each period of adaptation to a host. Daily samples of phage were assayed to determine when these changes occurred and which substitutions coincided with the growth-rate changes, focusing chiefly on host-specific changes in gene F (Figure 3). As with the endpoint isolates, there was little change in GR_S across the daily samples. Major changes in GR_C were closely associated with large frequency changes (selective sweeps) of substitutions at some gene F reversion sites. Substitutions at nucleotide site 1305 (amino acid residue F101) were the first gene F changes to arise during adaptation to either host, and selective sweeps at these sites always coincided with major changes in GR_C (Figure 3). Moreover, the A to G reversion at site 1305 seemed a requirement for recovery of growth on C, as this reversion was already present at high frequency in the first populations established on C. In contrast, populations could be readily established on S with a G at site 1305, though all populations grown on S did eventually evolve from

TABLE 2

Nucleotide sites experiencing host-correlated reversions

Site	No.	Gene	A. A.	A	S1	SC1	SCS1	SCSC1	S1	SC2	SCS2	SCSC2
4420	1	A	D → G	a	C	A	A	A	C	C	C	C
4700	1	A, A*	N → K	t	G	T	T	T	G	G	G	G
4805	1	A, A*	T → T	a	a	G	A	A	a	a	a	a
319	1	C	V → F	g	T	G	G	G	T	T	T	T
500	1	D	L → L	g	A	G	G	G	A	A	A	A
921	1	J	R → H	g	g	g	g	g	g	A	G	G
1305	6	F	G → D	g	A	G	A	G	A	G	A	G
1460	1	F	Q → E	c	c	c	G	C	c	c	c	c
2009	4	F	S → T	t	A	T	T	T	A	T	A	T
2093	1	F	L → F	c	c	c	T	C	c	c	c	c
2167	2	F	H → Q	t	G	T	T	T	G	T	T	T

Column 1 shows the site of substitution, column 2 the number of independent reversions occurring at that site, columns 3 and 4 the gene affected and the amino acid replacement, respectively. Column 5 (A) shows the nucleotide at this position in the ancestral genotype. Columns 6–9 and 10–13 show bases in the evolved isolates of replicates 1 and 2, respectively. Isolate S1 is depicted in both columns 6 and 10 since it is the starting isolate for both replicates. All evolved nucleotides are capitalized and nucleotide reversions to any previous nucleotide state are capitalized and underlined. Of the 20 total reversions occurring at the 11 different sites, 14 occurred in gene F, the major capsid gene.

G to A at site 1305. Other gene F substitutions swept through the populations after substitutions at 1305 and often coincided with detectable changes in GR_C (Figure 3).

Effects of specific substitutions on growth rate: The associations between GR_C and changes in the frequency of gene F substitutions shown in Figure 3 could be misleading because they neglect other substitutions that evolved during some of the selections. To more precisely identify the fitness effect of gene F changes, isolates differing only at particular gene F sites were obtained either directly from chemostat populations or were created by site-directed mutagenesis. Site 1305 from gene F was chosen as the primary focus of this analysis because it was the only site to show a perfect correlation with host, because the dynamics results suggested that substitutions at this site had a major impact on GR_C, and because it was always the first (or tied for first) gene F site observed to change on either host. In general, substitutions at site 1305 alone effected major changes in GR_C, consistent with the dynamics patterns (Figure 4). However, the magnitude of this effect differed between the two replicate genetic backgrounds, having stronger effects on GR_C in the genetic backgrounds of replicate 2 isolates than in those from replicate 1. Other sites in gene F were not evaluated as exhaustively as 1305, but some had large effects, in general supporting the patterns observed in the dynamics assay. In particular, whereas the introduction of base G at 1305 in SCS1 resulted in only a 26% recovery (on the log fitness scale), the additional introduction of base C at 2093 increased GR_C recovery to 85% of that observed in the SCSC1 isolate (data not shown).

