JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 2044–2050
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology
Vol. 178, No. 7

Exclusion of T4 Phage by the *hok/sok* Killer Locus from Plasmid R1

DOUGLAS C. PECOTA AND THOMAS K. WOOD*

Department of Chemical and Biochemical Engineering, University of California, Irvine, California 92717-2575

Received 25 September 1995/Accepted 29 January 1996

The *hok* (host killing) and *sok* (suppressor of killing) genes (*hok/sok*) efficiently maintain the low-copy-number plasmid R1. To investigate whether the *hok/sok* locus evolved as a phage-exclusion mechanism, *Escherichia coli* cells that contain *hok/sok* on a pBR322-based plasmid were challenged with T1, T4, T5, T7, and λ phage. Upon infection with T4, the optical density of cells containing *hok/sok* on a high-copy-number plasmid continued to increase whereas the optical density for those lacking *hok/sok* rapidly declined. The presence of *hok/sok* reduced the efficiency of plating of T4 by 42% and decreased the plaque size by ~85%. Single-step growth experiments demonstrated that *hok/sok* decreased the T4 burst size by 40%, increased the time to form mature phage (eclipse time) from 22 to 30 min, and increased the time to cell lysis (latent period) from 30 to 60 min. These results further suggest that single cells exhibit altruistic behavior.

Recent advances in the study of killer loci and phage exclusion suggest that the two areas may be closely related (22, 29, 39). Postsegregational killer loci (such as *hok* [host killing] and *sok* [suppressor of killing] genes [*hok/sok*], *parDE*, *doc/phd*, *ccd*, and *pem*) have been shown to stabilize plasmids (11, 15, 24, 26, 35) by killing plasmid-free daughter cells. Each killer system contains a gene that encodes a protein toxin and a gene that inhibits the toxin from being expressed (15). Antisense mRNA killer systems use fold-back inhibition and a small unstable antisense mRNA to prevent translation of the toxin in plasmid-bearing cells (44). A halt in transcription of antisense mRNA due to plasmid loss leads to a rapid decline in its concentration, allowing expression of the killer peptide (14). Proteic killer systems overexpress a labile antitoxin that masks the toxin. A halt in synthesis of antitoxin due to plasmid loss leads to a decline in antitoxin concentration, allowing the toxin to poison the cell.

Plasmid loss is not the only event that can prevent transcription of antisense mRNA and induce suicide systems. Temperature shock, amino acid deprivation (1), antibiotics (e.g., rifampin [16]), and phage infection all cause a sudden change in protein synthesis (23). This suggests that cell killing might be induced during phage infection. Furthermore, restriction-modification systems are generally believed to have evolved to inhibit phage infection; however, some plasmid-borne restriction-modification systems have been shown recently to mediate plasmid stability by postsegregational killing (22). This also suggests that plasmid-stabilizing killer loci may also function to exclude phage and might explain why the chromosome contains several killer loci (such as *relB* [1], *kicB/kicA* [11], *chpA* [26], *chpB* [26], and *gef/sof* [32]).

Phage-exclusion systems (other than superinfectivity) have been found in phage, plasmids, and cryptic phage elements (29, 42). The best-studied systems are exclusion of T4 rII mutants by *rex* of λ lysogens (2, 42), exclusion of T4 by *lit* of the cryptic DNA element e14 (3, 42), exclusion of polynucleotide kinase and RNA-ligase mutants of T4 by *prr* of cryptic DNA element prr (41, 46), and exclusion of T7 by *pif* of plasmid F (10, 29). The λ *rex* system consists of two proteins, RexA and RexB, which form a membrane complex; during infection, the RexA/RexB ratio is increased because of degradation of RexB, which probably causes the complex to form an ion channel that kills the cell (42). The T4 rII gene product overcomes this phage exclusion system by an unknown mechanism (42). The *lit* system excludes T4 by preventing late gene translation by encoding a protease of the essential-elongation factor Tu that becomes activated by T4 *gol* gene expression (3). The *prr* system has four proteins, PrrA, PrrB, PrrC, and PrrD, which act to exclude mutant T4. PrrC is an anticodon nuclease whose expression is masked by the other gene products but which becomes active upon T4 expression of peptide Stp (41). The nuclease then cleaves $tRNA^{Lys}$, preventing translation (46). To prevent T7 propagation, PifA interacts with T7 gene products 1.2 (dGTPase) and 10 (the major head protein of T7), causing membrane damage (12). Each of these phage-exclusion systems has much in common with killer loci: they require minimal cell resources (low expression levels), they are held inactive during normal cell growth, they have a method of detecting the presence of a phage, they respond without gene transcription after infection, and they have a toxin that attacks a highly conserved region of the cell (not the phage).

On the basis of this similarity of killer loci and phage-exclusion systems, exclusion of T1, T4, T5, T7, and λ-virulent phage was tested in *Escherichia coli* cells containing either the *parDE* or *hok/sok* killer loci (*hok/sok* are from R1 and are not related to T4 head proteins Hoc and Soc). Since cells containing high copies of *hok/sok* continued to grow in the presence of T4, plaque size, time to phage maturation, time to cell lysis by phage, and burst size were further characterized. This is the first report investigating the evolutionary significance of the *hok/sok* plasmid stability locus as a phage-exclusion system.

MATERIALS AND METHODS

Bacteria and plasmids. *E. coli* AMA1004 [Δ(*lacIPOZ*)*C29 lacY*$^+$ *hsdR galU galK strA*^{r} *leuB6 trpC9830*] (9) was used to prepare dead cells for plating in the presence of phage since it can be distinguished from BK6, a general P1 *kc* transductant derivative [AMA1004 Δ(*srl-recA*)*306*::Tn*10*] (51), by its tetracycline sensitivity and has the same phage-adsorption properties. AMA1004 and BK6 are *E. coli* K-12-derived strains (9) and contain the chromosomal killer loci *gef/sof* (30), *relB* (1), *kicA/kicB* (11), *chpAK/chpAI* (26), and *chpBK/chpBI* (26). BK6 was used to harbor all plasmids since it allows easy identification of cells containing a *lacZ*$^+$ plasmid. The plasmid pTKW106 (9.3 kb, *hok*$^+$/*sok*$^+$ Km^r *lacI*$^{q+}$ *ptac*::*lacZ*$^+$) (51) contains the wild-type *hok/sok* killer locus and *aphA* (Km^r) inserted into pMJR1750 (7.5 kb, Ap^r *lacI*$^{q+}$ *ptac*::*lacZ*$^+$) (43), a pUC18-derived plasmid that does not contain any killer loci or phage-exclusion systems. pTKW106 has kanamycin resistance for easy selection, *lacZ*$^+$ for convenient detection, and a high copy number (80 to 100 per cell [52]). The *hok/sok* gene pair was originally isolated from the R1 plasmid of *Salmonella paratyphi* B as well

