doi:10.1016/j.jmb.2007.07.064 J. Mol. Biol. (2007) 373, 308–314

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Eliminating the Requirement of an Essential Gene Product in an Already Very Small Virus: Scaffolding Protein B-free øX174, B-free

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Received 23 February 2007; received in revised form 15 June 2007; accepted 25 July 2007
Available online
10 August 2007

Unlike most viral assembly systems, two scaffolding proteins, B and D, mediate bacteriophage øX174 morphogenesis. The external scaffolding protein D is highly ordered in the atomic structure and proper function is very sensitive to mutation. In contrast, the internal scaffolding protein B is relatively unordered and extensive alterations do not eliminate function. Despite this genetic laxity, protein B is absolutely required for virus assembly. Thus, this system, with its complex arrangements of overlapping reading frames, can be regarded as an example of "irreducible complexity." To address the biochemical functions of a dual scaffolding protein system and the evolution of complexity, progressive and targeted genetic selections were employed to lessen and finally eliminate B protein-dependence. The biochemical and genetic bases of adaptation were characterized throughout the analysis that led to the sextuple mutant with a B-independent phenotype, as evaluated by plaque formation in wild-type cells. The primary adaptation appears to be the over-expression of a mutant external scaffolding protein. Progeny production was followed in lysis-resistant cells. The ability to produce infectious virions does not require all six mutations. However, the lag phase before progeny production is shortened as mutations accumulate. The results suggest that the primary function of the internal scaffolding protein may be to lower the critical concentration of the external scaffolding protein needed to nucleate procapsid formation. Moreover, they demonstrate a novel mechanism by which a stringently required gene product can be bypassed, even in a system encoding only eight strictly essential proteins.

Keywords: scaffolding protein; virus assembly; øX174; evolution; irreducible complexity

Edited by M. Gottesman

Introduction

The assembly of large icosahedral viruses is often dependent on a viral scaffolding protein to ensure morphogenetic fidelity and efficiency.[1] With the exception of Microviruses (canonical species øX174), small viruses generally do not require these proteins for assembly. Paradoxically, the Microviruses require two scaffolding proteins, an internal and external species, proteins B and D, respectively. Of very different character, the øX174 external scaffolding protein is highly ordered in the atomic structure and sensitive to mutation,[2–6] while the first ~40% of the internal scaffolding protein is unordered. Tolerant of substitutions, Microvirus B proteins with low sequence homology are cross-functional.[7] Moreover, proteins with N-terminal deletions constituting 44% of the protein retain function.[8] These observations suggest that the external scaffolding protein may be more critical for morphogenesis, as was observed in the two-scaffolding protein bacteriophage P4 system.[9] In contrast, the internal scaffolding protein may be an "efficiency protein" aiding several morphogenetic processes, but not strictly required for any one reaction.

The øX174 morphogenetic pathway is depicted in Figure 1. The first assembly intermediates are 9 S

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Abbreviation used: dsDNA, double-stranded DNA.

