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Coping with Inadvertent Lysis of *Escherichia coli* Cultures: Strains Resistant to Lysogeny and Infection by the Stealthy Lysogenic Phage Φ 80

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Abstract

Phage Φ 80 can infect *Escherichia coli* in a stealthy manner and persist by forming lysogens. Such Φ 80 lysogens are fairly common and often go undetected unless the host is grown at temperatures below 37°C. Since low growth temperatures are required for growing temperature-sensitive mutants and often preferred for large-scale applications such as protein production, Φ 80-resistant strains would be useful. We report the construction of *E. coli* strains that cannot be efficiently lysogenized or infected by bacteriophage Φ 80. These strains contain combinations of deletions or mutations in the bacterial attachment site for Φ 80 integration and/or deletions in the genes required for phage absorption to the host outer membrane. These strains should help contain and prevent Φ 80 infection of *E. coli* cultures in a laboratory or industrial setting.

Keywords

Phage; Φ 80; Lysis; Contamination; *tonA*; *fluA*; *tonB*; Lysogen

Most phages have a lytic (virulent) lifestyle in which they replicate by utilizing and subsequently killing their host. However, a subset of phages can shut down their lytic functions and integrate their genomes into a specific host chromosome site (the *att* site), where they are replicated as part of the host genome (Casjens 2003). Such integrated phage genomes are called prophages and the host that carries the prophage is called a lysogen. Prophages are surprisingly common, with one study finding 51 different functional phages released from 27 different *Escherichia coli* strains (Osawa et al. 2000) and another study showing that 83 of 107 *E. coli* strains tested, released at least one functional phage type (Schicklmaier et al. 1998). Ultimately, these phage elements can account for up to 20% of the genomic DNA of their hosts. (Casjens 2003)

In a given infected cell, the decision between becoming a lysogen or proceeding to lytic growth is dependent on the virus and numerous physiological factors (Casjens 2003). Lysogeny is generally reversed by DNA damage to the host, resulting in the excision of the prophage from the host chromosome and replication (Casjens and Hendrix 2015). This

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induction of phage lytic growth can be rationalized as “rats deserting the sinking ship”. The lysogeny versus lytic growth decision is best understood for phage λ (Casjens and Hendrix 2015; Gottesman and Weisberg 2004). Although a complex regulatory system underlies the lysis/lysogeny decision, the key λ protein is the cI repressor that shuts off transcription of the lytic genes of the prophage (Casjens and Hendrix 2015; Gottesman and Weisberg 2004). Induction of the prophage from the dormant state to the lytic mode is triggered by proteolytic cleavage of the repressor protein, which allows transcription of the lytic genes and production of 100–200 infectious phage particles/cell upon lysis. (Although the link between DNA damage and repressor cleavage is well understood, this is beyond the scope of this report.)

Phage λ has an impressive number of “cousin” phages (the lambdoid phages) that share the same general lifestyle and can often exchange genes (Campbell 1994; Casjens 2003). One of these lysogenic phages is phage $\Phi 80$ which has recently been shown to reside in many laboratory *Escherichia coli* strains (Rotman et al. 2010; Rotman et al. 2012). However, $\Phi 80$ differs markedly from λ in that it integrates into a different *att* site on the *E. coli* chromosome, lysogenizes more efficiently and as a lytic phage, propagates best at low growth temperatures $\sim 30^{\circ}\text{C}$: temperatures at which λ grows poorly (Rotman et al. 2010) (Rybchin 1984). Most importantly $\Phi 80$ lysogens are much more sensitive to DNA damage than λ (Devoret et al. 1983; Irbe et al. 1981; Rotman et al. 2012). Indeed, induction of $\Phi 80$ lysogens proceeds spontaneously without extrinsic DNA damaging agents (e.g., UV light) such as those needed to efficiently induce λ lysogens. Spontaneous induction of a subset of $\Phi 80$ lysogens does not result in lysis of the culture because the other cells of the culture remain lysogens and thus are immune to infection by the liberated phage (the prophage encoded cI repressor protein blocks transcription of any incoming phage) (Rotman et al. 2010). The $\Phi 80$ virus particles are stable to desiccation and aerosolization [4] and thus infectious virions can readily spread to other cultures. Indeed, $\Phi 80$ virions can waft up and down hallways and contaminate cultures of non-lysogens causing inexplicable lysis and growth inhibition (Rotman et al. 2010; Rotman et al. 2012).