* Corresponding author. Phone: (714) 824-3147. Fax: (714) 824-2541. Electronic mail address: tkwood@uci.edu.

as from *E. coli* (17). pOU82 parDE (*parDE*$^+$ Ap^r *lacZYA*$^+$) (19) is a low-copy-number mini-R1 plasmid (one to two plasmids per chromosome equivalent at 35°C [19]) that contains the proteic killer locus *parDE*. pOU82 parDE does not contain any other killer loci or phage-exclusion systems but undergoes runaway replication at 42°C; however, all cells were grown at 37°C. The naturally occurring source of *hok/sok*, R1 (Ap^r Cm^r Sm^r Su^r [Km^r spontaneously lost]) (4) was also used as a low-copy-number control. For all experiments, the presence of each plasmid was confirmed at the beginning and end by *lac*$^+$-dependent red colony formation on MacConkey agar or by growth with and without the appropriate antibiotics. In addition, the plasmid-bearing stocks (stored at −85°C) were verified by restriction endonuclease digestion (*Eco*RI) followed by electrophoretic visualization of the bands and growth on the appropriate antibiotics.

Phage. *E. coli* phage T1 wild type (ATCC 11303-B1), T4 wild type (ATCC 11303-B4), T4 rVII, T5 wild type (ATCC 11303-B5), T7 wild type (ATCC 11303-B7), and λ-virulent were chosen since they are well characterized, use different methods to disrupt the cell upon infection, and undergo only lytic growth. λ-virulent (a stable λ mutant that cannot integrate into the chromosome) and T4 rVII were kindly provided by John Keener. All phage stocks were prepared by growth on BK6 in Luria-Bertani (LB) medium (37) and stored at 4°C with chloroform.

Media. LB hard-agar plates were made by the addition of 1.5% Bacto agar to LB medium, and LB soft-agar overlay was prepared by the addition of 0.7% Bacto agar to LB medium. Tetracycline (15 μg/ml) was used to confirm the BK6 phenotype, kanamycin (50 μg/ml) was used for plating BK6/pTKW106 from −85°C stocks, and ampicillin (30 μg/ml) was used for plating BK6/pMJR1750 and BK6/pOU82 from −85°C stocks as well as for the single-step growth overnight cultures. Ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), and streptomycin (30 μg/ml) were used to plate BK6/R1 from −85°C stocks. Glycerol-Casamino Acids medium supplemented with 10 μg of tryptophan per ml (7) was used to grow AMA1004. All chemicals were purchased from Fisher Scientific (Pittsburgh, Pa.).

Infection and cell growth. To initially gauge the extent of phage exclusion, cell growth after infection was monitored by using a Spectronic 20D spectrophotometer (Milton Roy, Rochester, N.Y.). Each host was streaked from a −85°C glycerol stock onto a MacConkey agar plate with appropriate antibiotics. Plates were incubated at 37°C until colony color could be determined (24 to 36 h). A single colony was then picked and used to inoculate 20 ml of LB medium. The culture was grown overnight at 37°C and 250 rpm (series 25 shaker; New Brunswick Scientific, Edison, N.J.). Overnight cultures were diluted 50-fold and grown at 37°C and 250 rpm without antibiotics. When the optical density (OD_{600}) (600 nm used for all optical density measurements) reached 0.2 (~4×10^7 CFU/ml), the cells were added to four 250-ml Erlenmeyer flasks (20 ml each flask), and each flask was infected with a different PFU of phage (except for the no-phage control, which was used to determine the growth rates by least-squares fitting); therefore, three phage concentrations were used for each strain. The phage titer was such that a decrease in cellular OD_{600} would occur before the onset of stationary phase. The OD_{600} was monitored by sampling every 10 min until the control reached stationary phase ($OD_{600} > 1.6$). Stationary samples were plated on appropriate agar to confirm that cultures were plasmid bearing.

Efficiency of plating and plaque size. The enumeration of plaques was performed as outlined by Carlson and Miller (6). The overnight culture of each host was prepared from single colonies (as described above), diluted 100-fold (1 ml of culture into 100 ml of LB medium at 37°C), and grown at 37°C and 250 rpm. The phage was diluted to 1,000 to 3,000 PFU/ml with LB medium. When the host bacteria reached an OD_{600} of 1.0, 0.2 ml of cells was added to 0.1 ml of diluted phage; this was followed by the addition of 3 ml of soft agar (45 to 47°C). The mixture was immediately poured onto LB plates (five plates were used for each host). Plaques were examined and counted the following day. The efficiency of plating was calculated by computing the average PFU of each host and dividing it by the average number of plaques for BK6. Upon completion, the plating bacteria were spread on MacConkey agar for plasmid confirmation.

To determine T4 plaque size, the same procedure was used except that the cells were incubated with phage for 10 min to allow phage adsorption for more uniform plaque size (6). After 12 h of incubation at 37°C, plates were placed at 4°C to prevent further plaque growth, and the plaques were counted and measured with calipers. Twenty random plaques were measured from each of five plates.

Single-step growth. The single-step growth experiment is based on the procedure recommended by Carlson (5). An overnight culture (prepared from a single colony) was diluted 100-fold and grown to an OD_{600} of ~1.0, at which point a sample of cells was infected with phage at a multiplicity of infection (MOI) of 0.1 to 0.2 (to prevent superinfection) and the remaining cells were used for plating. After 4 min of phage adsorption, the cells were diluted 2×10^4 and 2×10^6 (to prevent further infection) and incubated at 37°C and 250 rpm. Samples were periodically taken from these two dilutions and mixed with chloroform (which lyses cells but does not harm phage) to determine total mature phage (TMP), the number of free phage plus all the mature phage inside infected cells. Total infective centers (TIC) were determined by taking periodic samples of the two dilutions and combining them with plating bacteria and soft agar. Plaques from the TIC samples indicated the number of free phage plus infected cells. From graphed data, the eclipse period was determined as the time when the average number of phage produced per cell is one (time of the initial intersection of the TMP and TIC curves [see Fig. 2]), and the latent period was determined as the time when the extrapolation of the steep increase of the TIC curve and its initial plateau intersect. The burst size was calculated by dividing the post-cell-lysis TIC plateau value by the initial prelysis TIC plateau value.