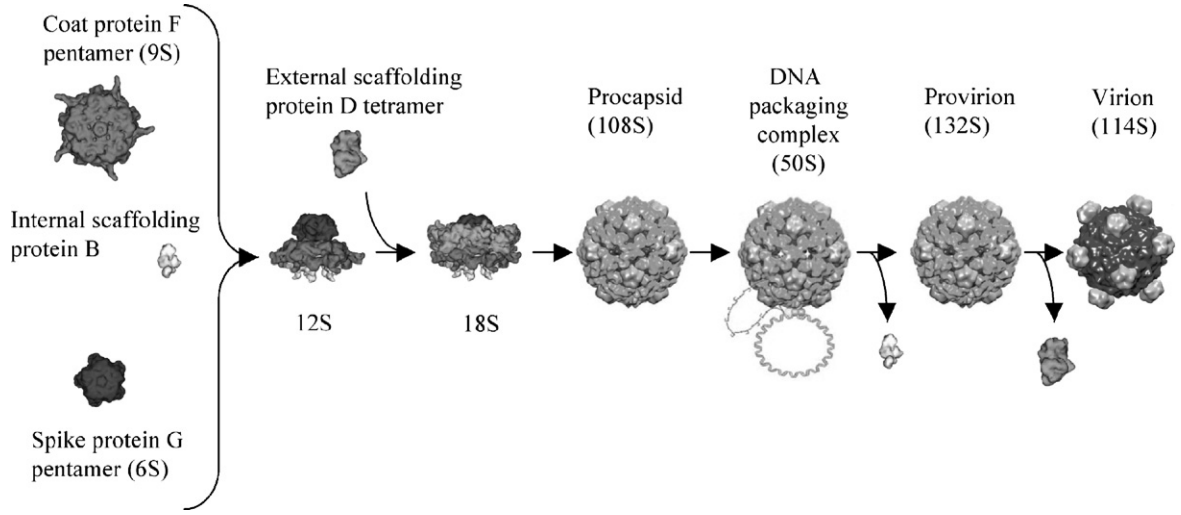

Figure 1. The øX174 assembly pathway.

and 6 S particles, pentamers of coat and major spike proteins, respectively. Five internal scaffolding proteins *via* their C termini, bind to the underside of a 9 S particle.[2,3,10–12] This triggers conformational changes, allowing subsequent interactions with the major spike G and external scaffolding D proteins. The internal scaffolding protein also facilitates, but is not absolutely required, minor spike protein H incorporation.[8] In the absence of the external scaffolding D protein, assembly halts at the formation of 12 S particles. Thus, the external scaffolding protein is responsible for arranging 12 pentameric subunits into procapsids,[13] in which the 12 coat protein pentamers make little or no contact with each other. The entire structure appears to be held together exclusively by external scaffolding protein contacts,[12] which contacts the major spike G as well as the major capsid protein.

If the internal scaffolding protein has evolved into an efficiency protein, playing a subsidiary role to the external scaffolding protein during morphogenesis, an inherent plasticity within the entire system could allow other proteins to compensate for reduced or eventually absent B protein function. A genetic protocol was developed to lessen and finally eliminate the morphogenetic requirement of the internal scaffolding protein. A series of N-terminal deletion B proteins were generated from cloned 5′deletion genes and used to complement the *nullB* (a nonsense mutation in codon 3) strain. Due to the presence of genomic overlapping reading frames, constructing the deletions directly in the genome would have disrupted three gene products. Hence, the cloned gene approach was taken. Since the C terminus is known to mediate most of the known contacts with the viral coat protein,[2,3,10] the N terminus was progressively removed. Proteins with progressively large N-terminal deletions were assayed for complementation on the level of plaque formation. Thus, all strains are capable of completing the entire viral life-cycle. The protein with the largest deletion that failed to complement the *nullB* strain was used to select a mutant strain capable of utilizing it. This mutant was then used for complementation assays with more truncated B proteins, and so on. Gene B contains no alternate start codons or detectable RBS sequences. Residual full-length B protein, perhaps made by nonsense codon read-through, has not been observed in any samples.

Results

As previously reported, internal scaffolding proteins lacking the first 53 of its 120 amino acid residues can support morphogenesis but assembly is very cold-sensitive,[8] and most particles produced at permissive temperatures lack infectivity due to the inefficient incorporation of the minor vertex protein H. At low temperatures, procapsids are not produced. Substitutions in the external scaffolding protein D (*ut*Δ*BD:* utilization of ΔB protein mutation located in protein D) suppress the cold-sensitive assembly defect, but do not appear to increase the efficiency of minor spike protein incorporation. The *ut*Δ*BD* residues make fivefold related interdimer D protein contacts.[2,3] Thus, the molecular effects are likely confined to pentamers, the basic øX174 assembly unit. Here, this strain is referred to as generation 1 (Table 1).