Attachment rates: Growth rate can be affected by many factors, from viral attachment and DNA entry into the cell, to the production of complete phage particles and their exodus from the cell. One plausible effect of the host-specific changes in F is on attachment rate. Attachment rate was modeled as an exponential decay process (rate α in $e^{-\alpha t}$) and was measured under conditions similar to those of the chemostat and the growth rate assays. All results presented here are for attachment to host C, since little variation was observed in attachment to S (data not shown). The correlation between attachment rate and GR_C of the evolved isolates was almost perfect (0.981): attachment rates of the S-adapted isolates were indistinguishable from $\alpha = 0$, whereas for C-adapted isolates attachment rates varied from $\alpha = 0.75$ to $\alpha = 1.0$ (Figure 5).

Substitutions at site 1305 strongly affected attachment rate (Figure 5). The introduction of A at site 1305 into an otherwise C-adapted lineage nearly abolished attachment (even though GR_C did not decline as much), whereas introduction of G at this site into an otherwise S-adapted lineage restored attachment rates to 40–60% of their final isolate values. The large effect of substitutions at site 1305 on attachment to C provides additional explanation for the highly repeatable initial sweep of reversions at this site during adaptation to C (Figure 3).

Location of host-specific residues in the protein structure: Locations of the amino acid residues from the five host-specific reversion sites in gene F were identified in the published three-dimensional structure of the major capsid protein (McKenna *et al.* 1994). From the outside, the capsid is shaped something like a rugged sphere with 12 mushroom-like spikes protruding from the icosahedral vertices (Figure 6A). Each spike consists of five