Phage propagation and cell growth. Each 100-ml host culture was grown to an OD_{600} of 0.2 as described previously. BK6 plating bacteria (50 ml) were grown separately to an OD_{600} of 1.0 as in the single-step growth experiment. Fifty milliliters of exponentially growing cells was removed and placed in another flask to which approximately 3.7×10^7 PFU of T4 was added (MOI, ~0.02). The remaining cells (~40 ml) were used as a no-phage control. The OD_{600} was monitored until the no-phage control reached stationary phase. The TIC was monitored by diluting and plating on BK6 every 15 to 20 min. The TMP was monitored by lysing the cells for at least 30 min with chloroform and then diluting and plating. The number of CFU was monitored by diluting the cells and phage solution in LB medium with the last 100× dilution performed by using dead AMA1004 to titrate phage. Dead *E. coli* was prepared by boiling for 10 min (7). The absence of viable cells was verified by plating on LB medium. To further ensure that only BK6 colonies developed (not AMA1004, used to titrate the phage), LB plates containing tetracycline (10 μg/ml) were used to determine CFU. Colonies of BK6/pTKW106 were tested for the plasmid by transferring to MacConkey agar plates.

RESULTS

For all of the following experiments, each culture was 100% plasmid bearing for the duration of each experiment. In addition, all experiments were repeated at least twice (except for the infection and cell growth experiments which used different phage concentrations to confirm the trends).

Infection and cell growth. The infection and cell growth experiment was used to screen for phage exclusion by the two killer locus systems *parDE* and *hok/sok* by monitoring the culture OD_{600} as a function of time after phage addition. Both T4 (Fig. 1) and T4 rVII (results not shown) exhibited phage exclusion by *hok/sok* but not *parDE* (results not shown); for the pTKW106 *hok*$^+$ cells, the absorbance continued to rise well after the optical density of the two controls which lack *hok* (BK6 and BK6/pMJR1750) dropped. As the MOI of T4 was increased, the OD_{600} of BK6/pTKW106 declined, indicating that T4 is able to cause cell lysis; but, for an MOI of up to 0.12, BK6/pTKW106 continued to grow whereas BK6 and BK6/pMJR1750 were lysed completely. BK6/R1 showed more limited T4 exclusion since the OD_{600} declined but not as rapidly as the two controls which lack *hok*. Growth in the absence of phage shows that BK6/pMJR1750 and BK6/pTKW106 have similar growth rates (Table 1).

At three concentrations of λ-virulent (MOI, 0.012, 0.12, and 1.2) and T5 (MOI, 0.1, 0.01, and 0.001), these phage were only slightly excluded by *hok* (maximum OD_{600} reached by BK6/pTKW106 in the presence of phage was higher relative to BK6/pMJR1750; results not shown). No exclusion was detected for T1 (MOI, 0.01, 0.1, and 1.0) and T7 (MOI, 10^{-6}, 10^{-5}, and 10^{-4}) with *hok*, and no phage exclusion was detected by *parDE* (results not shown for T1, T4 rVII, T5, and λ-virulent phage).

Efficiency of plating and plaque size. On the basis of the infection and cell growth experimental results, T4 exclusion by *hok* was investigated more rigorously. The efficiency of plating is a measure of the ability of a phage to infect a host compared with a control strain (7). The efficiency of plating of T4 on BK6/pMJR1750 was reduced slightly (14%) relative to host BK6, which may reflect the additional metabolic burden of the plasmid (plating comparison, PFU per milliliter, shown in Table 1). In contrast, the T4 efficiency of plating on BK6/pTKW106 (*hok*$^+$) was decreased by 42%. This suggests that 42% of the T4-infected BK6/pTKW106 cells die and produce no phage and that *hok/sok* excludes T4.

In addition to fewer plaques, the plaques on the BK6/pTKW106 plates were also significantly smaller than those of BK6 and BK6/pMJR1750. The presence of *hok/sok* on high-copy-number pTKW106 reduced the plaque size by 86% (1.30