The properties of $\Phi 80$ lysogens allow for stealthy but robust spreading in laboratory settings. One factor, noted above, is the unstable nature of the lysogens coupled with the $\Phi 80$ preference for lysogeny upon initial infection (Rotman et al. 2010). The second crucial factor is that $\Phi 80$ lytic development is very temperature dependent (Rotman et al. 2010). Phage production is rapid at $<30^{\circ}\text{C}$, slow at 37°C (the temperature at which *E. coli* strains are commonly grown), and is essentially blocked at 42°C (Rotman et al. 2010; Rybchin 1984). Indeed, lysis due to $\Phi 80$ is most commonly seen in cultures grown at $28\text{--}30^{\circ}\text{C}$, the permissive temperature of the temperature sensitive (*Ts*) strains used to study function of essential genes such as those of DNA replication and membrane lipid synthesis. Moreover, growth of *E. coli* strains at temperatures $<30^{\circ}\text{C}$ is often used in recombinant protein production where low growth temperatures can aid protein folding. Indeed, the ability of $\Phi 80$ to flourish at low growth temperatures was key to its detection (Rotman et al. 2010). We have observed lysis of *E. coli* cultures grown at 30°C by $\Phi 80$ phage in our laboratory and other laboratories report that this extends to some *E. coli* Genetic Stock Center strains (Rotman et al. 2010). We have endeavored to alleviate this problem by constructing and

characterizing a set of *E. coli* strains that cannot be infected or lysogenized. These otherwise “wild type” *E. coli* strains should allow effective control of the spread of $\Phi 80$.

Materials and Methods

Standard media and phage manipulation methods were used (Arber et al. 1983; Miller 1972). The strains and primers are given in Table 1. The Coli Genetic Stock Center (CGSC) was the source of two strains. Derivatives of *E. coli* strain BW25113 (Table 1) carrying deletions of the *fhuA* or *tonB* genes were constructed by replacing the genes with a kanamycin resistance cassette using the method of Datsenko and Wanner (Baba et al. 2006; Datsenko and Wanner 2000) and primer sets (Table 1) del tonA UP/del tonA DN and del tonB UP/del tonB DN, respectively. Strains SW113 and SW118 were constructed as described above using primer sets del att UP/del *yciI* DN and mut att UP/del *yciI* DN (Table 1, Figure 2A). Strain SW114 was constructed as above using primer sets del att UP/del *yciI* DN whereas strain SW115 was constructed as above using primers del *yciI* UP and del tonB DN. Stocks of $\Phi 80$ were prepared by growing a $\Phi 80$ lysogen (BW25113:: $\Phi 80$) at 37°C overnight and then lysing the culture by adding a few drops of chloroform and removal of the cell debris by centrifugation (Arber et al. 1983; Miller 1972). The supernatants were titered by plating serial dilutions on wild type BW25113 at 30°C to determine plaque forming units

We first considered blocking the adsorption of $\Phi 80$ to its *E. coli* host. Phage $\Phi 80$ is known to require the outer-membrane receptor FhuA, also known as TonA, for adsorption to the host (Braun 2009; Frost and Rosenberg 1975; Killmann et al. 1995). FhuA is coupled to the energized cytoplasmic membrane through TonB and both are required for $\Phi 80$ adsorption to be irreversible (Braun 2009; Frost and Rosenberg 1975; Killmann et al. 1995). To test the susceptibility of these strains to $\Phi 80$ infection, exponentially growing cultures were incubated with a $\Phi 80$ phage stock (titer 10^9 pfu/ml), plated on LB agar plates which were incubated at 30°C overnight. Both *fhuA* (*tonA*) and *tonB* strains were resistant to $\Phi 80$ infection whereas a control non-lysogenic BW25113 strain showed complete lysis (Figure 1). However, it should be noted that *fhuA* (*tonA*) and *tonB* mutant strains are deficient in the uptake of ferric enterochelin, ferric citrate and vitamin B12 (Braun 2009; Frost and Rosenberg 1975) and thus cannot be used to study iron metabolism and related processes such as Fenton reaction-mediated oxidative stress. In early work *tonB* mutations were often used to make strains resistant to phage T1 (hence the *ton* designation) which uses the same outer membrane receptor as $\Phi 80$ (many of the reported infections of cultures by the fully lytic phage T1 (Miller 1972) may well have been $\Phi 80$ infections given the stealthy nature of $\Phi 80$). However, a disadvantage of *tonB* strains was their sensitivity to the chromic acid then used to clean glassware (Postle 2007). For this reason, *tonB* mutations were replaced by the *fhuA* (*tonA*) mutations and such mutations are retained in several commercially available cloning and expression strains. However, the multiple hazards of chromic acid (a corrosive, toxic and carcinogenic agent) have since been recognized and chromic acid has been almost completely replaced (e.g., NOCHROMIX™). Hence, this development eliminated the disadvantage of *tonB* strains.

