APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2004, p. 4840–4847 Vol. 70, No. 8
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.8.4840–4847.2004

Protein Folding Failure Sets High-Temperature Limit on Growth of Phage P22 in *Salmonella enterica* Serovar Typhimurium

Welkin H. Pope,[1,2] Cameron Haase-Pettingell,[1] and Jonathan King[1]*

Massachusetts Institute of Technology, Cambridge,[1] and Woods Hole Oceanographic Institution, Woods Hole,[2] Massachusetts

Received 16 January 2004/Accepted 19 April 2004

The high-temperature limit for growth of microorganisms differs greatly depending on their species and habitat. The importance of an organism's ability to manage thermal stress is reflected in the ubiquitous distribution of the heat shock chaperones. Although many chaperones function to reduce protein folding defects, it has been difficult to identify the specific protein folding pathways that set the high-temperature limit of growth for a given microorganism. We have investigated this for a simple system, phage P22 infection of *Salmonella enterica* serovar Typhimurium. Production of infectious particles exhibited a broad maximum of 150 phage per cell when host cells were grown at between 30 and 39°C in minimal medium. Production of infectious phage declined sharply in the range of 40 to 41°C, and at 42°C, production had fallen to less than 1% of the maximum rate. The host cells maintained optimal division rates at these temperatures. The decrease in phage infectivity was steeper than the loss of physical particles, suggesting that noninfectious particles were formed at higher temperatures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a decrease in the tailspike adhesins assembled on phage particles purified from cultures incubated at higher temperatures. The infectivity of these particles was restored by in vitro incubation with soluble tailspike trimers. Examination of tailspike folding and assembly in lysates of phage-infected cells confirmed that the fraction of polypeptide chains able to reach the native state in vivo decreased with increasing temperature, indicating a thermal folding defect rather than a particle assembly defect. Thus, we believe that the folding pathway of the tailspike adhesin sets the high-temperature limit for P22 formation in *Salmonella* serovar Typhimurium.

Microorganisms have been detected in almost every climate on Earth, from the extreme heat and pressure of hydrothermal vents to the exposed rocks of frozen Antarctica. However, optimal growth is generally limited to a defined temperature regime characteristic of the habitat. Examination of the growth rates of microorganisms with respect to temperature reveals a clear pattern. The majority of organisms exhibit growth rates that slowly increase with rising temperature until a maximum growth rate is achieved. They then exhibit a sharp decline once the optimal temperature has been exceeded (24, 25). Many factors that contribute to the decline of the growth rate at higher-than-optimal temperatures have been identified, including lipid membrane stability (4, 33), rates of DNA synthesis and repair (10, 32), rate of protein synthesis (11), and protein misfolding (18, 28). However, it has generally been difficult to determine which factor sets the high-temperature growth limit. Environmental temperatures around the globe are increasing far more rapidly than documented rates of evolutionary change (see http://lwf.ncdc.noaa.gov/img/climate/research/anomalies/triad_pg.gif). Thus, it is becoming increasingly important to understand the processes that contribute to thermal limitation of growth.

A significant part of the thermal stress response is the induction of heat shock chaperones (12). These specialized proteins are capable of binding some species of misfolded and aggregated polypeptide chains, in some cases shifting folding from an unproductive aggregation pathway to the productive native pathway. However, as the temperature increases, chaperones become less capable of buffering the cell against the accumulation of misfolded proteins (12, 13, 24). Phage proteins, like cellular proteins, may or may not be recognized by the host's heat shock proteins. Temperature-sensitive-folding (*tsf*) mutants of the coat protein of phage P22 are rescued by overexpression of the GroEL and GroES chaperones (16, 17). However, P22 phage with similar *tsf* mutations in the tailspike do not appear to be aided by the GroEL/ES chaperone (8, 17). Some phage possess their own chaperones, which after infection are synthesized and provide nascent peptide chains with some measure of protection against aggregation induced by high intracellular protein concentrations and excessive heat (40). In *Escherichia coli*, phages T4 and λ both possess their own chaperones (23, 43). The ability of heat shock proteins to alleviate the aggregation of some polypeptide chains and not others increases the difficulty of determining which substrate pathway sets the growth limit at high temperatures.

Elucidation of the limiting process during high-temperature growth with respect to the folding of proteins cannot be attained by examination of the properties of mature proteins, since the properties of folding peptide chains are quite different from those of native folds. For example, the highly thermostable tailspike adhesin of phage P22 exhibits thermolabile folding intermediates (labeled "partially folded monomer" in Fig. 1A) (29, 39).

We have examined the issue of growth limitation at high temperature for *Salmonella enterica* serovar Typhimurium infected by phage P22. This model system has several characteristics that make it particularly appropriate for the study of this problem. The assembly of structural proteins of P22 has been

* Corresponding author. Mailing address: 77 Massachusetts Ave. 68-330, Cambridge, MA 02139. Phone: (617) 253-4700. Fax: (617) 252-1843. E-mail: jaking@mit.edu.