Overcoming defects in minor vertex protein H incorporation and DNA packaging

One-step selections with the generation 1 mutant yielded no mutant capable of utilizing the Δ58B protein, suggesting that multiple events were required. To enrich for multiple mutants, generation 1 phage were propagated in cells expressing the Δ53B gene. The progeny of this enrichment was then plated on cells expressing the Δ58B gene. The complete genomes of two isolates were sequenced and compared to the parental strain. The generation

Table 1. Summary of genotypes, phenotypes and selection conditions

Parental strain		Selected progeny strain	
Genotype and name[a]	Phenotype	Selection[b]	Mutations recovered and hypothesized functions
NullB[c]	Cold-sensitive plaque formation with the ΔB53 protein. At 28 °C, assembly is blocked before the procapsid. At 37 °C, virions and large particles lacking a full complement of protein H[d]	28 °C, ΔB53	*utΔB (D) T46I* or *D47H:* External scaffolding protein mediated conformational switching of coat protein to allow large particle formation.[d]
Generation 1 *nullB/utΔB(D)T46I*	No plaque formation with the ΔB58 protein. Aberrant empty particles produced; procapsids not filled when constructed with the ΔB58 protein (Figure 2(a) and (b))	37 °C, ΔB58[e]	*utΔB(A)N367D:* Mutated packaging protein A allows packaging of DNA in altered procapsids. *utΔB(H)T87A:* Mutations in H protein increases H protein incorporation efficiency.
Generation 2 *nullB, utΔB(D)T46I, utΔB(A)N367, utΔB(H)T87A*	No plaque formation with the ΔB67 protein. Virions are formed with the ΔB67 in lysis-deficient cells after an extended lag phase (Figure 3(a)) No virion formed in the absence of B protein	37 °C, ΔB67	*utΔBP1*: Promoter mutation up-regulating external scaffolding protein expression. Decreases the lag phase before virion formation.
Generation 3 *nullB, utΔB(D)T46I, utΔB(A)N367D, utΔB(H)T87A, utΔBP1*	Progressive cold-sensitive plaque formation with proteins ΔB72 through ΔB97. Produces virions without B protein in lysis-deficient cells, after an extended lag phase (Figure 3(b))	28 °C, ΔB97	*utΔB(F)T144A*, known substitution affecting coat-ex external scaffolding protein interaction s, may increase coat –external scaffolding protein affinity at lower temperature (5).
Generation 4 *nullB, utΔB(D)T46I, utΔB(A)N367D, utΔB(H)T87A utΔBP1, utΔB(F)T144A*	Cold sensitive with the ΔB110 protein. Produces virions in lysis-deficient cells, after an extended lag phase. Forms plaques without B protein in cells over-expressing the external caffolding protein.	28 °C, ΔB110	*utΔBP2*, Promoter mutation up-regulating external scaffolding protein expression.
Generation 5 *nullB, utΔB(D)T46I utΔB(A)N367D, utΔB(H)T87A utΔBP1, utΔB(F)T144A, utΔBP2*	Forms plaques without B protein above 33 °C. B-free øX174, be free!		

[a] Nomenclature: The letter after *utΔB* (*ut*ilizes Δ*B* protein) designates the mutated gene (A, D, H, F) or promoter (P). Letters and numbers designate the wild-type amino acid, residue position, and the substitution, respectively.
[b] Temperature of incubation and the deletion protein expressed for the selection.
[c] The starting strain contained an ochre mutation in the third codon of gene B. In one of the selections, the ochre mutation changed to an opal mutation.
[d] These results have been described.[8]
[e] An enrichment was performed before the selection; see the text for details.

2 mutants had acquired a substitution in the minor vertex protein H, *utΔBH* mutation, which most likely facilitates the incorporation of this protein. The other mutations conferred a substitution in the DNA-synthesizing/packaging protein A, *utΔBA*.

