Activation of a prophage-encoded tyrosine kinase by a heterologous infecting phage results in a self-inflicted abortive infection

David I. Friedman,[1]* Cara C. Mozola,[1†] Karen Beeri,[1‡] Ching-Chung Ko[2] and Jared L. Reynolds[1§]
[1]*Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109, USA.*
[2]*Pittsburgh Bacteriophage Institute, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA.*

Summary

Bacteria in their struggle for survival have evolved or acquired defences against attacking phage. However, phage often contribute to this defence through mechanisms in which a prophage protects the bacterial population from attack by another, often unrelated, phage. The 933W prophage, which carries Shiga toxin genes that enhance pathogenicity of enterohaemorrhagic *Escherichia coli* strain O157:H7, also carries the *stk* gene encoding a eukaryotic-like tyrosine kinase that excludes (aborts) infection by phage HK97. This exclusion requires the kinase activity of Stk. Little, if any, protein tyrosine phosphorylation can be detected in a 933W lysogen prior to infection with HK97, while extensive Stk-mediated tyrosine phosphorylation is evident following infection. This includes autophosphorylation that stabilizes Stk protein from degradation. Although increased levels of Stk are found following HK97 infection, these higher levels are not necessary or sufficient for exclusion or protein phosphorylation. An HK97 open reading frame, *orf41*, is necessary for exclusion and Stk kinase activity. We hypothesize that interaction with gp41 stimulates Stk kinase activity. Exclusion of HK97 appears to be specific since other phages tested, λ, φ80, H-19B, λ-P22dis and T4rII, were not excluded. Infection of the 933W lysogen with a non-excluded phage fails to induce Stk-determined phosphorylation.

Introduction

Bacteria, under constant threat of attack by phage, have evolved or acquired an array of defences against these viral as well as other assailants, e.g. restriction systems (Arber, 1971), CRISPR (Brouns *et al.*, 2008) and toxin–antitoxin systems (Fineran *et al.*, 2009). Prophage, through a variety of mechanisms referred to as exclusion also can contribute to the defence of their bacterial hosts (Duckworth *et al.*, 1981; Snyder, 1995; Gottesman, 1998; Chopin *et al.*, 2005). Some exclusion systems block entry of the infecting phage or destroy the infecting DNA, while others abort infection resulting in death of the infected bacterium before infectious phage can be produced, a dead end for the attacking phage. Although the infected bacterium dies, the failure in production of the attacking phage prevents spread to other members of the bacterial population. Hence, the resident prophage, while not protecting its own host bacterium, indirectly protects the remaining members of the population. Death of a fraction of a bacterial population to protect the remaining population has colloquially been referred to as altruistic behaviour (Shub, 1994; Ackermann *et al.*, 2008), a behaviour widely observed in nature (West and Gardner, 2010). Historically, Rex exclusion (Fig. 1A) expressed by λ prophage (Howard, 1967; Matz *et al.*, 1982; Parma *et al.*, 1992), which aborts infection of T4rII mutants, gained broad recognition through its use in classic molecular genetic studies (Benzer, 1966).

Coliphage 933W was isolated from the O157:H7 enterohaemorrhagic *Escherichia coli* (EHEC) strain EDL933 (O'Brien *et al.*, 1984). The phage genome includes genes encoding Shiga toxin 2 (*stx2 A* and *B*), the virulence factor responsible for the most serious sequelae of EHEC infection (Kaper *et al.*, 2004). 933W is a member of the lambdoid family of phages (Tyler *et al.*, 2005), so named because they have genomes arranged similarly to that of λ (Fig. 1A), with most genes shared from a large gene pool (Campbell and Botstein, 1983; Campbell, 1994; Hendrix, 2006). Thus, a lambdoid phage through recombination may share some genes with some members and other genes with other members of the family. For example, 933W (Fig. 1) has the same N transcription protein and
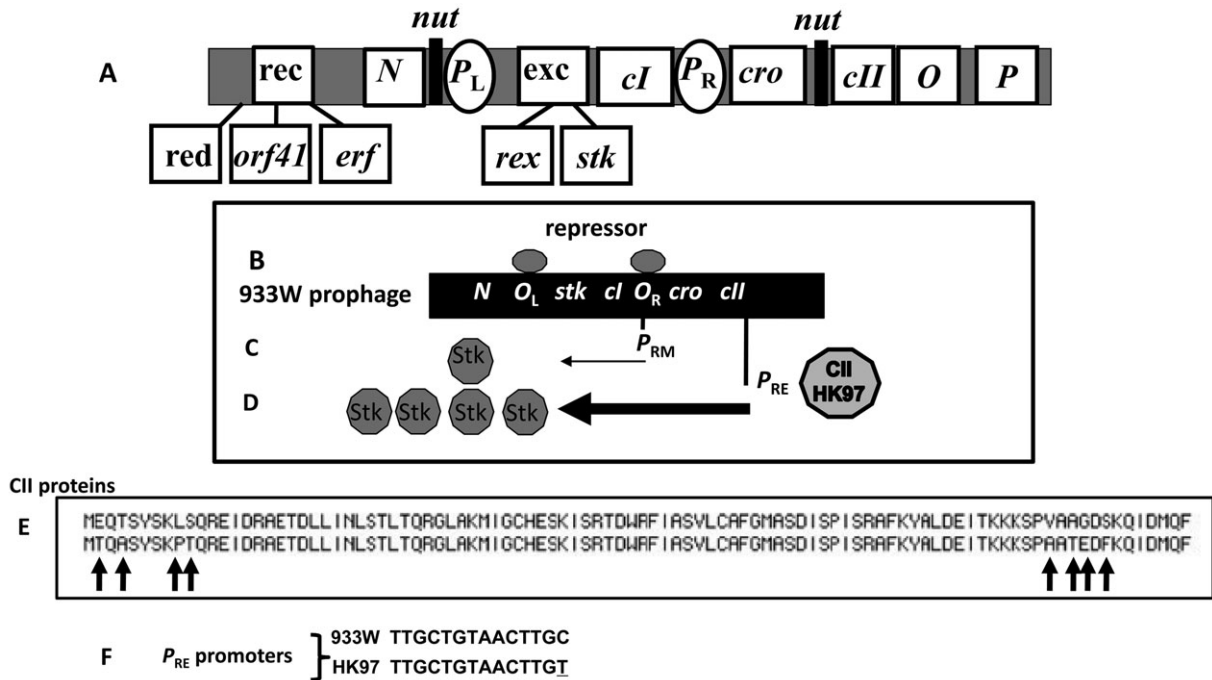
Fig. 1. Early regulatory region of lambdoid phages and control of Stk expression by infecting HK97.
A. Genetic map of early regulatory region of lambdoid phages showing position of selected genes. Shown are the similar locations (exc) of the *rex* gene of λ and *stk* gene of 933W and the similar locations (rec) of the *red* genes of λ, *erf* gene of P22, and *orf41*s of HK97 and HK022 (not drawn to scale).
B. 933W *cI* operon showing position of *stk* and other genes relevant to this study (not drawn to scale).
C. Transcription of *cI* operon (includes *stk*) during lysogeny.
D. Action of HK97 CII protein in stimulating transcription of 933W CI operon.
E. Comparison of 933W and HK97 CII proteins (arrows indicate positions of codon differences).
F. Similarity of P_{RE} promoters of 933W and HK97 (single nucleotide difference is underlined).