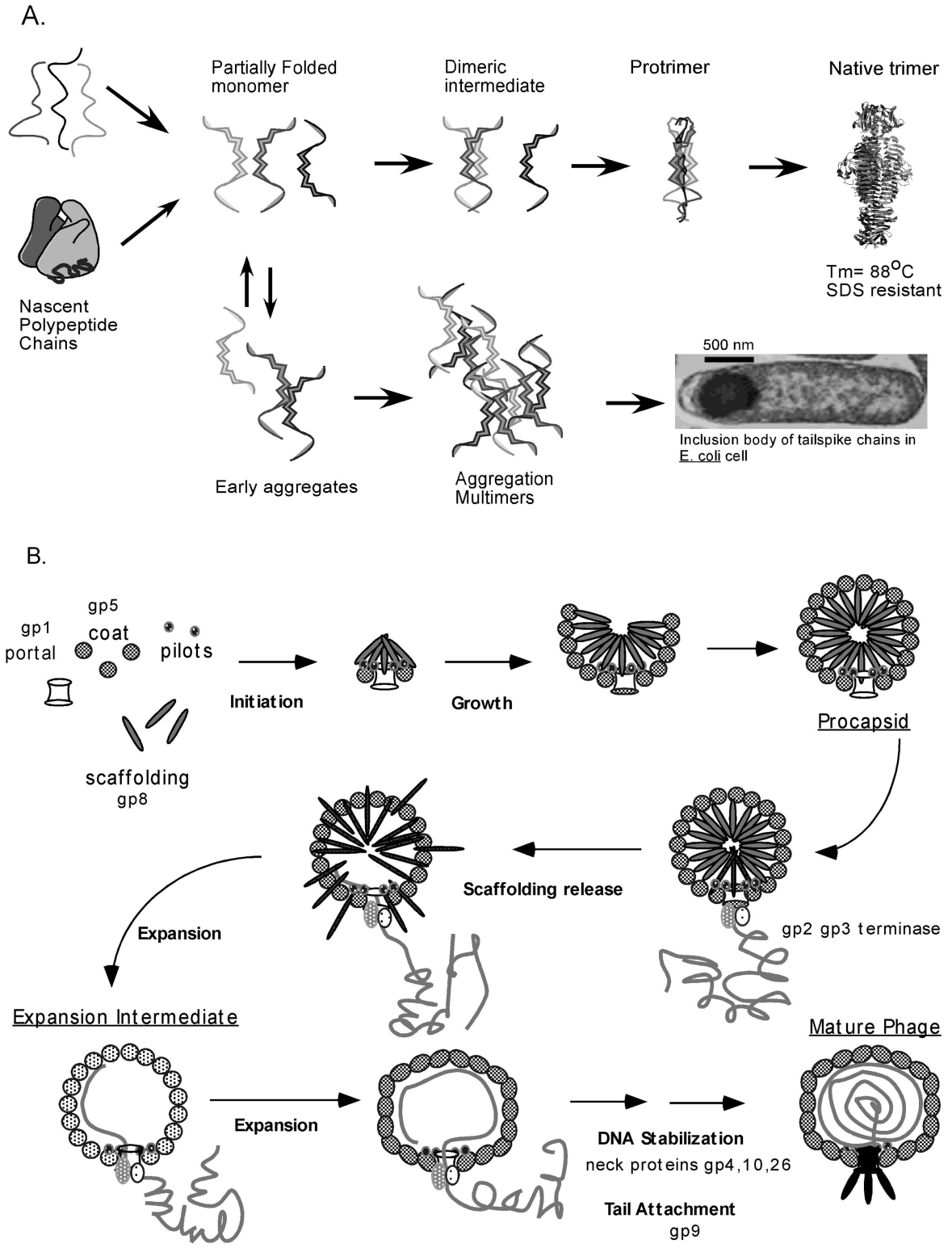

FIG. 1. Folding and assembly of the P22 tailspike and assembly of P22 virions. (A) Unfolded or nascent polypeptide chains proceed to some partially folded monomeric state. On the productive folding pathway, two such chains associate to form a partially folded dimer. The addition of a third chain creates a protrimer intermediate whose chains are associated but not fully folded. The protrimer undergoes chain rearrangement resulting in the native structure. At higher temperatures, the partially folded monomeric species is perturbed, preventing the formation of the productive dimer and allowing the formation of multimeric aggregates. These large aggregates form inclusion bodies within the cell. All species of tailspike, with the exception of the native trimer, are sensitive to SDS. Tm, thermal denaturation midpoint temperature. (B) After synthesis, the coat, scaffolding, portal, and pilot subunits form a nucleus of the procapsid shell. Coat and scaffolding subunits rapidly add on to the nucleus, forming the completed procapsid. Viral DNA, with the help of packaging proteins, is driven into the procapsid, and the scaffolding protein is ejected through the pores of the procapsid shell. The procapsid undergoes an irreversible expansion to the mature capsid, while DNA is pumped into the procapsid. After the encapsulation of the phage DNA, the neck and tailspike proteins attach to form the infectious mature virion.

well characterized (Fig. 1B). During the first stage, coat and scaffolding subunits and auxiliary proteins bind together to form a spherical procapsid. Phage DNA then enters the head through the portal complex, while the scaffolding protein exits the procapsid via pores in the shell. The DNA-filled procapsid undergoes an irreversible conformational change, expanding to the familiar icosahedral shape.

After attachment of the neck proteins, the tailspike trimer binds irreversibly to the capsid, conferring infectivity (26). The lateral surface of the tailspike recognizes and binds to the *Salmonella* lipopolysaccharide (LPS) projecting from the host cell surface (37). The intermediates in tailspike folding and assembly have been characterized (Fig. 1A). The tailspike adhesin, which has endorhamnosidase activity, binds the LPS in a cleft between two loop domains and cleaves it between rhamnose and galactose moieties (2, 27, 37).

Native tailspike homotrimers exhibit resistance to denaturation by sodium dodecyl sulfate (SDS) detergent, proteases, and temperatures up to 88°C, denaturing only in the presence of SDS at high temperatures. However, the tailspike folding process has been shown to be highly heat labile (15, 22, 36), resulting in the shifting of chains off the productive pathway at high temperatures to accumulate as inclusion bodies (Fig. 1A). These two properties, high thermostability of the native structure and thermolability of folding intermediates, make it possible to monitor the intracellular folding and assembly of the tailspike.

Early observations of P22 particles produced at high temperatures revealed a deficiency in the quantity of tailspike adhesins attached to phage heads (26). The in vivo and in vitro folding process of the tailspike's parallel β-helix motif has been demonstrated to be highly thermolabile, with significant loss of native tailspike protein beginning at temperatures as low as 35°C (22). This suggested that the loss of infectious particles at the high end of P22's physiological temperature range was due to the misfolding of the tailspike adhesin and its subsequent inability to assemble onto fully formed P22 heads.