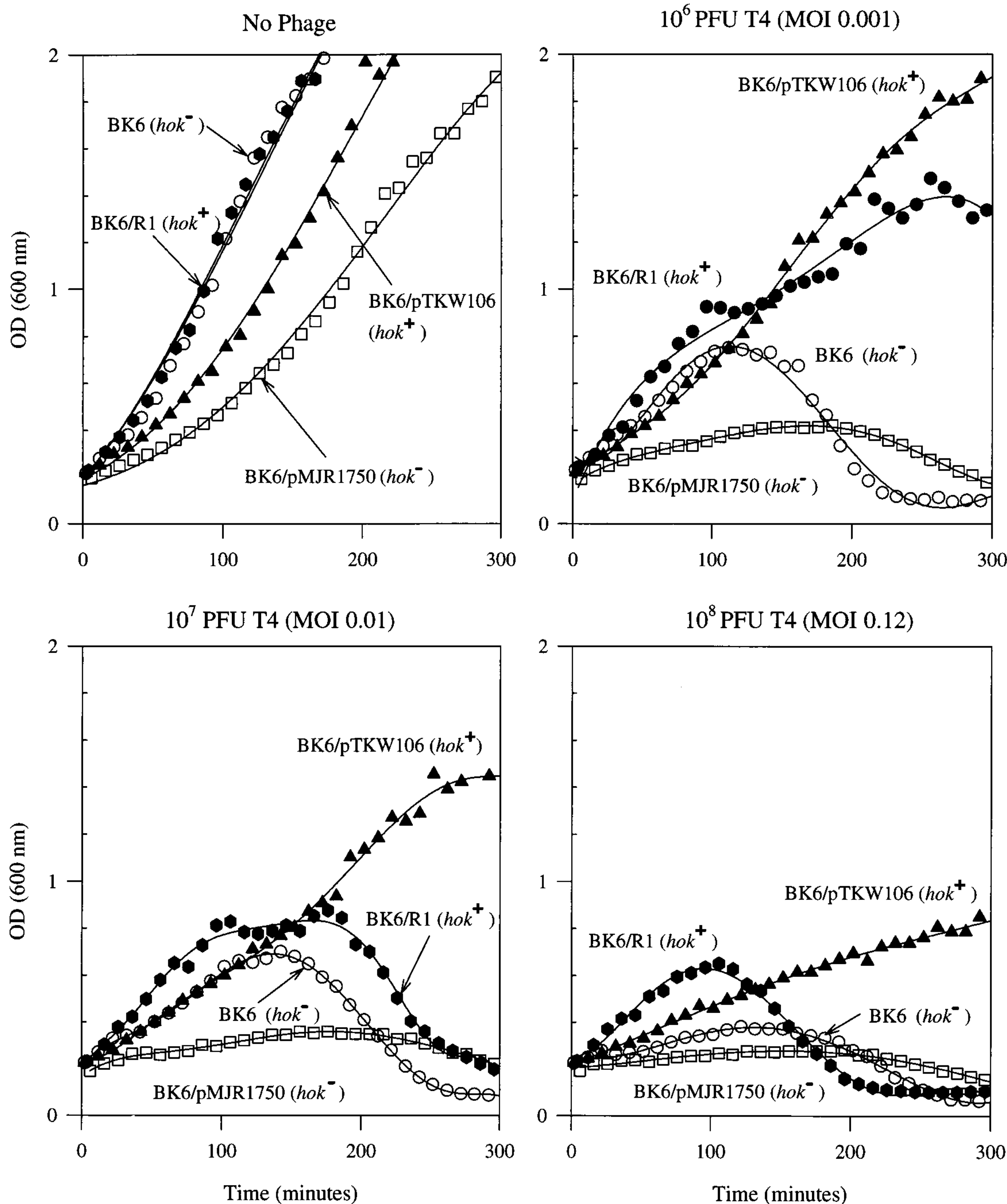

FIG. 1. OD_{600} of shake-flask cultures of BK6, BK6/pMJR1750, and BK6/pTKW106 after infection with bacteriophage T4.

versus 0.19 mm [Table 1]), and low-copy-number R1 reduced the plaque size by 22% (1.02 mm).

Single-step growth. The single-step growth of T4 shows that *hok/sok* interfere with T4 propagation. The eclipse time (average time to produce the first mature intracellular phage [5]) increased from 22 min in BK6 and BK6/pMJR1750 cells to 30 min in BK6/pTKW106 (hok^+/sok^+) cells (Fig. 2; Table 1). The latent period (average time to cell lysis [5]) is also much longer in *hok/sok* cells. It takes 60 min for T4 to lyse BK6/pTKW106 and only 28 min for BK6 and 31 min for BK6/pMJR1750. Surprisingly, the burst size (the average number of phage released at cell lysis [5]) of both plasmid-bearing cells was reduced from 210 ± 21 in BK6 to 89 ± 23 in BK6/pMJR1750 and 121 ± 40 in BK6/pTKW106.

Phage propagation and cell growth. The results of monitoring T4 propagation in BK6 and BK6/pTKW106 cells are shown in Fig. 3. The TIC consistently lagged behind the TMP in BK6/pTKW106 as expected from the single-step growth experiments. In addition, the total number of phage 150 min into the experiment was 10-fold greater for BK6 than for BK6/pTKW106. The OD_{600} of the hok^+ cells continued to rise as expected from the infection and cell-growth experiment (Fig.

TABLE 1. Single-step growth results, plating comparison, cell growth rates, and plaque sizes with phage T4 and host BK6[a]

Plasmid	Eclipse period (min)	Latent period (min)	Burst size (no. of phage/successfully infected cell)	Plating comparison (10^{10} PFU/ml)	Specific growth rate (1/h)	Plaque diameter (mm)
None	21 ± 3	28 ± 1	210 ± 21	7.7 ± 0.9	1.0 ± 0.12	1.3 ± 0.2
pMJR1750	22 ± 1	31 ± 9	89 ± 23	6.6 ± 0.6	0.63 ± 0.09	1.2 ± 0.3
pTKW106 (*hok*$^+$)	30 ± 1	60 ± 2	121 ± 38	4.5 ± 0.5	0.68 ± 0.14	0.19 ± 0.06

[a] Standard deviations are indicated.

1). Surprisingly, the number of CFU of BK6/pTKW106 did not drop after phage addition even though there were approximately 10^9 PFU of free phage per ml (Fig. 3). In contrast, the CFU of BK6 decreased by 3 orders of magnitude (Fig. 3).

DISCUSSION

The *hok/sok* killer locus at high gene dosage exclude phage T4 as shown by the growth of cells in the presence of T4 (MOI of up to 0.12), the 50% increase in time to make a mature phage, the 107% increase in time to cell lysis, the reduction in burst size, and the 86% reduction in plaque size. It appears that Hok disrupts the cell at the later stages of phage development by delaying cell lysis (Fig. 2). Therefore, phage exclusion is proposed as the evolutionary importance of the killer gene *hok* and chromosomal killer loci (*relB* [1], *kicB/kicA* [11], *chpA* [26], *chpB* [26], and *gef/sof* [32]).

Examination of the time required for T4 infection and killing by *hok/sok* indicates that there is sufficient time for the killer locus to disrupt cell metabolism before T4 directs cell lysis. Before Hok is translated, the pool of Sok mRNA, the Hok mRNA inhibitor, must be depleted. The half-life of Sok antisense mRNA in vivo is less than 30 s (16); therefore, within 3 min, 98% of the Sok mRNA will have been degraded once transcription is halted by T4. Inhibition of RNA polymerase and cessation of *sok* transcription start almost immediately when T4 injects 25 to 50 copies of the Alt protein with its DNA. The Alt protein ADP-ribosylates the host RNA polymerase (50), resulting in better recognition of some T4 promoters, and causes a twofold drop in RNA polymerase activity (23). Two minutes after infection, transcription is further blocked by the Mod protein, which further modifies the α subunit of RNA polymerase (50). Two other genes, *alc* and *asiA*, also act to ensure that host transcription is halted. The Alc protein aborts elongation on cytosine-containing DNA (host DNA contains cytosine but T4 DNA uses 5-hydroxymethylcytosine glycosylation), and AsiA inhibits σ^{70} initiation of RNA polymerase (23). T4 also disrupts the host DNA within 2 to 3 min by attaching it to the cell membrane (23) and degrading it with endonuclease II and endonuclease IV (8). Therefore, within 3 to 4 min almost all host transcription is stopped (33).