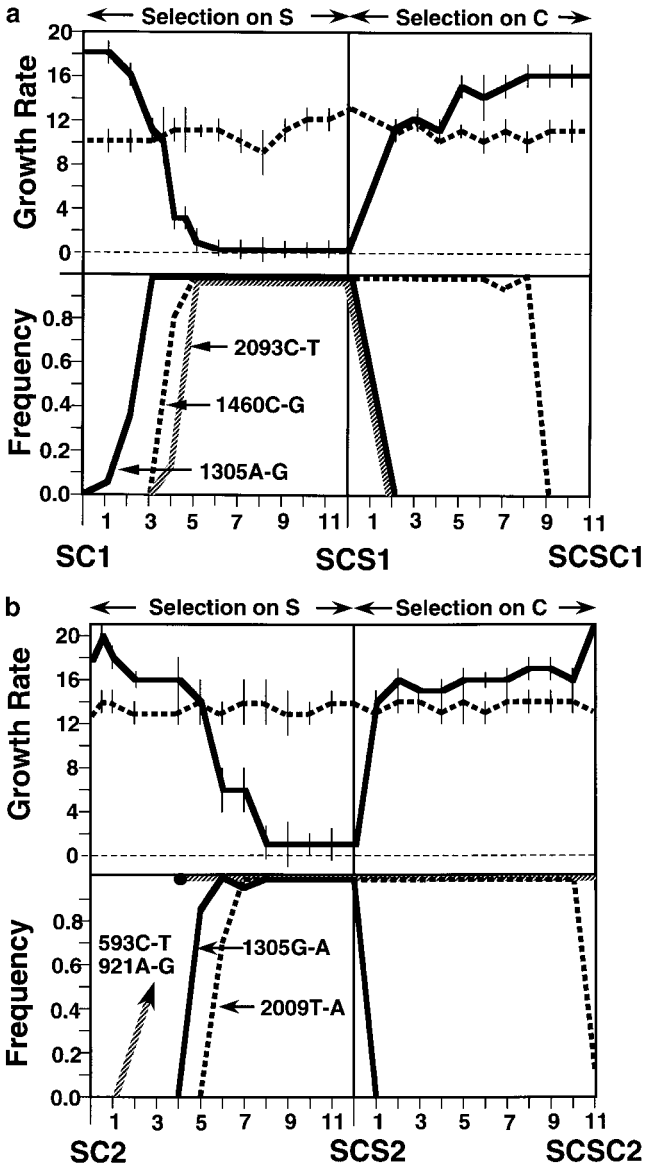

Figure 3.—Selective sweeps of gene F substitutions are correlated with evolutionary changes in growth rate. a is of replicate 1, b is of replicate 2. The top panel shows the growth rates for daily samples. GR_C is shown as a solid line, GR_S is dashed. Error bars represent 95% confidence intervals for the mean. The bottom panel shows the temporal change in the population frequency of the host-specific substitutions that accumulated in gene F. The profile for replicate 2 depicts all observed substitutions in the genome except for a silent substitution in D (site 551) and a 2-bp insertion into the H-A intergenic region during the terminal selection on C (these two changes were undetectable prior to day 10 in the culture consensus sequence, but were present in isolate SCSC2). Replicate 1 evolved three additional substitutions outside of gene F for the interval on S, and four additional substitutions during the terminal selection on C. In b, sites 593 and 921 are depicted with a gap because the specific rate at which they swept through between days 1 and 4 are unknown. The fifth gene F reversion site (2167, Table 2) only evolved during the initial selections on S and C; thus it does not appear here.

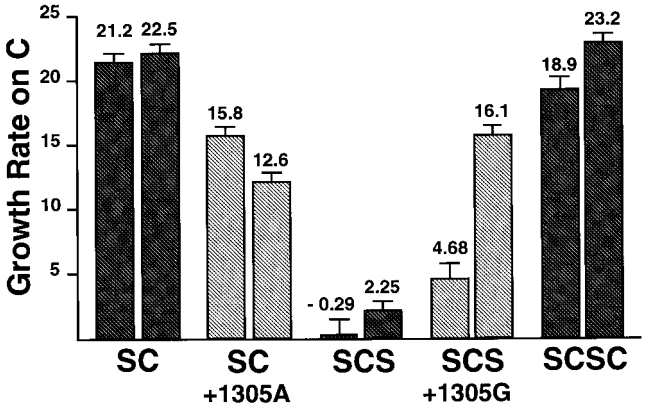

Figure 4.—Effect of substitutions at site 1305 on GR_C. Growth rates were measured on Escherichia for the SC, SCS, and SCSC isolates (dark hatching) and for site-directed mutants differing only at site 1305 (light hatching). For each pair of columns, replicate 1 is the left column, and replicate 2 is the right column. Site-directed mutants (+1305A and +1305G) differ from their respective evolved isolates (immediately to the left) only at site 1305. Vertical lines represent half of 95% confidence intervals of the mean.

copies of G protein surrounding one copy of H protein. Fanning out radially from underneath each spike are five copies of the F protein, forming an irregular disc. This pentameric disc of F proteins emanating from one vertex contacts adjacent discs of F, forming the main shell of the virion. The five host-specific reversion residues in gene F are exposed on the virion's outer surface and cluster near the perimeter of the G proteins, forming a loose ring around the outer edges of each spike (Figure 6B).

The major capsid protein (F) is the most conserved protein among ϕX174 and its related phages. Amino acid alignments of the capsid protein among the published sequences of five isometric bacteriophages (ϕX174, S13, ϕK, α3, and G4) revealed a zone on the protein's outer surface containing a high occurrence of radical amino acid differences among these isolates (Figure 6C). Our five host-specific switching residues not only fall into this region, but all five residues exhibit one or more radical amino acid differences among the five related phage isolates (Figure 6C).

DISCUSSION

Adaptation of the bacteriophage ϕX174 back and forth between two host species led to a consistent asymmetric phenotypic response. Adaptation to Salmonella yielded phage with low growth rates on Escherichia, but adaptation to Escherichia did not appreciably affect growth rates on Salmonella. The asymmetry in adaptation was repeated across multiple host-to-host transfers within a line as well as between replicate lines. Low growth rates were associated with poor rates of attachment to Escherichia cells. Each period of adaptation