MATERIALS AND METHODS

Salmonella serovar Typhimurium strain DB7155 [*sup*$^+$ *hisC525*(Am) *leuA414*(Am) SupE] was grown in M9 minimal medium supplemented with glucose (0.4%), yeast extract (0.01%), $MgSO_4$ (1 mM), $FeCl_3$ (1 μM), and $CaCl_2$ (1 μM); strain DB7136 [*hisC525*(Am) *leuA414*(Am)] was grown in the same medium additionally supplemented with histidine (0.0015%) and leucine (0.0015%). P22 strains 13_{H101}(Am) $C_1$7 (hereinafter referred to as P22) and 2_{H200}(Am)/13_{H101}(Am) $C_1$7 (hereinafter referred to as P22 2$^-$) were used. The amber mutation in gene 13 prolongs lysis of host cells, while the $C_1$7 mutation prevents lysogeny. The gene 2 amber mutation prevents packaging of DNA into newly formed capsids and upregulates tailspike synthesis (1).

Growth curves. An overnight (O/N) culture of strain DB7155 was inoculated (1:50) into minimal medium at 30, 37, 39, 40, 41, 42, or 43°C and was continuously aerated during incubation. Samples were withdrawn over a 6-h period, serially diluted with dilution fluid (tryptone [1%], NaCl [0.7%], and $MgSO_4$ [2 mM]), and plated on Luria-Bertani (LB) agar (1.2%) medium at 30°C. After O/N incubation, colonies were counted manually.

Burst size. An O/N culture of strain DB7155 was inoculated (1:50) ratio into medium at 37°C and were grown with constant aeration to a concentration of 2×10^8 cells/ml. Phage was added at a multiplicity of infection (MOI) of 10. After a 10-min adsorption period, infected cells were shifted to 30, 37, 39, 40, 41, 42, or 43°C. After cell lysis, which was evidenced by a significant loss of turbidity, phage were serially diluted in dilution fluid, mixed with soft LB agar and a few drops of plating bacteria, and plated on LB agar (1.2%) at 30°C. After O/N incubation, plaques were counted manually. Each titer was confirmed by three replicate experiments.

High-temperature P22 production and purification. An O/N culture of strain DB7136 (*sup*$^-$) was inoculated (1:10) into medium at 37°C and grown with constant aeration to a concentration of 2×10^8 cells/ml. Phage were added to the culture at an MOI of 10. After a 10-min adsorption period, the infected cells were moved to 30, 37, 39, 40, 41, 42, or 43°C and incubated for 2 or 3 h, depending on the length of time required to lyse strain DB7155 at the same temperature. Cells were harvested by centrifugation at $5,000 \times g$ for 10 min and then resuspended in 1 ml of 50 mM Tris (pH 7.6)–100 mM $MgCl_2$. Cells were lysed with $CHCl_3$, and DNase I and phenylmethylsulfonyl fluoride were added to the lysates. Phage were pelleted by a low-speed spin at $8,000 \times g$ overnight and then resuspended at 4°C in 50 mM Tris-HCl (pH 7.6)–100 mM $MgCl_2$. A 1-ml volume of each lysate was layered over a 16-ml CsCl step gradient (ρ = 1.3, 1.4, 1.5, and 1.7) and ultracentrifuged at 4°C and 28,000 rpm in an SW 28.1 rotor for 4 h. A milky-white phage-containing band was observed between ρ = 1.4 and ρ = 1.5. A 1-ml volume of purified phage preparation was harvested and, using a Pierce dialysis cassette, dialyzed against two changes of 100 mM Tris (pH 7.6)–50 mM $MgCl_2$.

Tailing assay. A 50-μl volume of purified P22 particle preparation (obtained as outlined above) was mixed with 50 μl (250 ng) of purified tailspike solution (purified as outlined by Haase-Pettingell et al. [20]) or 50 μl of dilution fluid and incubated at room temperature for 60 min. A 900-μl volume of dilution fluid was added. Samples were then serially diluted and plated for determination of PFU at 30°C. Each titer was confirmed by three replicate experiments.

SDS-polyacrylamide gel electrophoresis (PAGE) quantification. Purified P22 samples were boiled in SDS buffer and loaded onto a 10% acrylamide gel containing SDS. The gels were electrophoresed at a constant 20 mA until the dye front ran off the end of the gel. Gels were silver stained, and tailspike and portal bands were quantified using ImageQuant software (Molecular Dynamics).

DNA-packaging-deficient P22 lysate production and analysis. A 100-ml volume of minimal medium supplemented with histidine (0.0015%) and leucine (0.0015%) was inoculated with 5 ml of a strain DB7136 culture. Once the culture density reached $\sim 2 \times 10^8$ cells/ml, P22 2$^-$ phage were added at an MOI of 10. Phage were allowed to adsorb for 10 min, and then 10-ml volumes of the culture were shifted to each of the following temperatures: 30, 37, 39, 40, 41, 42, and 43°C. Cultures were incubated for 3 h, and then cells were harvested by centrifugation in an SS-34 rotor for 10 min at 7,000 rpm and 4°C. Cells were resuspended in 600 μl of lysis buffer B (50 mM Tris-HCl [pH 8], 25 mM NaCl, 2 mM EDTA, 0.1% Triton-X) and frozen. Once thawed, samples were sonicated with a Microson XL benchtop sonicator (Misonix) for 30 s each to disrupt DNA and any cells remaining after the freeze-thaw step. SDS sample buffer was added, and samples were electrophoresed at a constant 20 mA through a 10% acrylamide gel containing SDS until the dye front ran off the gel. The gel was stained with Coomassie blue stain, and bands were quantified using the software ImageQuant version 1.2 (Molecular Dynamics).

RESULTS

Effects of temperature on growth of *Salmonella* serovar Typhimurium and production of P22. Yields of infectious particles of P22 [13_{H101}(Am) $C_1$7] phage have been reported to decrease with increasing temperature above a plateau range of 30 to 39°C (26). This might reflect breakdown of heat-sensitive biosynthetic processes within the host cells. Comparison of the growth rates of the *Salmonella* serovar Typhimurium host cells and phage P22 particles produced was performed at 30, 37, 39, 40, 41, 42, and 43°C.