Gene B is fully contained within gene A. In acquiring the *utΔBA* mutation, the ochre mutation in codon 3 was changed to an opal mutation. A mutation in the DNA packaging protein A (*utΔBA*) suggests that the parental generation 1 mutant could assemble with the Δ58B protein into structurally altered procapsids that subsequently cannot interact with the packaging protein A. Regardless of the H protein content of those particles, it should be possible to isolate procapsids or degraded procapsids from infected cells. To test this hypothesis, the assembly intermediates synthesized by the parental generation 1 mutant was examined in cells expressing the Δ58B protein (Figure 2(a) and (b)). Empty capsids were produced. Empty capsids were also the most prevalent particle produced in the generation 2 mutant-infected cells. The production of virions was not high enough to detect by spectroscopy. Therefore each fraction was titered for infectious particles. Although the titer of the generation 2 mutant fractions were an order of magnitude lower than that obtained for the wild-type control, it was three orders of magnitude higher than the generation 1 infection (Figure 2(a)).

The wild-type protein becomes detrimental to the evolved strain

A second phenotype was identified amongst the progeny of the enrichment. These mutants formed very small plaques in the absence of exogenous B protein expression and complementation by the full-length protein lowered plating efficiencies below 10^{-2} at 30 °C. However, these mutants formed large plaques if the Δ58B protein was expressed. The complete genome of two isolates was sequenced. Like the generation 2 mutants, these strains had acquired the *utΔBH* mutation, but they had wild-type A and B genes. The ochre mutation was lost. Thus, the presence of the full-length B protein appears to detrimental. To further investigate this

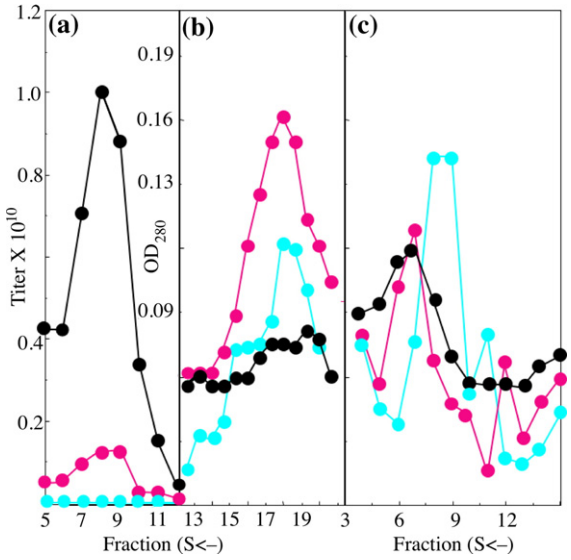

Figure 2. Particles produced by generation 1 and 2 mutants in cells expressing various internal scaffolding proteins. (a) and (b) Infectious virions and empty particles produced by wild-type (black), generation 1 (magenta), and generation 2 (cyan) mutants expressing the Δ58B protein. Particles were separated by sucrose-gradient sedimentation. S← indicates the direction of increasing S values. Data for fractions 5–12 are presented as titer, demonstrating the ability of the generation 2 strain to produce infectious virions with the Δ58B protein, which sediment at 114 S. For fractions 13–21 as A_{280} readings are presented to demonstrate the presence of empty particles, which sediment at 70 S. (c) The *B-damned* phenotype: particles produced by wild-type (black), generation 2 gene B nonsense revertants in cells expressing the Δ58B protein (magenta) and without co-expression of the deletion protein (cyan). A_{280} sedimentation profiles are aligned to each other by the most infectious fraction, which was fraction 7. Note that particle abundance as measured by A_{280}, fractions 8 and 9, does not co-sediment with infectivity in the absence of the Δ58B protein expression.

B-damned phenomenon, particles were characterized from B-*damned* infected cells expressing no exogenous scaffolding protein and cells expressing the Δ58B protein, which rescues the *B-damned* mutant (Figure 2(c)). Without the expression of the Δ58B proteins, the majority of the detected particles sediment slightly slower than infectious particles. The particles in this peak displayed a specific infectivity (plaque forming units/optical density) two orders of magnitude lower than phage produced either in the presence of the Δ58B protein or those contained in the most infectious fraction. At 30 °C in cells expressing the full-length B protein, no large particle was detected in lysates from *B-damned* infected cells (data not shown).