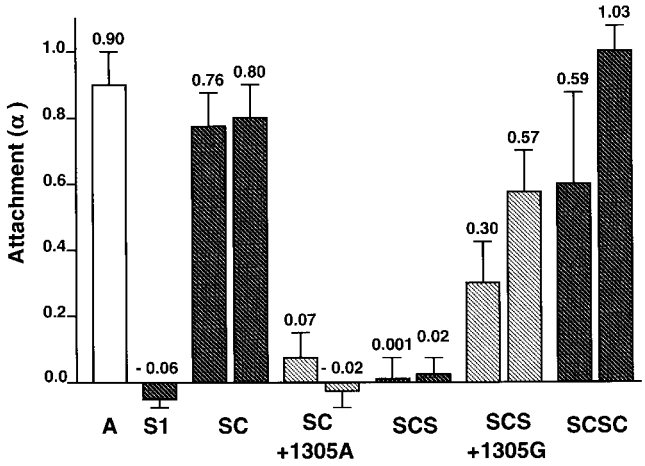

Figure 5.—Attachment rates to Escherichia cells of ancestor, evolved isolate (dark hatching), and site-directed mutants (light hatching). The vertical axis is attachment rate, α ($1 - e^{-\alpha t}$ is the fraction unabsorbed after t minutes). Where bars are paired, replicate 1 is on the left, and replicate 2 is on the right. Vertical lines represent half of 95% confidence intervals of the mean. Site-directed mutants (+1305A and +1305G) differ from their respective evolved isolates (immediately to the left) only at site 1305.

averaged 10 nucleotide changes throughout the ϕX174 genome, yet the ability/inability to grow efficiently on Escherichia was controlled largely by just two to three substitutions in the major capsid gene.

Patterns of protein evolution: Collectively the experiments identified five sites in the major capsid protein (F) and six sites in the remainder of the genome that might contribute to host-specific adaptation, although one site appeared to have the most consistent, large effect (base 1305, residue F101). These sites were identified initially among the many sites evolving in the experimental lineages because they evolved a new base when grown on Salmonella yet reverted in at least one of the lineages grown subsequently on Escherichia. The analysis here focused on the five sites in gene F.

All five host-specific gene F residues are exposed on the virion's surface and circumscribe the spikes. This location is consistent with the observed differences in viral attachment rates among the isolates and fits with early suggestions that the spikes become embedded in the cell wall during attachment (Brown *et al.* 1971; Bayer and Starkey 1972). However, the region demarcated by the five residues (Figure 6B, shown in yellow) is elevated and distinct from a proposed binding cleft (Figure 6B, shown in black) recognized by McKenna *et al.* (1994). The two regions could thus be involved in different steps of attachment, as is known for some tailed phages (Mathews 1977). Previous host-range mutants of ϕX174 have been genetically mapped to genes G and H, as well as F (Sinsheimer 1968; Newbold and Sinsheimer 1970; Weisbeek *et al.* 1973; Dowell *et al.* 1981). Summed across all evolved isolates we observed