recognition sites (*nut*) flanking the *cI* repressor gene as another lambdoid phage, HK97 (Friedman and Court, 2001). However, these two phages have different *cI* genes (Plunkett *et al.*, 1999; Juhala *et al.*, 2000). In contrast, HK97 and λ have different *N* genes and *nut* sites, but share the same *cI* gene. However, HK97 and λ both lack the *stx2* genes and the gene of our interest *stk*, both found in 933W.

The *stk* gene encodes a eukaryotic-like tyrosine kinase (Plunkett *et al.*, 1999; Tyler and Friedman, 2004). Although for many years thought to be limited to higher organisms, eukaryotic-like tyrosine as well as serine-threonine kinases have been identified in a number of bacteria (Kennelly and Potts, 1996; Leonard *et al.*, 1998; Grange-asse *et al.*, 2007; Pereira *et al.*, 2011) as well as phage (Robertson *et al.*, 1994). Because it is in the same operon as the *cI* repressor gene, *stk* is expressed by the prophage; i.e., Stk is constitutively expressed at low levels from the P_{RM} promoter (Fig. 1 B and C) (Tyler and Friedman, 2004).

We report that a lysogen of 933W through the activity of the Stk tyrosine kinase excludes infection by HK97 (Dhillon *et al.*, 1980; Juhala *et al.*, 2000). The 933W *stk* gene is positioned, like the *rex* exclusion genes of λ (Howard, 1967), downstream of the *cI* gene (Fig. 1A). Although

Stk-directed protein phosphorylation is not observed in extracts of the 933W lysogen, significant phosphorylation is observed following infection by the excluded HK97 phage. An HK97 open reading frame, *orf41*, is required to activate the Stk expressed from the 933W prophage that leads to exclusion of the infecting HK97 phage.

Results

Exclusion of HK97 by the 933W prophage

Efficiency of plating (eop) was used to obtain a quantitative measure of HK97 exclusion by the 933W prophage. The *E. coli* K12 strain K37 served as the standard (Tables 1 and 2). A reduction in eop of approximately five logs was observed when titres of an HK97 lysate measured on lawns of K9675 (a derivative of K37 with a 933W prophage) and K37 were compared (Table 1). This exclusion is due to the tyrosine kinase activity of the 933W *stk* gene product (gp) as shown by eop measurements with strain K11515, a derivative of K9675 in which the 933W *stk* gene has a single codon change that eliminates Stk kinase activity (Tyler and Friedman, 2004). A comparison

Table 1. Eop phage (titre on tested strain/titre on K37).

Tested Strain	933W prophage	*stk* allele	HK97	HK022	HK97 *orf41-1*	HK97Δ*cII*<>*cat*	HK97Δ*41*	HK97Δ*41*Δ*cII*<>*cat*
K37[b]	–	–	1	1	1	1	1	1
K9675	+	wt	~10^{-5}	0.5[a]	~1	~10^{-5}	~1	~1
K11515	+	*K42R*	~1	ND	ND	ND	ND	ND
K10567	+	*stk-c-Myc*	~10^{-4}	ND	ND	ND	ND	ND

a. Much smaller plaques on K9675.
b. Control.
See *Experimental procedures* for details.
ND, not done.

of HK97 titres on lawns of K37 and K11515 revealed no differences; i.e., the eop on K11515 using K37 as the standard was ~1. In addition, exclusion appears to be specific for HK97. A number of other phages tested were not excluded (Table 2). These included λ (Hendrix *et al.*, 1983), φ80 (Matsushiro, 1961), H-19B (Scotland *et al.*, 1983; Huang *et al.*, 1986) and λ-P22*dis* (a λ-P22 hybrid, see below) (Yamamoto *et al.*, 1977) as well as T4rII (Benzer, 1955). All of those phages plate with an eop of ~1 on K11541, a derivative of K9675 in which a second exclusion system that affects some phages, including λ and H-19B (unpublished results, this laboratory) was deleted. An exception was HK022 (Dhillon and Dhillon, 1976), which was partially excluded (see below).