Over-expression of the external scaffolding protein compensates for the loss of internal scaffolding protein function

The generation 3 mutant was selected on cells expressing the Δ67B protein. A mutation (ut*ΔBP1*) in one of the promoters from which the mutant external scaffolding gene is expressed was isolated (Tables 1 and 2). Computational analysis indicates that the substitution creates a better −10 consensus sequence.[14] It does not change the amino acid coding sequence of the overlapping A gene.

The acquisition of a promoter mutation suggests that higher concentrations of the external scaffolding protein, or some other phage product, may be required to nucleate assembly in the absence of protein B. The promoter mutations may expedite attaining this concentration, allowing it to occur before cell lysis. If this hypothesis is correct, the generation 2 mutant should produce progeny with the Δ67B protein. However, the appearance of progeny would be delayed relative to the generation 3 mutant. To test this hypothesis, progeny production was assayed as a function of time in lysis-resistant cells expressing the Δ67B protein.[15] Infections were synchronized so that $T=0$ represents the beginning of penetration. At set intervals, cells were chemically lysed and titers determined (Figure 3(a)). The appearance of wild-type progeny occurs quickly, titers above background are apparent at 10 min. There is almost an hour difference between the production of progeny in generation 2 and generation 3 infected cells. Generation 2 mutants produced no progeny in the absence of B protein expression. The isolation of the generation 4 and generation 5 mutants is discussed below. But, as can be seen in the Figure, the acquisition of further mutations appears to sequentially shorten lag times, which may distinguish between the ability to produce progeny in lysis-deficient cells and plaque formation. Burst size of the all the mutants is 10% of wild-type.

Table 2. Analysis of promoter sequences

Strain	Promoter sequence	Score[a]
WT	ATAGCTTGCAAAATACGTGGCCTTATGGTTACAGTATGCCCATCGCAGTT	0.91
Generation 3	T	0.92
WT	TCTTGTTGACATTTTAAAAGAGCGTGGATTACTATCTGAGTCCGATGCTG	0.99
Generation 5	T	0.99
WT	ATTTTGTTCATGGTAGAGATTCTCTTGTTGACATTTTAAAAGAGCGTGGA	0.48
Generation 5	T	0.89

[a] Promoter sequences were analyzed using the prokaryotic version Neural Network Promoter prediction.[14] Substitutions are given underneath the wild-type (WT) sequences.

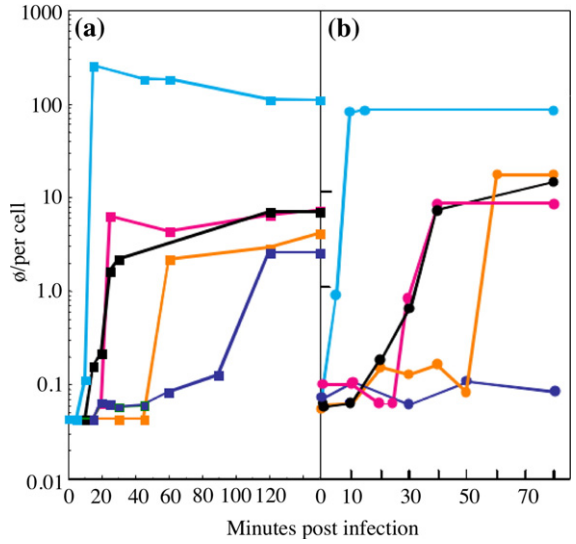

Figure 3. The kinetics of progeny production. Progeny production in lysis-resistant cell lines expressing (a) the Δ67B protein, and (b) cells expressing no B protein. Wild-type, cyan; generation 5, black; generation 4, magenta; generation 3, orange; generation 2, blue.