The upper thermal limit of vegetative growth for *Salmonella* serovar Typhimurium in this experiment was 42°C. The temperature regime of P22 production did not parallel the range favored by its host but, rather, sharply declined at 40°C, 3°C lower than the temperature at which *Salmonella* serovar Typhimurium growth became undetectable. As seen in Fig. 2, the optimal growth rate of *Salmonella* serovar Typhimurium was at 37°C. At this temperature, the burst size of P22 was approximately 150 phage/cell. At 40 and 41°C, the host's growth rate was approximately 95% of the maximum growth rate. In contrast, at 40°C, P22 PFU decreased 10-fold, while at 41°C a 100-fold decrease in PFU was observed.

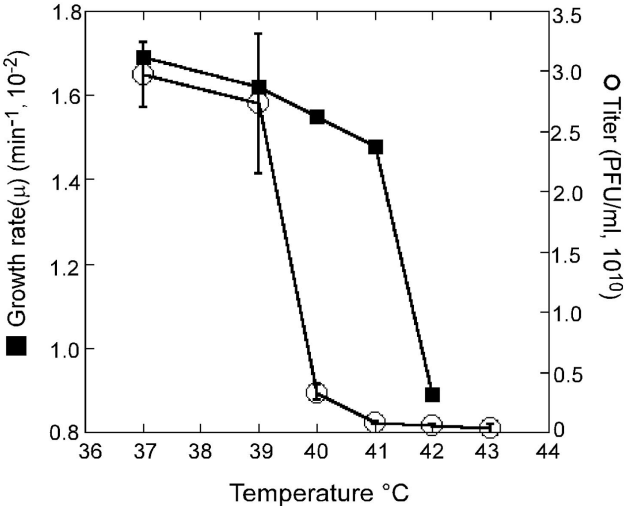

FIG. 2. *Salmonella* serovar Typhimurium growth rate and P22 titer versus temperature. The growth rate of *Salmonella* serovar Typhimurium at selected temperatures (closed squares) was monitored by enumeration of CFU. Growth rate (μ) was determined by applying the growth equation $N(t) = N_0e^{\mu t}$, where N_0 is the number of cells at time $(t) = 0$, N is the number of cells at time $(t) = t$, and solving for μ. R^2 values were all above 0.93, indicating a good fit for the regression. The titer of P22 infectious particles (open circles) was determined by plaque assay. Error bars reflect the standard error.

This result indicated that the observed deficit in phage production was not due to the thermally induced failure of some host process but rather resulted from the loss of an essential phage process, such as transcription, replication, protein production, DNA packaging, or virion assembly. As the initial steps of infection occurred at 37°C, with a subsequent shift to a target temperature, the loss of infectious particles at high temperatures was not due to a defect in the infection-absorption process.

Examination of phage formed at high temperatures. To identify the thermolabile process causing the loss of infectious particles at higher temperatures, a closer examination of particles formed at all temperatures was undertaken. Exponentially growing cultures at 37°C were infected with phage at an MOI of 10. After phage absorption, the cultures were shifted to 30, 37, 39, 40, 41, 42, or 43°C. Infected cells were collected by low-speed centrifugation and lysed, and phage particles were then purified by use of CsCl step gradients.

Figure 3a shows the results of SDS gel electrophoresis of purified particles. Coat protein, seen at a position corresponding to a molecular mass of 45 kDa, dominated the pattern. Also resolved were the products of gene 1 (portal protein), gene 9 (tailspike adhesin), and genes 16 and 20 (DNA injection proteins) (35). The ratio of these structural proteins was relatively constant at lower temperatures. However, samples produced from lysates of infected cells incubated at high temperatures exhibited decreased intensity of the tailspike band. Tailspike binding, the last step in phage assembly, yields from zero to six trimers per head (6, 26). To measure this process with greater sensitivity, the amount of tailspike per DNA-containing phage head was assessed.

The ring of P22 portal protein is required for DNA packaging and is present in the constant proportion of 12 protein subunits per DNA-containing phage head (3). Thus, the quantity of portal protein is directly proportional to the number of DNA-containing phage heads in the sample. The ratio of the amount of tailspike chains to the amount of portal protein chains, seen at 96 kDa, in each sample was determined. This ratio of tailspike chains to portal chains decreased as the temperature at which infection occurred increased, indicating that fewer tailspike adhesins were attached to the DNA-containing

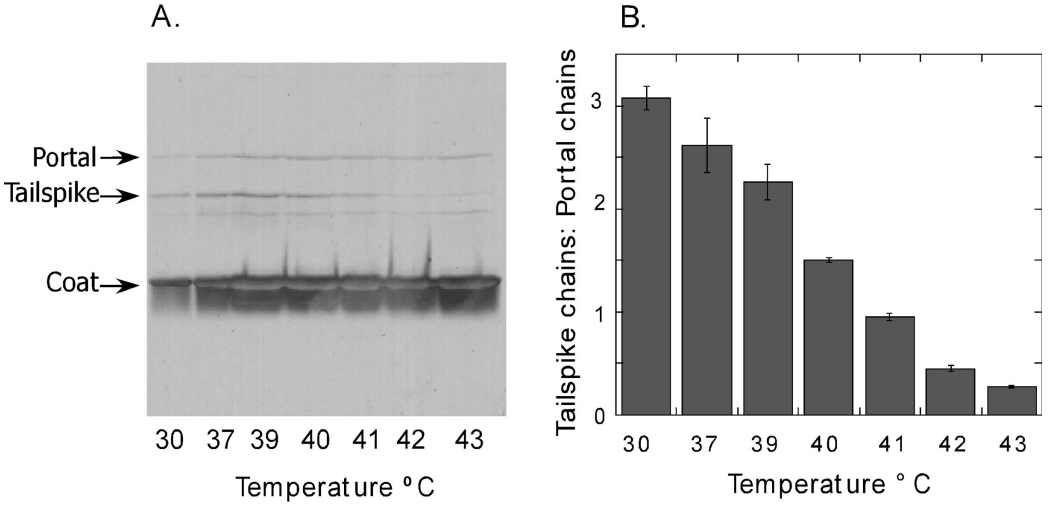

FIG. 3. SDS-PAGE of purified phage samples. (A) Phage particles, harvested by CsCl gradient purification from infected cells incubated at selected temperatures, were electrophoresed though an SDS–10% acrylamide gel. Protein bands were visualized with silver stain. Controls included previously purified P22 virions and P22 procapsids. (B) Analysis of tailspike chains versus portal chains in purified P22 samples. Relative quantities of tailspike chains and portal chains were determined by analyzing a silver-stained SDS-acrylamide gel with the ImageQuant software (Molecular Dynamics). Intensity of pixels was indicative of quantity of protein loaded in each lane. The ratio of the determined intensity of tailspike chains to portal chains in each sample was then plotted versus temperature. Error bars reflect the standard error as determined from three quantification replicates.