To activate Hok mRNA for translation, in addition to eliminating Sok antisense RNA, RNase III must truncate the full-length 441- and 398-nucleotide mRNAs to 361 nucleotides (16, 44). At this point in the lytic cycle, there should be relatively little mRNA in the cell since only the T4 early genes are active, leaving the full pool of host RNase III available for maturation of Hok mRNA. Since T4 mature phage does not appear until about 20 min and it takes ~5 min to stop transcription and degrade Sok antisense RNA, this gives 15 min to truncate full-length Hok mRNA, translate Hok mRNA (~3 s [13, 15]), and kill the cell. The rate-limiting step is Hok mRNA truncation since Gerdes et al. (16) show that only ~5% of initial Hok mRNA is truncated 10 min after transcription is halted. Once translated, the toxin acts quickly since the close relative of *hok*, *gef*, kills cells within a few minutes of induction (31); 10 min after shutdown of transcription, ghost cells start to appear, and by 20 min, most cells are dead (16). Therefore, within 15 min *hok/sok* should be able to disrupt the later stages of T4 development (assembly, packaging, and lysis).

Since T4 rapidly stops host mRNA translation (although the mechanism and extent of shutdown are not well understood [23]), Hok mRNA translation may be delayed. Five mechanisms of translational inhibition of host mRNA have been studied: modification of ribosomes, mRNA cleavage by RegB, inhibition of translation by binding of RegA to mRNA, modification of initiation factors, and inhibition of elongation by inhibition of tRNA (20). RegB and RegA bind to specific regions of mRNA (usually near the translation initiation region); however, their known recognition sequences are not found in the translation initiation region sites on Hok mRNA (38, 49), and its fold-back structure (44) would likely prevent binding to full-length Hok mRNA. In spite of T4 attempts to shut off host translation by the other three mechanisms, it appears that enough Hok protein is made to disrupt phage development.

Exclusion of T4 by *hok* is not due to a difference in growth rate between the cells which lack *hok/sok* and those that harbor the locus. Both plasmid-bearing strains, BK6/pMJR1750 (μ = 0.63 h^{-1}) and BK6/pTKW106 (*hok*$^+$; μ = 0.68 h^{-1}), have approximately the same growth rates; yet, the eclipse period, latent period, plaque diameter, plating efficiency, and growth in the presence of phage of BK6/pMJR1750 are dramatically different than those for BK6/pTKW106 (*hok*$^+$) and are much closer to those for faster-growing host BK6. Furthermore, the maximum cell density (OD_{600}) reached in the presence of phage for faster-growing BK6 was higher than that of plasmid-containing BK6/pMJR1750 for all phage tested (λ-virulent, T1, T4 [Fig. 1], T4 rVII, T5, and T7); hence, a reduction in growth rate makes the cell more susceptible to phage attack. This makes T4 exclusion by BK6/pTKW106 relative to faster-growing host BK6 even more significant.

The *hok/sok* killer locus system is found on a low-copy-number plasmid (R1) (15), as are most other killer loci (*ccd* [18], *doc/phd* [24], *parDE* [35], and *pemK/pemI* [45]) and phage-exclusion systems (29). Although *hok/sok* were placed on a high-copy-number, pBR322-derived plasmid for most of these experiments, the demonstration of T4 phage exclusion is still valid and should not be interpreted as an artifact of high copy number (low-copy-number R1 also showed slight exclusion). It was necessary to use a high-copy-number plasmid since, if *hok/sok* were very effective at one to two copies per chromosome equivalent against the phage tested, then their analogs on the chromosome *relB* and *gef/sof* would also exclude phage. However, only phage that could infect *E. coli* were used here; therefore, the phage used had evolved resistance to the chromosomal analogs of *hok/sok*. Furthermore, members of the *hok/sok* killer locus family may be found in most gram-negative bacteria (32); hence, because of the prevalence of antisense killer loci, resistant phage strains are expected.