Coat-external scaffolding protein interactions may further compensate for reduced internal scaffolding protein function

The generation 4 mutant was isolated on cells expressing the Δ97B protein. It acquired a coat protein F mutation (*ut*Δ*BF*), which is one of six known coat protein suppressors of cold assembly external scaffolding protein defects.[5] Located on the outer surface of the capsid, these residues may strengthen coat–external scaffolding protein interactions and may compensate for reduced B protein function. Over-expressing the wild-type D protein, but not other structural proteins, weakly rescues this mutant in the absence of B protein expression (plating efficiency $=10^{-2}$). Rescue is weak probably because the wild-type D protein is over-expressed from the plasmid, not the genome-encoded *ut*Δ*BD* D protein. Wild-type D protein over-expression does not rescue the generation 3 mutant, which does not contain the *ut*Δ*BF* mutation. Although øX174 transcripts are polycistronic,[15] these data indicate that mutant external scaffolding protein over-expression is needed for the B-independent phenotype seen in the next generation.

Further over-expression of the external scaffolding protein produces a full B-independent phenotype

Although the generation 5 mutant was isolated on cells expressing the Δ110B gene, this mutant has a B-independent phenotype, plating with an efficiency of 1.0 without the expression of an exogenous B protein. The generation 5 mutant acquired a mutation in the −35 region of the other external scaffolding gene promoter (*ut*Δ*BP2*). However, computational analysis indicates that the substitution may create a −10 region for a novel promoter.[14] Without the *ut*Δ*BP2* substitution, the predicted promoter is not detected even at the lowest stringency levels. The substitution does not alter protein C, encoded in an overlapping reading frame.

Again, the acquisition of a promoter mutation suggests that one function of the internal scaffolding protein may be to reduce the critical concentration of the external scaffolding protein D needed to nucleate assembly. Progeny production was assayed as a function of time in lysis-resistant cells as described above (Figure 3(b)). The appearance of generation 5 progeny, which contain two promoter mutations, occurs first. The generation 4 and 5 mutants differ by one promoter mutation, and generation 4 progeny production is delayed. The generation 3 and 4 mutants differ only by a mutation at a known coat–external scaffolding protein contact site. Generation 3 mutant progeny appear 50 min post infection. The generation 2 strain, which contains neither the coat protein nor promoter mutations, produced no progeny even 3 h post infection (data not shown). An examination of the soluble proteins from generation 5 infected cells extracts (Figure 4) indicate that the external scaffolding protein D is more abundant relative to structural proteins F, G and H when compared to the wild-type control. There is no evidence of protein B production. Aggregation was crudely examined by analyzing pellets made soluble (data not shown). No dramatic difference was detected between the ratios of phage proteins seen in the pellet and soluble generation 5 and wild-type fractions.

Discussion

The internal and external scaffolding proteins are found in all 47 øX174-like phages characterized to date.[16] Thus, there has been a strong evolutionary

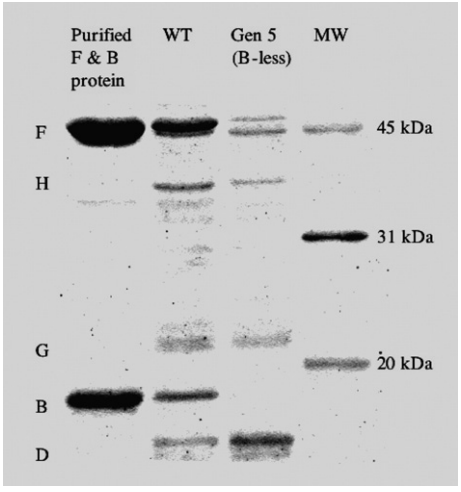

Figure 4. Proteins produced in wild-type and generation 5 infected cells. Proteins are stained with Coomassie brilliant blue.

pressure to maintain the internal scaffolding protein B despite the ability to form infectious particles in its absence. The growth characteristics of the B-free mutant suggest that the primary advantages of the two scaffolding protein system are a higher burst size and a shortening of the lag phase before the appearance of progeny. Four of the six mutations in the B-free strain most likely affect coat–external scaffolding protein interactions to shorten lag phases, allowing progeny to assemble before programmed cell lysis. However, burst sizes are only 10–20% of wild-type, even in lysis-resistant cells. Overcoming the delayed nucleation threshold would need to occur first. Whether the fitness of the B-free strain can be improved by acquiring mutations to elevate the total yield or if lower yields are the consequence of shortening lag phases in the absence of protein B remains to be determined.