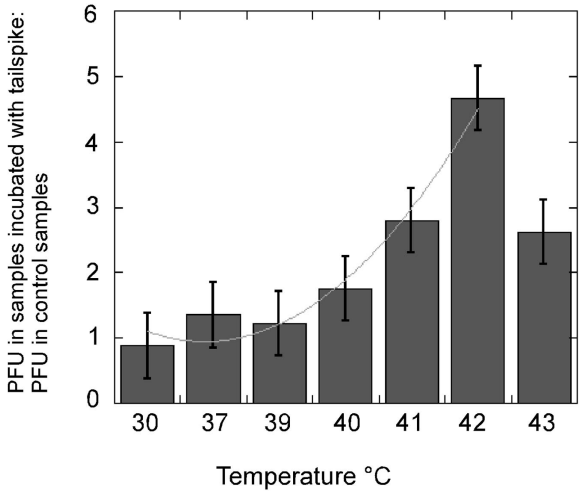

FIG. 4. Addition of purified exogenous native tailspike to samples. Native tailspike was added to purified P22 particles; after incubation for 60 min, the samples were plated to determine the phage titer. The ratio of PFU in P22 samples incubated with exogenous tailspike to PFU in P22 samples incubated with dilution fluid was determined and plotted versus the temperature at which the purified P22 particles were assembled. A trend line was added for clarity. Error bars reflect the standard error.

phage heads at high temperatures (Fig. 3B). This loss of bound tailspike was not due to thermal denaturation of mature tailspikes, as the thermal denaturation midpoint temperature for these structures is 88°C and they are resistant to intracellular degradation (39).

The tailspike deficit could reflect either a defect in their production at high temperatures or a defect in the portal vertex site to which they bind. This was resolved by determining whether the phage heads found at high temperatures could be converted to infectious particles by incubation with exogenous native tailspikes. In this tailing assay (Fig. 4), P22 infectious particles were generated upon mixing of tailspikeless DNA-containing phage heads with exogenous native tailspike (6, 26). The tailing assay indicated that addition of native tailspike to samples produced at high temperatures increased infectivity up to fivefold. Thus, the heads were competent to bind tailspike. The increase in number of infectious particles was an indication that many DNA-containing phage heads produced at higher temperatures were lacking sufficient tailspikes for infectivity.

In vivo, the lack of tailspikes at higher temperatures may be due to (i) misfolding and aggregation of the adhesins, (ii) slower synthesis of these adhesins, or (iii) a failure of these adhesins to successfully attach to the phage heads. To distinguish between translational and folding defects, we quantified the amount of native tailspike and partially folded tailspike intermediates produced within the cell at each temperature. This is possible through SDS-PAGE analysis, as native tailspike is not denatured by SDS unless it is boiled (14, 39). Thus, in unboiled samples on SDS gels, native tailspike electrophoreses with a mobility different from that of partially folded chains, which form conventional SDS-polypeptide chain complexes (14).

The lysates described above accumulated very little free native tailspike, presumably due to the rapid assembly of native trimer onto mature phage heads formed within infected cells. This suggested that the high-temperature-related problem was not failure of the assembly of native tailspike onto phage heads. Although native tailspike is thermostable, the presence of a thermolabile intermediate in the folding pathway has been well documented (21, 22). To examine chain folding and assembly in vivo, we used a P22 strain [2_{H200}(Am)/13_{H101}(Am) $C_1$7] containing an amber mutation in the DNA-packaging protein encoded by gene 2 (1). This mutation prevents the packaging of phage DNA into newly formed procapsids, thereby preventing addition of the neck protein and prolonging lysis. As a result, all newly synthesized tailspike chains remain in the cytoplasm (30). In addition, unpackaged DNA also is involved in upregulating the total amount of tailspike synthesized.

Exponentially growing cells were infected with P22 2^- at 37°C. Samples of the culture were incubated at 30, 37, 39, 40, 41, 42, and 43°C, harvested by low-speed centrifugation, and lysed. Using SDS-PAGE analysis and Coomassie blue staining, we were able to quantify the amount of native tailspike, folding intermediates, and coat protein within each lysate (Fig. 5). The native trimers are easily distinguishable from partially folded species in unboiled samples electrophoresed through SDS-acrylamide gels, as only the native trimer is resistant to SDS denaturation and, consequently, migrates toward the top of the gel (14). All other tailspike chains—misfolded, aggregated, and folding intermediates—denature in the presence of SDS and migrate to a position corresponding to a molecular mass of 72 kDa (14, 22).

For each sample, the ratio of native tailspike to SDS-sensitive tailspike species and the ratio of total tailspike (native plus SDS sensitive) to coat protein were determined (Fig. 5B). The total-tailspike sample values were normalized to the amount of coat protein present. Similar quantities of tailspike were present in all lysates tested except for that produced at the highest temperature, 43°C. At that temperature, there was a substantial decrease in the amount of coat protein synthesized. The high ratio of total tailspike to coat protein may have been the result of this large decrease in coat protein. This result implies that the high-temperature (up to 42°C) defect in P22 formation is not the result of a decrease in the rate of tailspike polypeptide synthesis.

To rule out the possibility of an assembly defect at high temperatures, we examined the ratio of native tailspike to partially folded or assembled SDS-sensitive tailspike species in each lysate. An observed increase in this ratio in cells incubated at higher temperatures would indicate that the protein was capable of folding at higher temperatures but was unable to attach to phage heads, while a decrease in this ratio would indicate a thermally induced folding defect in the adhesin.