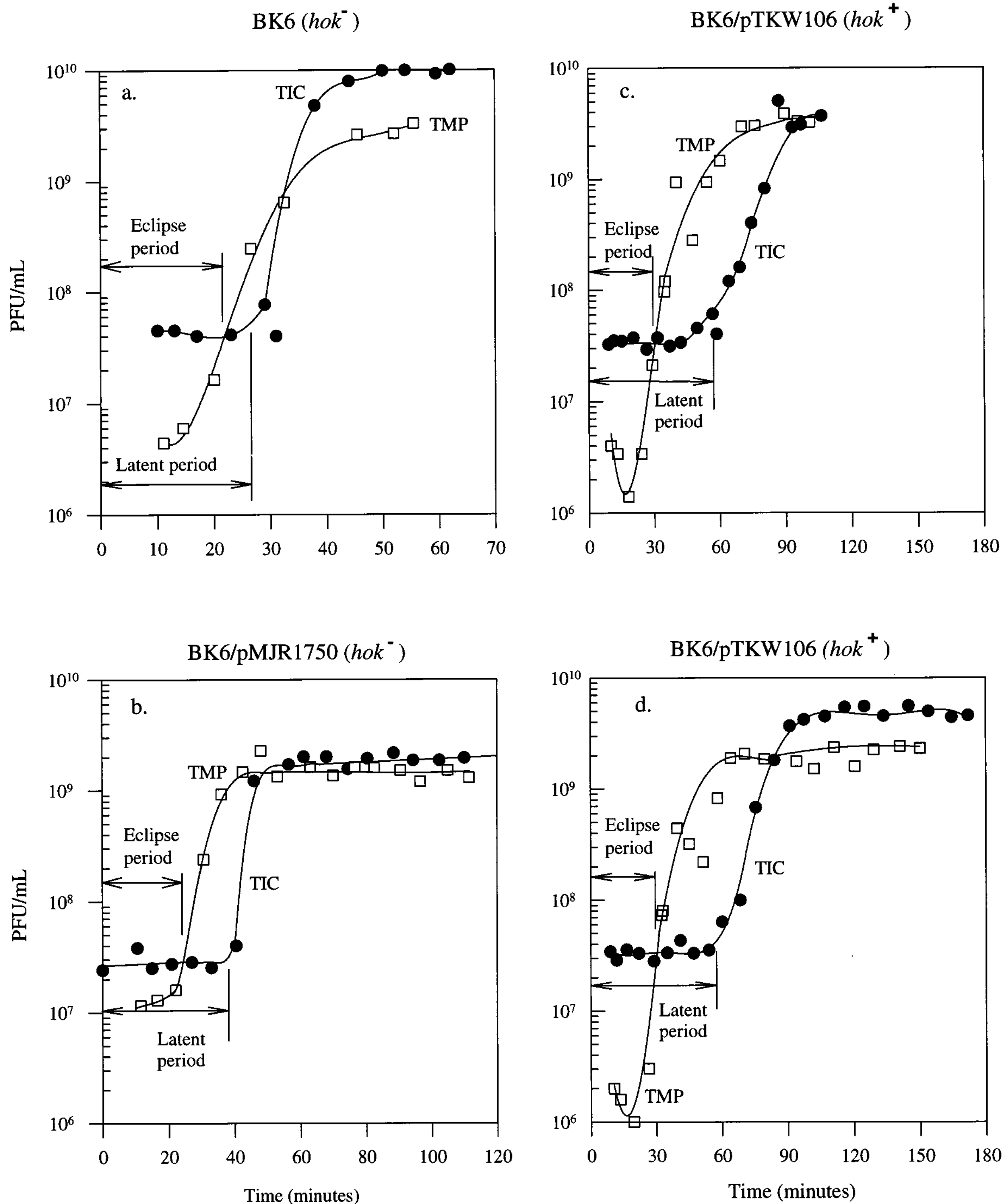

FIG. 2. Single-step growth results with T4 phage (MOI, 0.1 to 0.2) and host BK6 (a), BK6/pMJR1750 (b), and BK6/pTKW106 (c and d [duplicate experiments]). Symbols: ●, TIC (i.e., nonlysed samples); □, TMP (i.e., lysed samples).

Therefore, high gene dosage of *hok* is required to study the evolutionary importance of this locus since all the phage tested have coevolved with the killer loci (recall that two of the well-studied phage-exclusion systems [*rex* and *prr*] function only to exclude mutant phage [40]), and *hok* is not being studied early in its evolutionary development.

The *hok/sok* killer locus did not prevent phage other than T4 from successfully infecting the cell because of the differences in how each virus takes over the cell. During λ infection, host transcription is not shut down; hence, Sok was continually made so Hok was not translated. Phage T1 blocks translation of Hok upon injection by decreasing the proton motive force of the membrane (48). Host transcription declines by 60% 2 min after infection and gradually is reduced by 74 to 99% immediately before cell lysis after 13 min (27). This gradual reduction in host transcription, block of translation, and quick lysis

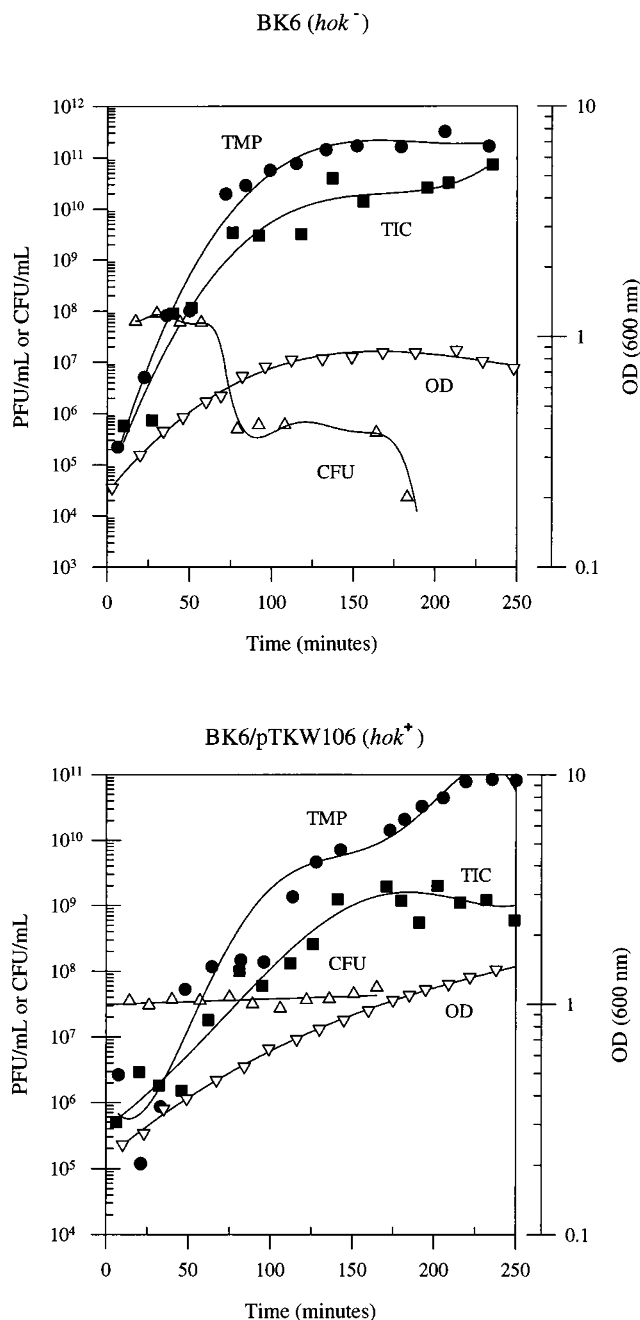

FIG. 3. Infection with 3.7×10^7 PFU of T4 phage (MOI, 0.02) and cell growth of BK6 and BK6/pTKW106.

probably does not allow adequate time for *hok* expression. Surprisingly, phage T5 was not excluded even though it stops host gene transcription and does not stop translation (28). Transcription is blocked by degradation of host DNA and RNA polymerase modification (27). DNA degradation starts within the first 2 min of infection and leads to completely nonfunctional DNA within 8 min (27). There should also be sufficient time before lysis (45 min) for *hok* to be expressed; however, T5 has not been as well studied, so it is possible that it inhibits *hok* expression by some other mechanism. Phage T7 shuts down the host RNA polymerase by phosphorylation at 4 min (gene 0.7, an ATP protein phosphotransferase which phosphorylates serine and threonine residues) (34) and direct inhibition (gene 2) (21). The host DNA is degraded by genes 3 and 6 (27), after which host transcription cannot occur. Gene 0.7 also modifies over 90 proteins (36), several of which are important translational proteins. The activity of RNase III is also elevated by the kinase. The Hok protein contains four serines and three threonines (14) which might also be modified by the kinase. These complex modifications to the translational machinery combined with the short latent phase (12 to 15 min) (21) of T7 prevent *hok* expression and phage exclusion.