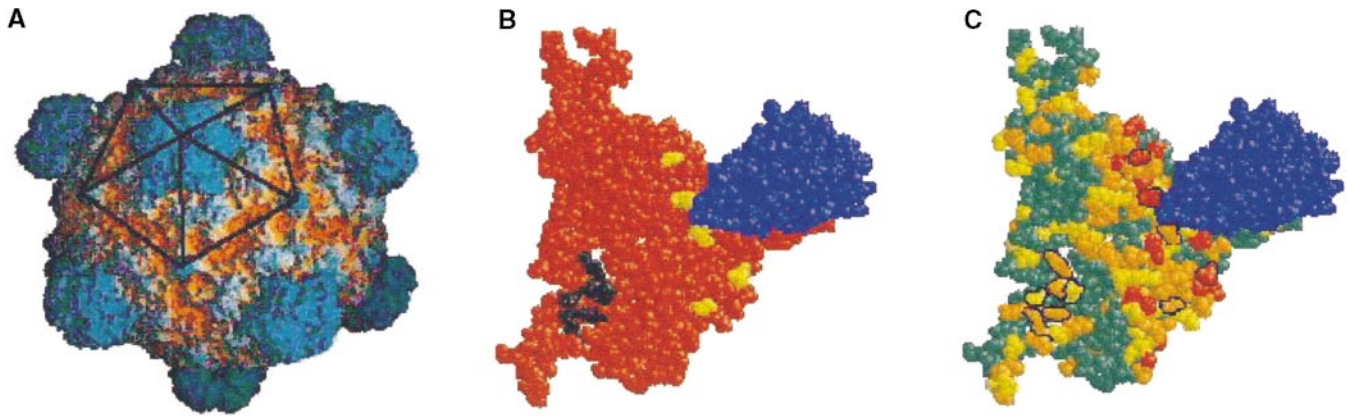

Figure 6.—(A) External view of the ϕX174 virion. The shell of the virion forms an icosahedron, comprised of 12 pentameric units (one is outlined in black). Each pentameric unit consists of five copies of the major capsid protein F, five copies of the major spike protein G, and one of the minor spike protein H (but the structure of H does not resolve in the electron density map). At each vertex, a spike protrudes from the surface, comprised of five copies of G and one copy of H. Of the 12 spikes, 8 are visible here as large blue protrusions. One triangle in the pentameric unit contains a single copy of G and a single copy of F. A is modified with permission from M. G. Rossmann. (B) Space-filling model of a single copy of the F (red) and G (blue) proteins. All five gene F host-specific changes (shown in yellow) affect surface residues in the major capsid protein. The residues in the putative carbohydrate binding site (McKenna *et al.* 1994) are shown in black. (C) All five host-specific F protein residues have undergone radical amino acid replacements within the microviridae. F and G proteins are shown. Residues in F are color coded to indicate the degree of evolutionary conservation among the five published sequences of icosahedral bacteriophage, ϕX174, S13, ϕK, α3, and G4 (GenBank accession nos.: V01128, M14428, X603223, X60322, V00657). Sites that were conserved among all related isolates are shown in green; sites at which only conservative amino acid substitutions were observed are shown in yellow; sites with one radical amino acid substitution are shown in orange, and those with two or more radical substitutions are shown in red. Classification of radical and conserved amino acid differences was taken from Miyata *et al.* (1979; see materials and methods). Reversion sites from this study and the previously identified carbohydrate binding residues are outlined in black.

16 substitutions in gene H and a single substitution in gene G, yet we found no host-correlated reversions in either of these genes in our lineages.

Sequence comparisons among gene F from the five available isolates of isometric phages (ϕX174, S13, ϕK, α3, and G4) revealed that the five host-specific residues identified in this study are located in regions exhibiting unusually high levels of amino acid variation relative to other parts of the protein (Figure 6B). These residues are clustered in the protein's three-dimensional structure, rather than in the linear gene sequence. Nonetheless, F is the most conserved of the 11 proteins in these viruses, and it aligns unambiguously across these five phages, with only ϕK showing any variation in length (four additional amino acids near the carboxy terminus).

From sequence comparisons among species, Goldman *et al.* (1998) estimated a twofold excess in the substitution rate of surface *vs.* internal residues for a wide variety of globular proteins. They proposed that the rate difference resulted from fewer constraints on surface regions than on interior regions of these proteins. Our results likewise reveal more rapid amino acid evolution on the (outer) surface of the major capsid protein, although the surface of a virion is probably not directly comparable to the surface of a globular protein in this context. In the absence of other data, one might assume that this elevated rate of evolution demarcates a region of the virus that is less critical to viral function, and thus less constrained, than the more conserved regions of the virus. However, our experimental results support the hypothesis that this elevated rate of surface residue evolution has been favored by selection (*e.g.*, Andrade *et al.* 1998). Given the demonstrated involvement of this region in host recognition, it is tempting to speculate that the rapidly evolving band of amino acids in protein F surrounding the viral spikes is a signature of past adaptations to different hosts. Of course, a higher rate of adaptive substitution is also compatible with a relaxation of constraints, so the two models are not mutually exclusive.