The results of an SDS-PAGE analysis of DNA-packaging-defective lysates are presented in Fig. 5B. A decrease in the ratio of native tailspike to SDS-sensitive tailspike species is evident. This indicates that a thermal folding defect is the cause of the loss of infectious particles at elevated temperatures. As the quantity of tailspike chains synthesized remained relatively constant at temperatures up to 42°C, a synthesis defect could also be ruled out. Therefore, it is likely that the thermolability of a tailspike folding intermediate is responsible for the decrease in infectious particles at high temperatures.

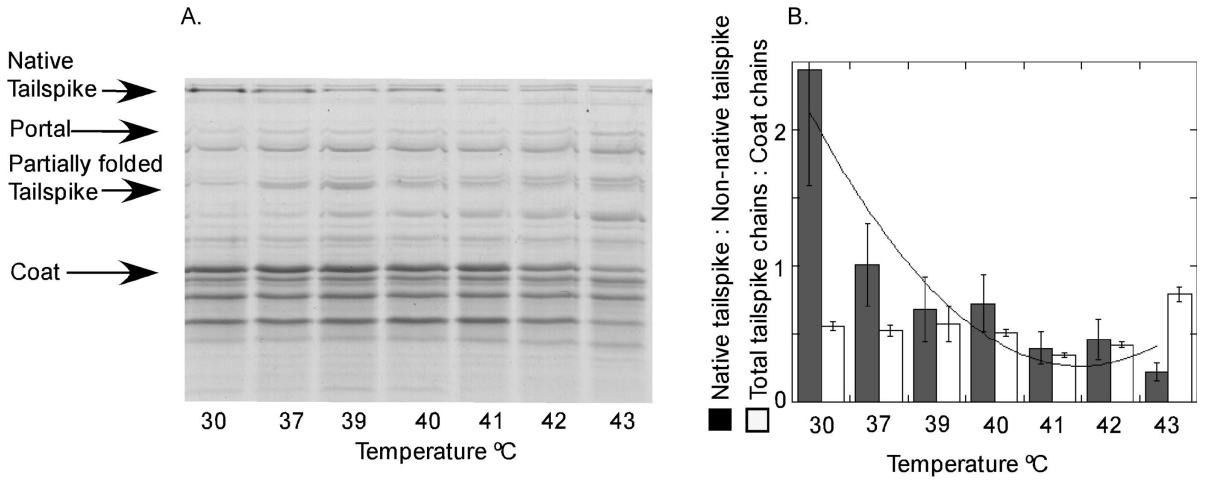

FIG. 5. SDS-PAGE analysis of P22 2⁻ DNA-packaging-defective lysates. (A) Cells were incubated for 2 to 3 h at selected temperatures, pelleted, and lysed by sonication. Lysates were DNase I treated and loaded, without boiling, on a 10% acrylamide gel containing SDS. Protein bands were visualized using Coomassie blue stain. (B) Analysis of native and partially folded tailspike chains (gray bars). Relative quantities of native tailspike, partially folded tailspike, and coat protein (white bars) were determined by analyzing a Coomassie blue-stained SDS-acrylamide gel with ImageQuant software (Molecular Dynamics). The ratio of native tailspike chains to partially folded tailspike chains versus temperature is in gray, and the ratio of total tailspike chains to coat chains versus temperature is in white. A trend line was added for clarity. Error bars reflect the standard error, as determined from three gel quantification replicates.

DISCUSSION

Identification of the thermolabile steps in those microbial processes which limit growth at high temperatures has been difficult. For phage P22 propagating in *Salmonella* serovar Typhimurium, the yield of infectious P22 particles produced declined at a lower temperature than did the reproductive capacity of the uninfected host cells. A significant loss of infectious P22 particles was observed for lysates incubated at temperatures of 40°C and above, while *Salmonella* serovar Typhimurium was capable of growth at temperatures up to 42°C. This suggested that the formation of some phage component was limiting the yield at higher temperatures. By infecting cells at the permissive temperature of 37°C and shifting them to higher target temperatures, we were able to control for potential thermal defects in adsorption of the phage.

Close examination of purified particles produced at high temperatures revealed a decrease in the amount of tailspike adhesin attached to DNA-containing phage heads. The heads that lacked tailspike were capable of binding exogenous active tailspike, indicating that the defect was not in the capsid structure.

SDS-PAGE analysis of crude lysates of cells infected with P22 terminase mutants showed that similar quantities of tailspike chains were produced at all temperatures, indicating that the loss of tailspike at high temperatures was not the result of a thermal defect in transcription or translation. The ratio of native tailspike to total tailspike chains decreased with increasing temperature, an indication that the lack of tailspike adhesins was due to misfolding of these polypeptide chains rather than to a defect in their synthesis or in the ability of the native trimer to bind the phage capsid.

The tailspike folding pathway, which has been well characterized experimentally, proceeds through several intermediates before achieving the native structure (7, 29). This pathway proceeds through a thermolabile folding intermediate, which aggregates into an inclusion body if folding occurs at high temperatures (21, 22) and prevents the production of native tailspike (Fig. 1A).

The native tailspike is a homotrimer. Each of the three chains has a parallel β-helix region that terminates in a triple β-helix formed by the wrapping of the three peptide chains around each other. Using the extended lateral surface of the parallel β-helix, native tailspike binds the host's LPS (37). Kreisberg et al. showed that the triple-β-helix motif gives the native trimeric protein extra stability (31). The most likely thermolabile motif within the tailspike adhesin is the parallel β-helix formed by each chain within the structure (21, 29, 34, 39).

It is not clear why the folding of the tailspike adhesin is the most thermally sensitive process in the production of mature P22 particles. The tailspike structure contains two relatively rare protein motifs: the parallel β-helix (38) and the triple-stranded β-helix (42). It is possible that the folding intermediates for these domains are intrinsically thermolabile. Mutational studies of the tailspike have revealed the existence of a substantial number of single-amino-acid, temperature-sensitive-folding mutations within the parallel β-helix (21).