In a study unbiased by experimental protocols, over 40 new Microvirus coliphages were isolated and determined to represent approximately 3.0% of the isolated phages.[16] Considering the niche in which the øX174-like viruses must compete, the fast kinetics of the viral infection may be critical to Microvirus persistence within this marauding horde of large double-stranded (dsDNA) phages. The larger genomes of dsDNA phages often encode enzymes and proteins that participate in host cell takeover and/or super-infection exclusion. For example, early in phage T4 infections, early proteins alter host cell transcriptional processes and degrade host cell DNA. While the cessation of host cell gene expression is an obvious result, this would inhibit the replication of a super-infecting phage. Similarly, the inactivation of host cell RNA polymerases by early phage T7 proteins would have similar effects. Such evolutionary mechanisms may have allowed dsDNA phages to evolve more elaborate temporal gene expression and delay actual progeny production, which would occur after late gene expression, usually 10–15 min after penetration. Microviruses have evolved neither elaborate temporal gene expression nor particularly effective super-infection exclusion mechanisms. Thus, progeny production may have to be rapid. Infectious progeny can be detected at least as early as 5 min after penetration, a time when many dsDNA phages may just be making the transition to middle or late gene expression.

Evolutionary studies with the Microviruses suggest that the external scaffolding protein, or its most recent incarnation, may be the newest of the Microvirus genes.[16] This gene is not found in the Gokushoviruses, a subfamily of the Microviridae, in which there appears to be a deep evolutionary divide. The øX174-like phages, or Microviruses, infect free-living bacteria. The Gokushoviruses, represented by the Chlamydiaphage CHP2,[17] infect obligate intracellular bacteria, in which very few dsDNA phages have been isolated. In fact, all six known phages of Chlamydia are Gokushoviruses. It is tempting to speculate that the Gokushoviruses, like the Coelacanths, have persisted by occupying a niche free of competition from more modern phage species. Conversely, a Microvirus may have entered this unoccupied niche. No longer needing a kinetically fast assembly pathway, it lost one of its scaffolding proteins. Regardless of the precise evolutionary history of the Microviridae, our results illustrate a novel mechanism by which a stringently required gene product can be bypassed, address the evolution of complexity, and demonstrate the inherent ability of even the smallest organisms to adapt, albeit in measured steps, to an overall large selective pressure.

Materials and Methods

Bacterial cells and phage

Cell growth and phage plating protocols have been described.[11] The cloning and expression of the ΔB constructs were as described.[8] For the growth curve experiments, *slyD* cells, which are resistant to øX174 E protein-mediated lysis,[15] were grown to a concentration of 1.0×10^8 cells/ml. Cells were concentrated, washed twice, and resuspended in HFB buffer[11] containing 10 mM $MgCl_2$ and 5.0 mM $CaCl_2$, a starvation buffer, at a concentration of 1.0×10^9 cells/ml. Cells were infected at a multiplicity of infection of 3.0 and infections were incubated at 35 °C for 15 min to allow pre-adsorption. At time zero, infections were diluted 1/100 (v/v) with TK medium. Thus $t=0$ marks the beginning of penetration. At the indicated time-points, aliquots were removed and diluted 1/100 (v/v) with iced HFB buffer containing 5.0 mg/ml of T4 lysozyme, and titered for infectious virions in cells expressing the Δ58B protein.

Sedimentation and protein analyses

Sucrose gradient sedimentation and PAGE were as described.[6] To generate the sedimentation profiles, 5.0 ml 5%–30% sucrose gradients were separated into approximately 50 fractions. Particles were detected either by direct plating assays (infectious virions) or spectroscopy (non-infectious particles).

Acknowledgements

This research was supported by the National Science Foundation (MCB 054297 to B. A. F.). The authors thank Drs H. A. Wichman and D. Rokyta for critical comments and independently verifying the genome sequence of the B-independent strain. The authors thank Dr Gisela Mosig for many years of discussion and encouraging us to generate the B-free mutant.

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