HK97 infection and Stk-directed protein phosphorylation

Tyrosine phosphorylation of proteins was examined by immunoblot analysis using an anti-phosphotyrosine antibody. To begin, we consider two limitations regarding the use of immunoblots in this paper. (1) A large number of proteins are phosphorylated by Stk and therefore the immunoblots are used to provide a measure of total protein phosphorylation without regard to individual proteins. (2) Although small differences in intensity in different regions of the immunoblots were observed from experiment to experiment, the overall patterns of large or small amounts of total phosphorylation were consistently observed. These small differences likely reflect the lack of specificity of Stk phosphorylation activity.

Our immunoblot analyses showed very little tyrosine phosphorylation in the uninfected 933W lysogen K9675 (Figs 2 and 4). However, HK97 infection of 933W lysogens K9675 and K10567 results in extensive tyrosine

Table 2. Eop (titre on K11541/titre on K37).

HK97	λ	HK022	λ-P22*dis*	H-19B	φ80	T4*rII*
< 10^{-4}	~1	0.5[a]	~1	~1	~1	~1

a. Much smaller plaques on K11541.
See *Experimental procedures* for details.

phosphorylation (Figs 2–5). This phosphorylation was shown to result from the kinase activity of Stk using K11515, the derivative of K9675 with a single base change at *stk* codon 42 that eliminates kinase activity (Tyler and Friedman, 2004). HK97 infection of this strain failed to direct protein phosphorylation (Fig. 2).

Timing of Stk synthesis and protein phosphorylation following HK97 infection

We followed Stk production and protein phosphorylation after HK97 infection of K10567, a lysogen in which the 933W prophage has a c-Myc tagged *stk* gene that retains close to wild-type function (Fig. 3 and Table 1). The tagged Stk was used because our anti-Stk antibody was ineffective at identifying Stk at levels expressed during infection with HK97. Stk levels were detected at 10 min post infection and preceded protein phosphorylation which was not observed until ~15 min following infection. Significant phosphorylation was not observed until ~20 min.
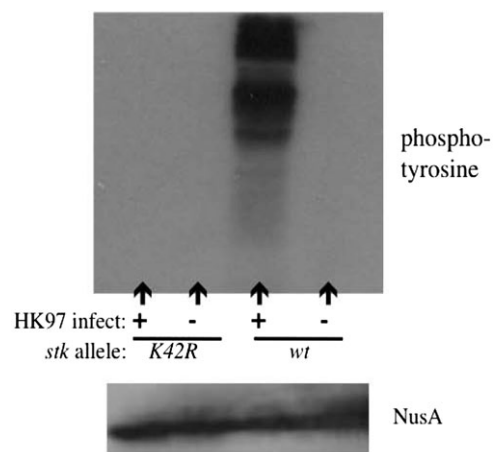

Fig. 2. Immunoblot showing Stk-directed tyrosine phosphorylation following infection of 933W lysogens with HK97. Strain K9675 (*stk*+): lanes: 3 (infected) and 4 (uninfected). Strain K11515 (*stkK42R*): lanes: 1 (infected) and 2 (uninfected). NusA probed as a loading control. See *Experimental procedures* for details.
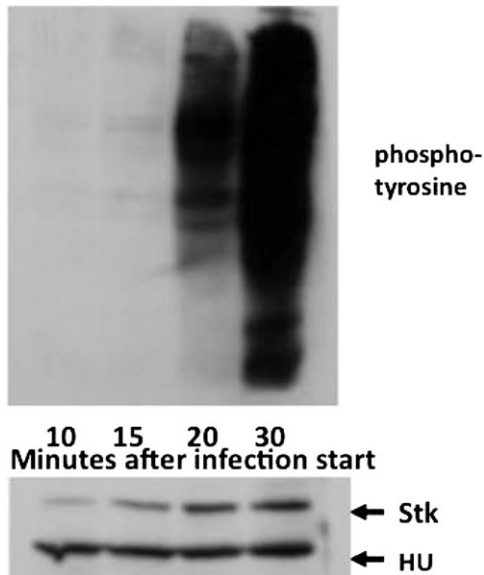
Fig. 3. Timing of Stk production and protein phosphorylation following HK97 infection of 933W lysogen (K10567). Ten-millilitre aliquots were removed at indicated times and samples prepared for immunoblot analysis. The commercial anti-c-Myc used to identify Stk was fortuitously contaminated with anti-HU, which served as a loading control. See *Experimental procedures* for details.

HK97 CII protein and Stk synthesis

Because *stk* is in the same 933W operon as *cI* repressor (Tyler *et al.*, 2004), we reasoned that the HK97 function responsible for activating Stk both in its roles of excluding HK97 and promoting protein phosphorylation would likely increase transcription of *cI* and genes in that operon from the low levels expressed from the 933W prophage,
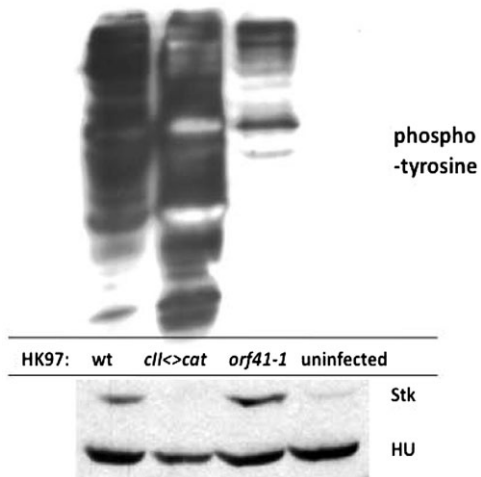
autogenously regulated at P_{RM} by repressor (Ptashne *et al.*, 1976) (Fig. 1 B–D). The λ CII protein and its homologues in other lambdoid phage act at promoters, P_{RE} (Fig. 1D) to direct high-level transcription of *cI* (Reichardt and Kaiser, 1971; Wulff and Rosenberg, 1983; Ho and Rosenberg, 1988). This led us to hypothesize that the HK97 and 933W CII proteins might be so similar that the CII protein expressed by the infecting HK97 binds at the P_{RE} of the 933W prophage directing high levels of transcription of the 933W *cI* operon with its downstream *stk* gene (Fig. 1D). Comparison of CII and P_{RE} sequences of HK97 and 933W showed they are indeed very similar (Fig. 1E and F).