An alternative explanation for the thermolability inherent in the folding pathway is the lack of a helper chaperone. Brunschier et al. (8) showed that tailspike intermediates, unlike the P22 coat protein, were not rescued by *E. coli*'s GroEL/ES chaperone system (8). Gordon et al. (17) examined several temperature-sensitive-folding mutants of both the tailspike and coat proteins in conjunction with overexpression of GroEL/ES. The coat protein mutants were rescued from thermally induced aggregation by chaperone overexpression, while the tailspike mutants could not be rescued in this manner (17). The 666-amino-acid tailspike chain may be too large for the lumen of the GroEL/ES chaperone. The decline in native tailspike yield is presumably a consequence of both the intrinsic ther-

molability of the partially folded intermediates and the inability of GroEL to chaperone the species. There is likely to be a strong selection for host recognition adhesin function within the host-phage ecosystem. We suspect that the stability and efficacy of the tailspike's native state balance the folding and assembly disadvantages of this motif at higher temperatures.

In the above discussion, we assume that the folding problem is intrinsic to the biochemistry of the parallel β-helix motif. Alternatively, this loss of tailspike yield could be an evolved response of P22 that allows for a reduction of the phage population when the host is experiencing stress. For example, under nutrient-poor conditions, the T4 coliphage Wac protein will bind the long tail fibers of the phage into an upright position, thereby preventing infection of the host (9). It is possible that the sensitivity of the folding of the parallel β-helix to elevated temperatures is a similarly evolved response preserved to prevent phage propagation in times of host stress.

It is likely that prior to the evolution of warm-blooded organisms, the early ancestors of *Salmonella* spp. were adapted to a soil or an aquatic environment. In the aquatic regime, temperatures rarely (if ever) reach 40°C, while a soil environment may experience large temperature fluctuations over brief periods of time. Both scenarios would select for a mature protein motif that is highly stable and resistant to external stresses such as proteases and temperature. Modern *Salmonella* spp. are capable of propagating outside the body and are exposed to diverse temperature regimes. With the evolution of warm-blooded organisms and subsequent adaptation of *Salmonella* spp. to the guts of these animals, the parallel β-helix may have been retained through selection for native properties.

In the marine environment, primary producers such as cyanobacteria are subjected to a very limited range of environmental temperatures. Phages whose morphologies resemble those of enteric phages have been isolated from marine synechococcal strains (41). We are presently examining whether the formation of phage structural proteins is a rate-limiting step in the production of cyanophages of the marine genus *Synechococcus*.

ACKNOWLEDGMENTS

We thank Peter Weigele for helpful discussions.

This work was funded by NIH grant GM17980 and NSF grant EIA0225609 to J.K.