The *parDE* killer locus did not exclude any virus tested. Plasmid pOU82 is present in one to two copies per chromosome (19), and this gene dosage may not have been enough to kill the cell before the virus could reproduce. Furthermore, *parDE* is a proteic killer locus whose mechanism has not been determined, although the degradation of *parE* probably requires a protease (35). *ccdB/ccdA* and *doc/phd*, two well-studied proteic systems, require proteases Lon (47) and ClpXP (24), respectively. Lon is used to degrade abnormal proteins such as those made by a virus. The only known substrates of ClpXP besides Phd are bacteriophage proteins (24). It has been observed that the protein antidotes tend to have a half-life of one to two generations (24), which allows sufficient time for viral replication. Jensen et al. (19) have shown that it takes two to three generations for *parDE* to stop growth, though it is very efficient.

Since *hok/sok* excludes T4, this is another form of bacterial altruism in that suicide of the infected cell protects the whole bacterial population (40, 41). This behavior is of no benefit to an isolated cell and can be explained only by examination of bacterial populations (40).

Phage exclusion could be particularly important for cells that clump together or grow in a biofilm. When a virus infects cells on the outside layer of a biofilm, the dead cells form a protective layer which may prevent the internal cells from becoming infected as well as act as a decoy for other phage. In a biofilm, plasmid-borne *hok/sok* could also be conjugated (R1, the native plasmid of *hok/sok*, is able to conjugate) to nearby cells, which would further enhance the population's chance of surviving a viral attack. Hence, phage exclusion provides selective pressure for the presence of killer loci, which helps to explain why these loci are found on both bacterial plasmids and chromosomes.

Models for explaining the evolution of phage and bacterial populations have been proposed, and most are based on the supposition of random mutations (25). However, the evolution of *hok/sok* and their related family of killer loci (15, 32) is an example in which simple mutation is unlikely to have occurred. The complexity of the system, which includes overlapping genes, adjacent ribosome-binding sites, sense and antisense promoters on opposite DNA strands, and fold-back inhibition, all controlling a toxic peptide (44), is too sophisticated to have been created by stepwise mutation. It probably is an example of existing genes being put to a new use by recombination. Killer loci also autoselect against their own mutation or loss. Resistance to this killing action cannot be achieved by a small shift in phage metabolism since phage proteins do not interact with any part of the killer locus. Some of the phage studied have avoided this killer system possibly by making new enzymes or by no longer solely stopping host transcription (at the expense of reduced phage productivity).

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation under grant BES-9224864.

We thank John Keener for kindly providing both λ-virulent and T4 rVII phage, Edward Goldberg for suggesting the single-step growth

experiments and helpful comments, Kenn Gerdes for providing pOU82 parDE, and Stanley Cohen and Chris Miller for providing R1.