Infection with HK97 resulted in a substantial increase in Stk, while a mutant HK97 with a deletion substitution for the *cII* gene, HK97*cII<>cat*, failed to show evidence of increased synthesis (Fig. 4). This is consistent with our hypothesis that HK97 CII acts to direct high-level expression of Stk. Surprisingly, despite the failure to increase synthesis of Stk, HK97 *cII<>cat* was excluded by the 933W prophage (Table 1) and directed levels of protein phosphorylation comparable to those directed by the wild-type HK97 (Fig. 4). Hence, HK97-directed protein phosphorylation and associated exclusion do not require high-level expression of Stk.

Identifying the HK97 function(s) required for exclusion

To identify the HK97 function(s) involved in exclusion by Stk, we selected for and isolated a mutant of HK97 not excluded by the 933W prophage (Table 1). Comparison of

Fig. 4. Immunoblot showing Stk-directed protein phosphorylation and production following infection of K10567 with HK97 variants and an uninfected control (K10567). The commercial anti-c-Myc used in this experiment as discussed in the legend to Fig. 3 allowed identification of Stk and HU. See *Experimental procedures* for details.
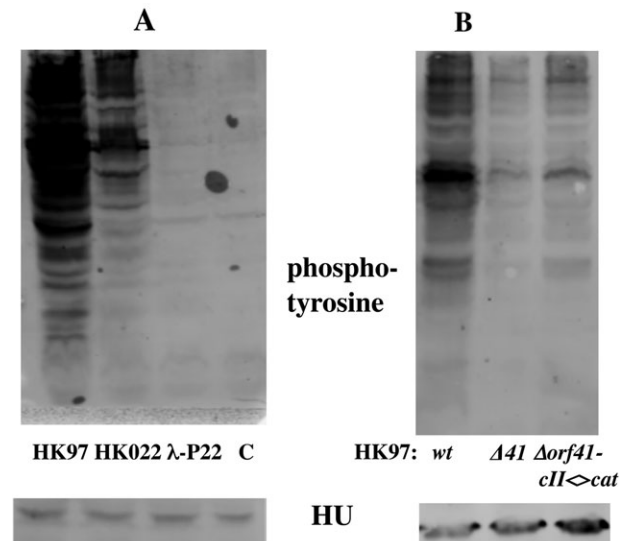

Fig. 5. Immunoblots showing protein phosphorylation by Stk following infection of K10567 by phage with *orf41*-like genes. HU served as loading control.
A and B. Comparison following infections with different phages and uninfected control (C).

the DNA sequences of the mutant and parental HK97 phages showed a difference in only one gene, *orf 41*. This gene has partial sequence homology with the *erf* gene of phage P22 (Juhala *et al.*, 2000; Hendrix, 2006). The P22 *erf* gene encodes a recombination function essential for phage growth in a *rec⁻* host (Botstein and Matz, 1970). The genome position of *orf41* is analogous to that of *erf* (Botstein and Matz, 1970) as well as the λ *red* recombination genes (Oppenheim and Court, 1983) (Fig. 1A). Taken together, these observations suggest that *orf41* encodes a recombination function. The HK97 *orf41* mutation allowing growth in a 933W lysogen causes a frameshift resulting in premature translation termination. Based on this observation, we have named the mutant phage HK97*orf41-1*. DNA sequencing showed that the *cII* gene in this mutant phage is wild-type. Infection with HK97*orf41-1* results in increased levels of Stk comparable to those observed in the wild-type infection (Fig. 4). Notwithstanding the elevated levels of Stk, infection with HK97*orf41-1* results in a significantly lower level of protein phosphorylation (Fig. 4).

To confirm that *orf41* is necessary for the Stk-directed exclusion by the 933W prophage, we constructed a derivative of HK97 with *orf41* deleted. This phage, HK97Δ*orf41*, exhibits the same phenotypes as HK97*orf41-1* during infection of a 933W lysogen; i.e., is not excluded and promotes only low observable levels of protein phosphorylation by Stk (Table 1 and Fig. 5B).

With these results in mind, we return to the observation that infection of a 933W lysogen with HK97*cII<>cat* mutant results in phosphorylation levels similar to those seen with wild-type HK97 infection without a concomitant increase in Stk levels. This led us to the question whether the *cII* mutant relies on a different pathway for activating Stk, one independent of gpOrf41. To answer this question, we constructed HK97Δ*orf41-cII<>cat*. This phage exhibits precisely the same phenotypes as HK97Δ*orf41*; on infection of a 933W lysogen shows no defect in plating (Table 1) and exhibits the same weak protein phosphorylation (Fig. 5B). Hence, the gporf41 is responsible for the exclusion and the significant level of protein phosphorylation observed following infection of the 933W lysogen with HK97*cII<>cat*.