REFERENCES

1. **Adams, M. B., H. R. Brown, and S. Casjens.** 1985. Bacteriophage P22 tail protein gene expression. J. Virol. **53:**180–184.
2. **Baxa, U., S. Steinbacher, S. Miller, A. Weintraub, R. Huber, and R. Seckler.** 1996. Interactions of phage P22 tails with their cellular receptor, *Salmonella* O-antigen polysaccharide. Biophys. J. **71:**2040–2048.
3. **Bazinet, C., J. Benbasat, J. King, J. M. Carazo, and J. L. Carrascosa.** 1988. Purification and organization of the gene 1 portal protein required for phage P22 DNA packaging. Biochemistry **27:**1849–1856.
4. **Beney, L., and P. Gervais.** 2001. Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. Appl. Microbiol. Biotechnol. **57:**34–42.
5. **Benton, C. B., J. King, and P. L. Clark.** 2002. Characterization of the protrimer intermediate in the folding pathway of the interdigitated beta-helix tailspike protein. Biochemistry **41:**5093–5103.
6. **Berget, P. B., and A. R. Poteete.** 1980. Structure and functions of the bacteriophage P22 tail protein. J. Virol. **34:**234–243.
7. **Betts, S., and J. King.** 1999. There's a right way and a wrong way: in vivo and in vitro folding, misfolding and subunit assembly of the P22 tailspike. Struct. Fold. Des. **7:**R131–R139.
8. **Brunschier, R., M. Danner, and R. Seckler.** 1993. Interactions of phage P22 tailspike protein with GroE molecular chaperones during refolding in vitro. J. Biol. Chem. **268:**2767–2772.
9. **Conley, M. P., and W. B. Wood.** 1975. Bacteriophage T4 whiskers: a rudimentary environment-sensing device. Proc. Natl. Acad. Sci. USA **72:**3701–3705.
10. **Corry, P. M., S. Robinson, and S. Getz.** 1977. Hyperthermic effects on DNA repair mechanisms. Radiology **123:**475–482.
11. **Dean, R. G., and E. J. McGroarty.** 1979. Protein and ribonucleic acid syntheses in heat-damaged and heat-killed *Escherichia coli*. J. Bacteriol. **138:**492–498.
12. **Ellis, R. J., and S. M. van der Vies.** 1991. Molecular chaperones. Annu. Rev. Biochem. **60:**321–347.
13. **Fink, A. L.** 1999. Chaperone-mediated protein folding. Physiol. Rev. **79:**425–449.
14. **Goldenberg, D. P., P. B. Berget, and J. King.** 1982. Maturation of the tailspike endorhamnosidase of *Salmonella* phage P22. J. Biol. Chem. **257:**7864–7871.
15. **Goldenberg, D. P., and J. King.** 1981. Temperature-sensitive mutants blocked in the folding or subunit of the bacteriophage P22 tailspike protein. II. Active mutant proteins matured at 30°C. J. Mol. Biol. **145:**633–651.
16. **Gordon, C. L., and J. King.** 1993. Temperature-sensitive mutations in the phage P22 coat protein which interfere with polypeptide chain folding. J. Biol. Chem. **268:**9358–9368.
17. **Gordon, C. L., S. K. Sather, S. Casjens, and J. King.** 1994. Selective in vivo rescue by GroEL/ES of thermolabile folding intermediates to phage P22 structural proteins. J. Biol. Chem. **269:**27941–27951.
18. **Gragerov, A. I., E. S. Martin, M. A. Krupenko, M. V. Kashlev, and V. G. Nikiforov.** 1991. Protein aggregation and inclusion body formation in *Escherichia coli rpoH* mutant defective in heat shock protein induction. FEBS Lett. **291:**222–224.
19. **Greene, B., and J. King.** 1996. Scaffolding mutants identifying domains required for P22 procapsid assembly and maturation. Virology **225:**82–96.
20. **Haase-Pettingell, C., S. Betts, S. W. Raso, L. Stuart, A. Robinson, and J. King.** 2001. Role for cysteine residues in the in vivo folding and assembly of the phage P22 tailspike. Protein Sci. **10:**397–410.
21. **Haase-Pettingell, C., and J. King.** 1997. Prevalence of temperature sensitive folding mutations in the parallel beta coil domain of the phage P22 tailspike endorhamnosidase. J. Mol. Biol. **267:**88–102.
22. **Haase-Pettingell, C. A., and J. King.** 1988. Formation of aggregates from a thermolabile in vivo folding intermediate in P22 tailspike maturation. A model for inclusion body formation. J. Biol. Chem. **263:**4977–4983.
23. **Hashemolhosseini, S., Y.-D. Stierhof, I. Hindennach, and U. Henning.** 1996. Characterization of the helper proteins for the assembly of tail fibers of coliphages T4 and λ. J. Bacteriol. **178:**6258–6265.
24. **Herendeen, S. L., R. A. VanBogelen, and F. C. Neidhardt.** 1979. Levels of major proteins of *Escherichia coli* during growth at different temperatures. J. Bacteriol. **139:**185–194.
25. **Ingraham, J.** 1996. Effect of temperature, pH, water activity, and pressure on growth, p. 1543–1554. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society of Microbiology, Washington, D.C.
26. **Israel, J., T. Anderson, and M. E. Levin.** 1967. *In vitro* morphogenesis of phage P22 from heads and baseplate parts. Proc. Natl. Acad. Sci. USA **57:**284–291.
27. **Iwashita, S., and S. Kanegasaki.** 1973. Smooth specific phage adsorption: endorhamnosidase activity of tail parts of P22. Biochem. Biophys. Res. Commun. **55:**403–409.
28. **Jaenicke, R.** 1991. Protein folding: local structures, domains, subunits, and assemblies. Biochemistry **30:**3147–3161.
29. **King, J., C. Haase-Pettingell, A. S. Robinson, M. Speed, and A. Mitraki.** 1996. Thermolabile folding intermediates: inclusion body precursors and chaperonin substrates. FASEB J. **10:**57–66.
30. **King, J., C. Hall, and S. Casjens.** 1978. Control of the synthesis of phage P22 scaffolding protein is coupled to capsid assembly. Cell **15:**551–560.
31. **Kreisberg, J. F., S. D. Betts, C. Haase-Pettingell, and J. King.** 2002. The interdigitated beta-helix domain of the P22 tailspike protein acts as a molecular clamp in trimer stabilization. Protein Sci. **11:**820–830.
32. **Landry, J., and P. Chretien.** 1983. Relationship between hyperthermia-induced heat-shock proteins and thermotolerance in Morris hepatoma cells. Can J. Biochem. Cell Biol. **61:**428–437.
33. **Marr, A. G., J. L. Ingraham, and C. L. Squires.** 1964. Effect of the temperature of growth of *Escherichia coli* on the formation of β-galactosidase. J. Bacteriol. **87:**356–362.
34. **Miller, S., B. Schuler, and R. Seckler.** 1998. A reversibly unfolding fragment of P22 tailspike protein with native structure: the isolated beta-helix domain. Biochemistry **37:**9160–9168.
35. **Prevelige, P. E., Jr., and J. King.** 1993. Assembly of bacteriophage P22: a model for ds-DNA virus assembly. Prog. Med. Virol. **40:**206–221.
36. **Smith, D. H., P. B. Berget, and J. King.** 1980. Temperature-sensitive mutants blocked in the folding or subunit assembly of the bacteriophage P22 tailspike protein. I. Fine-structure mapping. Genetics **96:**331–352.
37. **Steinbacher, S., U. Baxa, S. Miller, A. Weintraub, R. Seckler, and R. Huber.**

1996. Crystal structure of phage P22 tailspike protein complexed with *Salmonella* sp. O-antigen receptors. Proc. Natl. Acad. Sci. USA **93:**10584–10588.
38. **Steinbacher, S., R. Seckler, S. Miller, B. Steipe, R. Huber, and P. Reinemer.** 1994. Crystal structure of P22 tailspike protein: interdigitated subunits in a thermostable trimer. Science **265:**383–386.
39. **Sturtevant, J. M., M. H. Yu, C. Haase-Pettingell, and J. King.** 1989. Thermostability of temperature-sensitive folding mutants of the P22 tailspike protein. J. Biol. Chem. **264:**10693–10698.
40. **van der Vies, S. M., A. A. Gatenby, and C. Georgopoulos.** 1994. Bacteriophage T4 encodes a co-chaperonin that can substitute for *Escherichia coli* GroES in protein folding. Nature **368:**654–656.
41. **Waterbury, J. B., and F. W. Valois.** 1993. Resistance to co-occurring phages enables marine *Synechococcus* communities to coexist with cyanophages abundant in seawater. Appl. Environ. Microbiol. **59:**3393–3399.
42. **Weigele, P. R., E. Scanlon, and J. King.** 2003. Homotrimeric, β-stranded viral adhesins and tail proteins. J. Bacteriol. **185:**4022–4030.
43. **Wood, W. B., F. A. Eiserling, and R. A. Crowther.** 1994. Long tail fibers: genes, proteins, structure, and assembly, p. 282–290. *In* J. D. Karam, J. W. Drake, K. N. Kreuzer, G. Mosig, D. H. Hall, F. A. Eiserling, L. W. Black, E. K. Spicer, E. Kutter, K. Carlson, and E. S. Miller (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.