Rates and patterns of nucleotide evolution: These results parallel those of many experimental adaptations of parasites to *in vitro* propagation, which have shown rapid adaptation to the (novel) culture conditions (Ebert 1998). Our initial period of propagation was novel for ϕX174 in at least three respects: host, high temperature, and growth in a chemostat. ϕX174 was originally isolated on an Escherichia host (Sinsheimer 1959) and is typically propagated on *E. coli* C in the lab. ϕX174 can infect only rough strains of Salmonella (Hayashi *et al.* 1988), and its growth on Salmonella probably represents a novel environment for this virus. Likewise, high temperature is inhibitory to this phage (Godson 1978; Dowell 1980). Since the initial phase of adaptation involved these three novel factors, we would expect the highest rate of adaptation to have occurred early and that many of the later changes were specifically in response to host. To the extent that there are host-by-temperature interactions, the second period of adaptation (S1 → SC) would also have been novel. The per day substitution rate was indeed (significantly) greater in early isolates (S1 and SC) than in late isolates (SCS and SCSC) for both replicates. Substitutions in later isolates were also predominantly more host specific (those at sites in Table 2, as a proportion of the totals in Table 1), but the trend is not strong enough given the limited sample sizes to reject the null model of no difference (in a permutations test).

One surprising feature of the adaptations was that substitutions that evolved on Salmonella often failed to produce a measurable improvement of Salmonella growth rate. How, then, did these substitutions evolve? There are two plausible explanations for this result: (i) the substitutions improved some fitness component other than growth rate, or (ii) growth rate was the only fitness component affected, but the changes in growth rate were too small to be detected in our assays. With respect to the first explanation, growth rate may not capture some aspects of fitness in the chemostat. For technical reasons, and because overall growth on Escherichia was so depressed in Salmonella-adapted phages, our assays measured the fitness component of growth rate: the ability of the phage to reproduce at a low multiplicity of infection (MOI). The chemostat likely maintained relatively high phage/cell ratios, at least during some stages of selection. Turner and Chao (1998) demonstrated elegantly that low-moi assays can misrepresent total fitnesses of viruses evolved at high moi. The second explanation stems from the huge span of fitness represented on our scale. If growth rate is equivalent to fitness in the chemostat, a growth rate increase of only 0.35 on our scale is sufficient to drive a substitution fully across the range of our ability to detect polymorphism in just 24 hr, yet such small fitness increments are statistically undetectable in our growth rate assays. Some substitutions that evolved on S (*e.g.*, the G-A substitution at 1305 during the SC1-SCS1 selection on Salmonella) were polymorphic on 2 days during their sweep on S, consistent with a relatively small benefit.

Evolution of host range in other viruses: A vast literature exists on the genetics of viral host-range variation, which extends to variation in tissue tropism and virulence. Not uncommonly, mutations in coat proteins underlie host-range alterations (see below), although changes in nonstructural proteins are sometimes responsible instead (*e.g.*, Yin and Lomax 1983; Subbarao *et al.* 1993). Tissue specificity in the human immunodeficiency virus (HIV) has received special attention recently because of the association between different stages of infection with tissue tropisms. Infection by HIV-1 requires the cellular CD4 receptor and one of at least four cellular coreceptors (Bieniasz *et al.* 1997). Macrophage-tropic viruses establish the infection and

use the CCR5 coreceptor, whereas the T-cell tropic viruses characteristic of advanced stages use the CXCR4 coreceptor. Coreceptor usage is affected by the V3 loop of the envelope protein. Single amino acid changes in the V3 loop can alter coreceptor usage, but the effect varies with genetic background, and the association in natural isolates of V3 sequence with coreceptor usage is complicated (Bieniasz *et al.* 1997; Speck *et al.* 1997).

The tailed, T-even phages are exceptionally well-studied with respect to the genetics of host range (Montag *et al.* 1987; Hashemolhosseini *et al.* 1994a,b; Kutter *et al.* 1995). Host-range mutations typically reside in hypervariable regions of the tail-fiber genes. These regions appear extraordinarily variable, tolerating changes in length and recombining with divergent tail-fiber genes of other phages, although the structural effects of that variation are unknown. In one remarkable case, host range was restored by a mutation in a phage-encoded chaperone protein that was absent from the mature virion (Hashemolhosseini *et al.* 1994b).