Other phages and protein phosphorylation

To further determine if there is a correlation between the effect of Stk on exclusion and protein phosphorylation, we examined protein phosphorylation following infection with a weakly excluded phage, HK022, and one that is not excluded by this system, λ-P22 *dis*, a hybrid phage that includes the P22 *erf* gene (Yamamoto *et al.*, 1978). Neither of these phages is affected by the second 933W exclusion system discussed above (unpublished results,

this laboratory). Results of these experiments are consistent with the previously discussed eop results (Table 1). Infections with λ-P22*dis* failed to result in observable protein phosphorylation (Fig. 5A). Infection with HK022, whose *orf41* shares extensive homology with HK97 *orf41* (Juhala *et al.*, 2000), results in protein phosphorylation. However, the levels are less than those observed following HK97 infection (Fig. 5A).
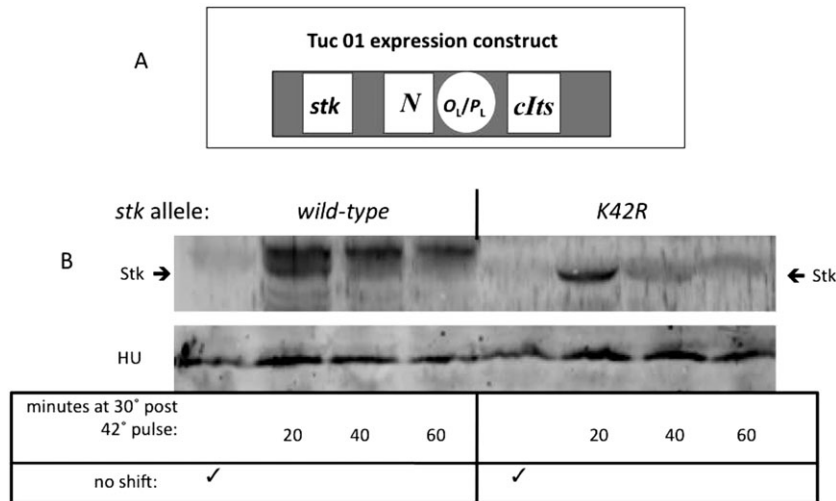
Functional relation of the orf41 and P22 erf gene products

The HK97 *orf41* was annotated as *erf*-like (Juhala *et al.*, 2000) based on the observation that there is significant identity in its 5′ region with the 5′ region of the P22 *erf* gene (Hendrix, 2006). The P22 *erf* (essential recombination function) gene was so named because its product is required for phage growth in a RecA deficient host. Under that condition, Erf is required for circularization of the terminally redundant P22 genome, a step required for phage growth (Botstein and Matz, 1970). We found that HK97Δ*orf41* growth is not significantly reduced in a *recA* mutant, K3701 (data not shown) providing compelling evidence that gpOrf41, although possibly encoding a recombination function, is not essential for HK97 growth in the absence of host recombination activity. The infecting HK97 DNA most likely circularizes by a mechanism similar to that of λ (Yarmolinsky, 1971).

Autophosphorylation and Stk stability

We examined Stk stability and autophosphorylation using strains K11569, encoding the c-Myc-tagged *stk* gene, and K11580, isogenic except for the *stkK42R* mutation that eliminates kinase activity. These strains have these *stk* genes cloned downstream of the λ P_L promoter in the TUC 01 strain (Datta *et al.*, 2008) (Fig. 6A). This places the *stk* genes under control of the strong P_L promoter (Shatzman *et al.*, 1983) and temperature sensitive λ CI857 repressor (Sussman and Jacob, 1962), which denatures at high temperature (e.g. 42°) and renatures at low temperature (Fig. 6A). Hence, Stk will only be expressed during the time the bacteria are grown at high temperature.

The following briefly outlines the experimental design we used to examine Stk stability (see *Experimental procedures* for details). Bacteria were grown at 30° to early log phase, where Stk is not expressed, shifted for 20 min to 42°, where Stk is expressed, and, following treatment in an ice bath to insure that repressor renatures, shifted to 30° where again Stk is not expressed. Stk levels and physical properties were measured by immunoblot from aliquots removed at times indicated in Fig. 6B after the shift back to 30°.

Fig. 6. *In vivo* stability of Stk.
A. Relevant features of chromosomally located TUC 01 construct (Datta *et al.*, 2008); the *stk* gene is placed downstream of the λ P_L promoter under control of the temperature sensitive renaturable CI857 repressor.
B. Immunoblot showing fate of wild-type Stk, strain K11569 (left) and K42R mutant, strain K11580 (right). Stk was expressed for 20 min by a shift to 42°, where CI857 repressor is denatured (Sussman and Jacob, 1962), followed by shift to 30° for indicated times, where renatured CI857 repressor blocks new synthesis of Stk.

The results of the experiment are shown in Fig. 6B. Two differences are apparent between Stk in the strains expressing wild-type (K11569) and those expressing K42R (K11580) Stk. First, at 20 min post shift, wild-type Stk appears as two distinct, but closely migrating bands. In contrast, Stk-K42R appears as a single band that comigrates with the lower, faster migrating, wild-type Stk band. This suggests that the slower migrating band from the wild-type results from autophosphorylation. This is consistent with our previous study showing evidence of Stk autophosphorylation (Tyler and Friedman, 2004). Second, the faster-migrating (non-autophosphorylated) bands of both wild-type and Stk-K42R origin are relatively unstable. By 40 min post shift to 30°, loss of Stk is substantial in isolates from K11580. In contrast, at 60 min post shift there is still significant Stk of the slower migrating species from K11569. From the fact that the slower migrating band is the stable form, we conclude that autophosphorylation contributes to Stk stability.