REFERENCES

1. **Bech, F. W., S. T. Jørgensen, B. Diderichsen, and O. H. Karlström.** 1985. Sequence of the *relB* transcription unit from *Escherichia coli* and identification of the *relB* gene. EMBO J. **4:**1059–1066.
2. **Benzer, S.** 1966. Adventures in the rII region, p. 157–165. *In* J. Crains, S. Stent, and J. D. Watson (ed.), Phage and the origins of molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Bergsland, K., J. Kao, Y. N. Yu, R. Gulati, and L. Snyder.** 1990. A site in the T4 bacteriophage major head protein gene that can promote the inhibition of all translation in *Escherichia coli*. J. Mol. Biol. **213:**477–494.
4. **Blohm, D.** 1979. Structural elements of the R1 plasmid and their rearrangement in derivatives of it and their miniplasmids, p. 233–241. *In* K. N. Timmis and A. Pühler (ed.), Plasmids of medical, environmental and commercial importance: developments in genetics, vol. 1. Elsevier/North-Holland Biomedical Press, New York.
5. **Carlson, K.** 1994. Single-step growth, p. 434–437. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.
6. **Carlson, K., and E. S. Miller.** 1994. General procedures, p. 427–429. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.
7. **Carlson, K., and E. S. Miller.** 1994. Working with T4, p. 421–426. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.
8. **Carlson, K., E. A. Raleigh, and S. Hattman.** 1994. Restriction and modification, p. 369–381. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.
9. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100:**293–308.
10. **Cram, H. K., D. Cram, and R. Skurray.** 1984. F plasmid *pif* region: Tn*1725* mutagenesis and polypeptide analysis. Gene (Amsterdam) **32:**251–254.
11. **Feng, J., K. Yamanaka, H. Niki, T. Ogura, and S. Hiraga.** 1994. New killing system controlled by two genes located immediately upstream of the *mukB* gene in *Escherichia coli*. Mol. Gen. Genet. **243:**136–147.
12. **García, L. R., and I. J. Molineux.** 1995. Rate of translocation of bacteriophage T7 DNA across the membranes of *Escherichia coli*. J. Bacteriol. **177:**4066–4076.
13. **Gausing, K.** 1972. Efficiency of protein and messenger RNA synthesis in bacteriophage T4-infected cells of *Escherichia coli*. J. Mol. Biol. **71:**529–545.
14. **Gerdes, K., F. W. Bech, S. T. Jørgensen, A. Løbner-Oleson, P. B. Rasmussen, T. Atlung, L. Boe, O. Karlström, S. Molin, and K. von Meyenburg.** 1986. Mechanism of postsegregational killing by the *hok* gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli relB* operon. EMBO J. **5:**2023–2029.
15. **Gerdes, K., L. K. Poulsen, T. Thisted, A. K. Nielsen, J. Martinussen, and P. H. Andreasen.** 1990. The *hok* killer gene family in gram-negative bacteria. New Biol. **2:**946–956.
16. **Gerdes, K., T. Thisted, and J. Martinussen.** 1990. Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1: *sok* antisense RNA regulates formation of *hok* mRNA species correlated with killing of plasmid-free cells. Mol. Microbiol. **4:**1807–1818.
17. **Jacob, A. E., J. A. Shapiro, L. Yamamoto, D. I. Smith, S. N. Cohen, and D. Berg.** 1977. Appendix B, p. 601–670. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Jaffé, A., T. Ogura, and S. Hiraga.** 1985. Effects on the *ccd* function of the F plasmid on bacterial growth. J. Bacteriol. **163:**841–849.
19. **Jensen, R. B., E. Grohomann, H. Schwab, R. Díaz-Orejas, and K. Gerdes.** 1995. Comparison of *ccd* of F, *parDE* of RP4, and *parD* of R1 using a novel conditional replication control system of plasmid R1. Mol. Microbiol. **17:** 211–220.
20. **Kano-Sueoka, T., and N. Sueoka.** 1968. Characterization of a modified leucyl-t-RNA of *Escherichia coli* after bacteriophage T2 infection. J. Mol. Biol. **37:**475–491.
21. **Krüger, D. H., and C. Schroeder.** 1981. Bacteriophage T3 and bacteriophage T7 virus-host cell interactions. Microbiol. Rev. **45:**9–51.
22. **Kulakauskas, S., A. Lubys, and S. D. Ehrlich.** 1995. DNA restriction-modification systems mediate plasmid maintenance. J. Bacteriol. **177:**3451–3454.
23. **Kutter, E., T. White, M. Kashlev, M. Uzan, J. McKinney, and B. Guttman.** 1994. Effects on host genome structure and expression, p. 357–368. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.
24. **Lehnherr, H., and M. B. Yarmolinsky.** 1995. Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **92:**3274–3277.
25. **Lenski, R. E., and J. E. Mittler.** 1993. The direct mutation controversy and neo-Darwinism. Science (Washington, D.C.) **259:**188–194.
26. **Masuda, Y., K. Miyakawa, Y. Nishimura, and E. Ohtsubo.** 1993. *chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for stable maintenance of plasmid R100. J. Bacteriol. **175:**6850–6856.
27. **McCorquodale, D. J.** 1975. The T-odd bacteriophages. Crit. Rev. Microbiol. **4:**100–159.
28. **McCorquodale, D. J., and Y. T. Lanni.** 1970. Patterns of protein synthesis in *Escherichia coli* infected by amber mutants in the first-step-transfer DNA of T5. J. Mol. Biol. **48:**133–143.
29. **Molineux, I. J.** 1991. Host-parasite interactions: recent developments in the genetics of abortive phage infections. New Biol. **3:**230–236.
30. **Poulsen, L. K., A. Refn, S. Molin, and P. Andersson.** 1991. The *gef* gene from *Escherichia coli* is regulated at the level of translation. Mol. Microbiol. **5:**1639–1648.
31. **Poulsen, L. K., A. Refn, S. Molin, and P. Andersson.** 1991. Topographic analysis of the toxic Gef protein from *Escherichia coli*. Mol. Microbiol. **5:**1627–1637.
32. **Poulsen, L. K., N. W. Larsen, S. Molin, and P. Andersson.** 1989. A family of genes encoding a cell-killing function may be conserved in all gram-negative bacteria. Mol. Microbiol. **3:**1463–1472.
33. **Rabussay, D.** 1983. Regulation of gene expression transcription: phage-evoked changes in RNA polymerase, p. 167–173. *In* C. Mathews, E. M. Kutter, G. Mosig, and P. B. Berget (ed.), Bacteriophage T4. American Society for Microbiology, Washington, D.C.
34. **Rahmsdorf, H. J., S. H. Pai, H. Ponta, P. Herrlich, R. Roskoski, Jr., M. Schweiger, and F. W. Studier.** 1974. Protein kinase induction in *Escherichia coli* by bacteriophage T7. Proc. Natl. Acad. Sci. USA **71:**586–589.
35. **Roberts, R. C., A. R. Ström, and D. R. Helinski.** 1994. The *parDE* operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. J. Mol. Biol. **237:**35–51.
36. **Robertson, E. S., L. A. Aggison, and A. W. Nicholson.** 1994. Phosphorylation of elongation factor G and ribosomal protein S6 in bacteriophage T7-infected *Escherichia coli*. Mol. Microbiol. **11:**1045–1057.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Sanson, B., and M. Uzan.** 1993. Dual role of the sequence-specific bacteriophage T4 endoribonuclease Reg B: mRNA inactivation and mRNA destabilization. J. Mol. Biol. **233:**429–446.
39. **Schmitt, C. K., and I. J. Molineux.** 1991. Expression of gene 1.2 and gene 10 of bacteriophage T7 is lethal to F plasmid-containing *Escherichia coli*. J. Bacteriol. **173:**1536–1543.
40. **Shub, D. A.** 1994. Bacterial altruism? Some strains of *Escherichia coli* harbor genes that trigger cell death upon infection by bacteriophage T4; these may provide examples of the evolution of altruistic behavior in bacteria. Curr. Biol. **4:**555–556.
41. **Snyder, L.** 1995. Phage-exclusion enzymes: a bonanza of biochemical and cell biology reagents? Mol. Microbiol. **15:**415–420.
42. **Snyder, L., and G. Kaufmann.** 1994. T4 phage exclusion mechanisms, p. 391–396. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.
43. **Stark, M. J. R.** 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene (Amsterdam) **51:**255–267.
44. **Thisted, T., N. S. Sørensen, and K. Gerdes.** 1995. Mechanism of post-segregational killing: secondary structure analysis of the entire Hok mRNA from plasmid R1 suggests a fold-back structure that prevents translation and antisense RNA binding. J. Mol. Biol. **247:**859–873.
45. **Tsuchimoto, S., Y. Nishimura, and E. Ohtsubo.** 1992. The stable maintenance system *pem* of plasmid R100: degradation of PemI protein may allow PemK protein to inhibit cell growth. J. Bacteriol. **174:**4205–4211.
46. **Tyndall, C., J. Meister, and T. A. Bickle.** 1994. The *Escherichia coli prr* region encodes a function type IC DNA restriction system closely integrated with an anticodon nuclease gene. J. Mol. Biol. **237:**266–274.
47. **Van Melderen, L., P. Bernard, and M. Couturier.** 1994. Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. Mol. Microbiol. **11:**1151–1157.
48. **Wagner, E. F., H. Ponta, and M. Schweiger.** 1980. Development of *Escherichia coli* virus T1: the role of the proton-motive force. J. Biol. Chem. **255:**534–539.
49. **Wiberg, J. S., and J. D. Karam.** 1983. Processing and translation: translational regulation in T4 phage development, p. 193–201. *In* C. K. Mathews, E. M. Kutter, G. Mosig, and P. B. Berget (ed.), Bacteriophage T4. American Society for Microbiology, Washington, D.C.
50. **Wilkens, K., and W. Rüger.** 1994. Transcription from early promoters, p. 132–141. *In* J. D. Karam (ed.), Molecular biology of bacteriophage T4. American Society for Microbiology, Washington, D.C.
51. **Wood, T. K., R. H. Kuhn, and S. W. Peretti.** 1990. Enhanced plasmid stability through post-segregational killing of plasmid-free cells. Biotechnol. Tech. **4:**36–41.
52. **Wood, T. K., and S. W. Peretti.** 1991. Effect of chemically-induced, cloned-gene expression on protein synthesis in *E. coli*. Biotechnol. Bioeng. **38:**397–412.