A close parallel to our experimental ϕX174 results lies with the natural evolution of host range in canine parvovirus (Truyen *et al.* 1996; Parker and Parrish 1997). There is no detectable sequence homology between ϕX174 and parvovirus, but the genomes of both are short, single-stranded DNA molecules. Moreover, both virions are tailless icosahedrons, each with 60 copies of the major repeating structural motif, and the major capsid proteins of both viruses contain an eight-stranded β-barrel (McKenna *et al.* 1994; Parker and Parrish 1997). Canine parvovirus first appeared in the late 1970s as a host-range variant of a feline parvovirus, and the host shift appears to have resulted from two to three substitutions in the capsid protein (Parrish *et al.* 1985; Truyen *et al.* 1995). Although the initial dog parvovirus was not infectious for cats, subsequent variants spread worldwide throughout the dog population that had regained the ability to infect cats. No fitness improvements could be detected in dogs for this new variant, either *in vitro* or *in vivo* (Truyen *et al.* 1996). The few substitutions responsible for changes in parvovirus host range map to a similar part of the viral capsid surface as do the host-specific mutations in ϕX174 (Truyen *et al.* 1995, 1996). Results from parvovirus and ϕX174 thus contrast with those of the T-even phages in that the regions affecting host range appear to be much more variable in the tailed viruses.

Asymmetric attenuation: It has often been observed that adaptation to one environment compromises adaptation to other environments (Caspari 1952; Wright 1968; Lenski 1988a; Novella *et al.* 1995; Ebert 1998; though not in Novella *et al.* 1999). One mechanism proposed to explain this principle is antagonistic pleiotropy, *i.e.*, that most substitutions have effects on multiple phenotypes, and those phenotypes optimal in one environment will often not be optimal in other environments. This principle underlies the long-standing practice in virology of producing live, attenuated vaccines by adapting a virus to novel hosts or tissues, thereby diminishing its virulence to humans. In the present study, ϕX174 virtually lost the ability to grow on Escherichia after adaptation to Salmonella. Yet no corresponding loss of growth rate on Salmonella was detected following adaptation to Escherichia (of course, adaptation to Escherichia may have compromised fitness on Salmonella in nonassayed components).

Many different systems have revealed that the detrimental effect of a fixed substitution can be rectified not just by a reversion of that substitution, but also by various changes at one or multiple second sites (compensatory changes: Lenski 1988b; Borman *et al.* 1996; Domingo and Holland 1997; Schrag *et al.* 1997; Bjorkman *et al.* 1998; Burch and Chao 1999; Escarmis *et al.* 1999). Levin *et al.* (1999) discussed the paradox that, when reversions of a single deleterious change would restore full fitness, compensatory changes of lesser benefit often evolved instead. They proposed that compensatory changes as a class arose more often than reversions; hence reversions should consistently ascend only in large populations (see also Johnson *et al.* 1996). Using a bacterial mutation for antibiotic resistance that was deleterious in the absence of antibiotic, Levin *et al.* confirmed that large population size facilitated the evolution of reversions. Importantly, however, an earlier study (using the same system) showed that a reversion was favored only if antibiotic selection was relaxed soon after the first resistance mutation evolved, before second, compensatory changes evolved. Once a compensatory change was also present, the two substitutions acted synergistically on fitness, so that neither substitution was selected to revert (Schrag *et al.* 1997). In ϕX174, initial recovery of growth rate on Escherichia was observed only via reversions of host-specific changes. In contrast to what might have been expected in light of the Schrag *et al.* results, reversions arose even when adaptation to Salmonella proceeded long enough that substitutions accumulated at multiple host-specific sites.

An experimental evolutionary approach, such as that used here, has potential applications for a variety of viral systems where the molecular and mechanistic bases of host-specificity, or variation in viral host range are unknown. Similar approaches could be used to study viral adaptation to various drugs or therapeutic agents. This approach is beginning to be applied to the evolution of drug resistance by examining molecular evolution in the presence, and then in the absence, of a drug [Boucher *et al.* (1993); Borman *et al.* (1996); Schrag *et al.* (1997); Bjorkman *et al.* (1998); these studies examined single cycles of resistance and recovery to a drug]. For example, multiple drug cocktails are commonly used to combat HIV infection. A similar experimental approach, but based on adaptation to alternating drugs instead of hosts, could lead to insights about mechanisms of drug resistance, trade-offs between resistance

to different drugs, and potential approaches for preventing the future development of drug resistance.

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