High expression of Stk and bacterial viability

High levels of Stk can be lethal for *E. coli* as shown by studies with the TUC 01 *stk* construct (Fig. 6A). Cultures of K11569 (contains the TUC 01 *stk* construct) and K11580 (contains the TUC 01 *stk-k42R* construct) were grown at 30°. Each culture was divided into two aliquots; one aliquot maintained at low temperature while the other pulsed for 30 min at 42°. As discussed, the shift to 42° denatures the λ CI857 ts repressor allowing expression of Stk or Stk-K42R. Following the pulse, aliquots were cooled in ice to insure repressor renaturation. Dilutions of each aliquot were spread on LB plates that were incubated overnight at 32° (a temperature at which the CI857 repressor is stable) to measure viable bacterial count. Under these conditions the construct expressing func-

tional Stk shows extensive protein phosphorylation (data not shown).

Results of the experiment are shown in Table 3. Comparing growth of the two strains at 30°, we find both had the same titre, $\sim 2 \times 10^8$. However, at 42°, where the bacteria grow faster, the titre of the bacteria with the *stk-K42R* allele is greater than 50-fold higher than the titre of the bacteria with the wild-type *stk* allele (Table 3). Moreover, the survivors of 42° growth took longer to recover. We conclude that expression of Stk can be lethal to the bacterium. Second, we infer that the lethal effect is due to protein phosphorylation. However, that conclusion must be tempered considering the results discussed above that less Stk is present in bacteria expressing Stk-K42R.

Discussion

Bacterial protein kinases, like those in higher organisms, modulate activity of many proteins including a large number of those involved in regulatory processes (Krell *et al.*, 2010). Many of these are histidine kinases (Mascher *et al.*, 2006; Pereira *et al.*, 2011). Bacterial tyrosine kinases are rare (Grangeasse *et al.*, 2007), with eukaryotic-like tyrosine kinases even less abundant (Leonard *et al.*, 1998). Hence, it was surprising to find a

Table 3. Survival following 20 min expression of Stk.

Strain	*stk* allele	Shift to 42°[a]	Titre
K11569	wt	No	2×10^8
K11569	wt	Yes	5×10^{6}[b]
K11580	*K42R*	No	2×10^8
K11580	*K42R*	Yes	3×10^8

a. Stk only expressed during 20 min at 42°.
b. Survivors took longer to recover.
See *Experimental procedures* for details.

eukaryotic-like tyrosine kinase encoded in the genome of a phage. Although phage-encoded protein kinases are uncommon, one of the best studied is the serine/threonine kinase of phage T7 and, like Stk, the T7 kinase phosphorylates a large number of proteins (Robertson *et al.*, 1994).

Because Stk is similar to eukaryotic tyrosine kinases, is found in the same phage genome as the *stx* genes, and the prophage is found in pathogenic EHEC strains (e.g. O157:H7), it is plausible to consider that Stk is a virulence factor. Moreover, eukaryotic-like serine/threonine kinases play important roles in virulence of a number of Gram-positive pathogens (Burnside and Rajagopal, 2011). However, we have not found any supporting evidence for this idea, nor had we been able to identify a phenotype for Stk until we found that Stk was responsible for the exclusion of HK97 by the 933W prophage.

Based on the studies reported here, we can conclude that the tyrosine kinase activity of Stk is responsible for exclusion of phage HK97 by the 933W prophage. Less obvious, however, is the mechanism that activates Stk to catalyse protein phosphorylation. Curiously, even though HK97 infection results in increased production of Stk, this increased production is neither necessary nor sufficient for HK97-directed stimulation of Stk phosphorylation activity. Key to understanding the role of HK97 in this process are experiments with HK97 mutants. Infection with an HK97 lacking *cII* (*cII*◇*cat*) showed that increased levels of Stk are not necessary and HK97 derivatives missing *orf41* showed that the increased levels, similar to those observed with HK97 wild-type infection, are not sufficient. The observation that λ-P22dis, which is not excluded, does not activate Stk confirms that HK97 plays an essential role in its own exclusion during infection of the 933W lysogen.

The 933W prophage through action of its *stk* gene product is not *sui generis* in exhibiting specificity in excluding another lambdoid phage. The HK022 prophage through action of its *nun* gene product only excludes the few lambdoid phages that have the *N* gene of λ (Robert *et al.*, 1987).

Although infection of the 933W lysogen with either HK97*orf41-1* or HK97Δ*orf41* fails to result in exclusion, the infections do result in some tyrosine phosphorylation, albeit significantly reduced from that observed with HK97 infection. This information does not lead us to question whether *orf41* is necessary for exclusion, but it does raise the possibility that the *orf41* gene product may not be sufficient for exclusion; an additional function expressed by HK97 may be required for effective exclusion.

How might proteins act to alter Stk to a form that catalyses extensive protein phosphorylation and act to exclude HK97? Our studies indicate that autophosphorylation plays an essential role. Although an activator protein (e.g.

gp41) is not required for autophosphorylation when Stk is at high concentration, it is required for activity and presumably autophosphorylation when Stk is at the low concentration levels of the lysogen. Such requirements for activation are not unique to Stk. By structure and function Stk is grouped with the extensively studied eukaryotic kinases (Plunkett *et al.*, 1999; Tyler and Friedman, 2004). Consistent with our observations regarding Stk, activity of many of these kinases require autophosphorylation (Smith *et al.*, 1993) that occurs either through dimerization or interaction with effector proteins (Roskoski, 2004; Pike *et al.*, 2008). We propose that the HK97 gp41 serves as an activator stimulating Stk autophosphorylation resulting in exclusion. We have not yet identified any specific proteins whose phosphorylation causes exclusion, nor have we ruled out the possibility that the large number of proteins phosphorylated by Stk leads to a loss of cellular activity necessary for phage production. However, our finding that a short exposure to high levels of Stk affects bacterial viability is consistent with such a possibility.

Stimulation of Stk autophosphorylation is not limited to gp41 and possibly another protein encoded by HK97. In a previous *in vitro* study, we showed that histone protein II, which is phosphorylated by Stk, stimulates Stk autophosphorylation (Tyler and Friedman, 2004). However, in the same study we found that myelin basic protein, which also is phosphorylated by Stk, does not stimulate autophosphorylation. We postulate that the high levels of Stk synthesized in the isolation of rStk (an epitope tagged, but active Stk) allow dimerization resulting in incomplete but sufficient auto-phosphorylation of Stk to stimulate enzymatic activity. However, interaction with histone protein II, presumably similar to the interaction with gp41, supports further autophosphorylation. This raises the question as to whether there is a condition(s) in which a bacterial protein is expressed that stimulates Stk activity. Two observations make such a scenario plausible. First, Stk is constitutively expressed by the 933W prophage (Tyler and Friedman, 2004) and second, these constitutive levels are sufficient to promote significant protein phosphorylation in the presence of the gp41. However, the experiments with the TUC 01 construct show that when Stk is expressed at high levels it can autophosphorylate.

Our observation that expression of activated Stk interferes with cell growth suggests a possible function similar to toxin–antitoxin (programmed cell death) systems (Yarmolinsky, 1995; Van Melderen & Saavedra De Bast, 2009). As discussed above, such a system has been implicated in protection against phage infection (Fineran *et al.*, 2009). In these systems, the toxin, which is inhibitory to cell growth, is held in check by the antitoxin. In the usual case, the toxin is more stable than the antitoxin and thus when concentrations of antitoxin are reduced below those of the toxin, cell death or stasis ensues. Many roles

Table 4. Relevant genotype and source of bacteria.

Strain	Relevant genotype	Reference/source
K37	K12 lab strain	This lab
TUC 01	W3110 *lac U169Δ gal490 pgl8Δ attL {int<>cat sacB} λcl857 Δcro bioA*	Don Court (Datta *et al.*, 2008)
K9675	K37 (933W)	Tyler and Friedman (2004)
K11515	K37 (933W *stk-K42R*)	This lab
K10567	K37 (933W *stk-c-Myc*)	This lab
K11541	K37 (933W *Δori ΔL0095*)	This lab
K11569	TUC 01 (*int-cIII*)<>*stk-c-Myc*	This lab
K11580	TUC 01 (*int-cIII*)<>*stkK42R-c-Myc*	This lab
K2701	K37(*Δsrl-recA*)	This lab

have been proposed for these systems, e.g. plasmid maintenance by destroying bacteria that lose the plasmid, population survival by death of the many to sustain the few, and growth stasis, called persistence, of a fraction of the population resulting in escape from action of harmful agents such as antibiotics. A case in point is HipA, the toxin in the HipA-B system, which like Stk is a eukaryotic-like kinase, but in this case a serine kinase (Correia *et al.*, 2006; Schumacher *et al.*, 2009). Unlike Stk, HipA phosphorylation appears directed to one protein, transcription factor EF-Tu, in this way inhibiting protein synthesis and establishing a surviving (persistent) subpopulation in the presence of antibiotic. Stk differs from classical toxins by remaining quiescent until activated, suggesting a pretoxin whose activity requires not loss of an antitoxin but gain of a protoxin. If Stk serves in some type of toxin role, we speculate that the bacterium encodes the Stk activator, protoxin, which is expressed under specific stress conditions at levels that limit bacterial growth of a subpopulation until more favourable conditions are encountered. The protoxin that activates Stk causing aborted HK97 infection is the gp41 made by the infecting HK97. This leads to a dead end for HK97 blocking further growth and in this way protecting the remaining bacterial population from further infection.

Here we list a few unanswered questions we consider most compelling. Is the origin of Stk eukaryotic? Is phage

exclusion driving selection or is that activity a fortuitous addition to Stk's main activity? If the latter, what is that activity? The immunoblots showed a large number of proteins phosphorylated, raising the following question: does this promiscuity have physiological significance? Is phosphorylation of a specific protein(s) the cause of exclusion or is it the wide range of proteins phosphorylated?

Experimental procedures

Bacteria and phage

See Tables 4 and 5.

Media

LB broth, LB sucrose, top agar and TB have previously been described (Livny and Friedman, 2004). Antibiotics were used at the following concentrations: ampicillin 100 μg ml⁻¹, chloramphenicol 8 μg ml⁻¹ and kanamycin 30 μg ml⁻¹.

Phage infection

Cultures were grown overnight in LB maltose (0.4%) and diluted into LB maltose, grown to ~ 10⁸ ml⁻¹, and centrifuged. Sedimented bacteria were resuspended in buffer (yielding a 10-fold concentration) appropriate for adsorption of the phage: for HK97, 10 mM Tris pH 8.0 and for all other phages,

Table 5. Relevant information and source of phage.

Phage	Relevant information	Source/reference
HK97	Wild-type	Roger Hendrix (Dhillon *et al.*, 1980)
HK97*cII*<>*cat*	Deletion substitution of *cII*	This lab
HK97*orf41-1*	Plates on 933W lysogen	This lab
λ	λ+	NIH collection
HK022	Lambdoid phage	Robert Weisberg (Dhillon and Dhillon, 1976)
λ-P22*dis*	λ P22 hybrid	Yamamoto *et al.* (1977)
H-19B	Lambdoid phage	O'Brien *et al.* (1984)
T4rII	Does not plate on λ lysogen due to action of Rex	Robert Greenberg (Benzer, 1955)
HK97*Δorf41*	In frame deletion	This lab
HK97*Δorf41-cII*<>*cat*	Double mutant	This lab
φ80	Lambdoid phage	NIH collection

0.01 M Mg^{++}. Phage were added at a multiplicity (moi) of ~5 and allowed to adsorb for 10 min. Infected bacteria were microfuged. Supernatants were titred for unadsorbed phage (allowing calculation of per cent adsorbed) and sediments were resuspended in 10 ml LB and grown for 20 min at 37°. Infected bacteria and uninfected controls were centrifuged and sedimented cells resuspended in 1 ml of buffer, microfuged, and prepared for electrophoresis as described below.

Timing of Stk synthesis and protein phosphorylation after HK97 infection

Following adsorption, infected bacteria were resuspended in LB and grown at 37°. Ten-millilitre aliquots were removed at times indicated in Fig. 3 for immunoblot analysis as described below.

Immunoblots

Procedure was essentially as previously described (Tyler and Friedman, 2004). Cell extracts were prepared in two ways. (1) Samples were resuspended in 90 μl of loading buffer (Sambrook *et al.*, 1989), boiled for 20 min, supernatants were collected following a 10 min spin in the microfuge, and either used immediately or frozen for later use. (2) Bacteria were sonicated, filtered and concentrated prior to preparation for gel loading as above (Fig. 2). Proteins were separated using SDS-PAGE electrophoresis. Lanes were overloaded with protein to maximize sensitivity of assay.

Stk levels were assessed using either of two antisera: (1) a rabbit antiserum raised against Stk protein (Tyler and Friedman, 2004), which is only effective when Stk is overexpressed, and (2) a commercially (Santa Cruz Biotechnologies) obtained mouse HRP conjugated anti-c-Myc that identifies Stk that was c-Myc tagged and is effective when Stk-c-Myc is expressed at lower levels. The tagged Stk maintained the observed phenotypes of excluding HK97 (only slightly less effective, Table 1) and showed similar protein phosphorylation following HK97 infection. Tyrosine phosphorylation was assessed using an HRP conjugated anti-phosphotyrosine antibody (Santa Cruz Biotechnology). HU loading control was detected using HRP conjugated anti-HU antibody as a known contaminant of an early preparation of the commercial anti-c-Myc antibody (Santa Cruz Biotechnologies) or anti-HU kindly supplied by Dr Sankar Adhya. Anti-NusA was from this laboratory. Where required, blots were treated with secondary alkaline phosphatase conjugated donkey anti-rabbit antiserum (GE Healthcare UK Limited). Bands were identified using ECL (General Electric) and viewed using either film or the Typhoon Trio Variable Mode Imager.

Genetic constructs

The λ Red system (recombineering) was used to make all genetic substitutions and changes (Datsenko and Wanner, 2000; Court *et al.*, 2002). When a selection was not available, a two-step procedure was used; first a double-stranded *cat-sacB* cassette (CSB) was inserted at the designed location

(Yu *et al.*, 2000) and subsequently replaced with double-stranded DNA or single-stranded oligonucleotide with the designed change.

The TUC 01 strain (a derivative of SIMD30) (Datta *et al.*, 2008), used for controlled expression of Stk, has a portion of a λ prophage that includes the *cI857* (ts) gene and the genes of the λ P_L operon under control of the strong λ P_L promoter. This promoter has 8–10 times the strength of the P_{lac} (Shatzman *et al.*, 1983). Inserted in the *int* gene is a CSB. The defective prophage itself can supply the Red functions for recombineering when shifted to 42° as in a standard recombineering reaction (Datta *et al.*, 2008). The Stk construct, which has the entire region from *int* through *cIII* replaced with the *stk* gene (Fig. 6A), was obtained by selection on LB sucrose plates at 42°.

PCR and DNA sequencing were used to confirm that constructs conformed to the original design. Sequencing to confirm constructs and mutants was performed by the University of Michigan DNA Sequencing Core.

Sequence of the HK97 mutant determined to have the *orf41-1* mutant was performed at the University of Pittsburgh (Pittsburgh Phage Institute). A comparison of the DNA sequence of the mutant HK97 with the published HK97 sequence alerted us to a number of *orfs* possibly indicating mutant genes. Each of the identified *orfs* from both the mutant and the parental HK97 from our laboratory were sequenced by the University of Michigan Sequencing Core facility. Comparison of these sequences revealed that only the *orf41* pair differed.

Assessing stability of Stk (Fig. 6)

Cultures of K11569 and K11580 were grown to early log phase at 30° in LB. Ten-millilitre aliquots of each were maintained at 30° with shaking for the duration of the experiment and then prepared for immunoblot analysis. The rest (45 ml) of the two cultures were shifted to and maintained at 42° for 20 min with shaking, cooled in an ice bath to renature ts repressor, and placed at 30° with shaking. Ten-millilitre aliquots of the heat-treated aliquots were removed at 20 min intervals following the shift back to 30° and prepared for immunoblot analysis. This procedure provided a means to assess the *in vivo* stability of Stk; i.e., the Stk in cultures subsequently grown at 30° largely will have been synthesized during the prior incubation at 42°.

Measuring of exclusion (eop)

Dilutions of phage were added to 2.5 ml of top agar with 100 μl of overnight culture of K37 and plated on TB plates that were incubated at 37°. The efficiency of plating (eop) is determined by dividing the phage titre on lawns of the tested strain by the titre on lawns of the control strain (K37).

Survival following expression of Stk

K11569 and K11580 were grown to early log phase at 30° and each divided into two aliquots. One aliquot of each culture was kept a 30° and the other shifted to 42° for 30 min.

Following the shift, cultures were cooled on ice and dilutions were plated on LB plates that were incubated at 32°.

Acknowledgements

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