We next considered blocking the ability of $\Phi 80$ to lysogenize *E. coli*. The primary site where $\Phi 80$ integrates into the *E. coli* chromosome (*attB80*) was mapped near the *trp* and *tonB* loci (Leong et al. 1985) (Figure 2A). Alignment of the *attB80* core sequence (Figure 2B) (Leong et al. 1985) to the genome sequence of the *E. coli* K-12 strain MG1655, located it to the last 19 nucleotides of a gene of unknown function called *yciI* (Figure 2A), which lies directly adjacent to the divergently transcribed *tonB* gene. We first tested the JW1243 *yciI759::Kan* strain of the Keio collection of *E. coli* deletion strains (Baba et al. 2006) (Table 1) for its ability to be lysogenized by $\Phi 80$. The lysogeny test is based on the fact (noted above) that $\Phi 80$ lysogens are immune to superinfection by $\Phi 80$ phage. Exponentially growing cultures of the wild type strain BW25113 and strain JW1243 *yciI759::Kan* were inoculated with a $\Phi 80$ phage stock (multiplicity of infection <1) and allowed to grow for 15 min at 37°C before being plated on LB agar plates and incubated at 37°C overnight. Surviving colonies were grown to OD₆₀₀ of 0.2 at 37°C and these cultures were cross-streaked against a high-titer $\Phi 80$ phage stock. As expected, the wild type strain grew beyond the phage cross streak indicating that $\Phi 80$ lysogens had formed (Figure 3B). We had expected that the *yciI759::Kan* strain JW1243 would not form lysogens because the *yciI759* deletion would have removed the *attB80* site. However, this was not the case. Strain JW1243 could be infected lytically (Figure 3D) and the $\Phi 80$ lysogens that were formed clearly protected it from lysis by the phage in the cross-streak (Figure 3B). Moreover, the growth medium of strain JW1243 was also found to contain very high phage titers ($>10^7$), comparable to those of the wild type lysogen (Figure 3A). This result was unexpected until we realized that in order to accommodate overlapping genes, the Keio deletion strains were constructed with retention of the last seven codons of each ORF (Baba et al. 2006) and thus JW1243 precisely retained the *attB80* integration site. We therefore abandoned strain JW1243 and proceeded to construct strains that could not be lysogenized.

Two different strains (Figure 2A) were constructed in order to block lysogeny (Table 1). In strain SW118 the chromosomal attachment site required to form lysogens was deleted whereas in strain SW113 the attachment site core sequence was altered (*attB80**) without altering the YciI carboxyl terminal amino acid sequence (Figure 2C). When tested for phage titers after infection, strains SW113 and SW118 showed remarkably lower phage production (at least 1000-fold lower) (Figure 3A). Why should the lack of functional *attB80* site inhibit phage production? This seems likely due to efficient formation of abortive lysogens such as those formed in phage λ infections in which either the bacterial *attB* site (Shimada et al. 1972) or the phage *attP* site (Gottesman and Yarmolinsky 1968) have been deleted. The linear phage genome enters the cell where it is converted to a covalently closed and supercoiled form by host functions. Such abortive lysogens are lost during host cell division because the lack of *att* site-specific recombination blocks phage integration into the host chromosome and *cI* repressor blocks phage replication (Gottesman and Yarmolinsky 1968; Shimada et al. 1972). A plausible explanation for the marked reduction in phage production seen in strains SW113 and SW118 is that the prophage genome of abortive lysogens is a circular molecule (Matsubara and Kaiser 1968; Signer 1969) whereas in normal lysogens the prophage genome becomes linearized by site specific recombination between the host and phage *att* sites (Gottesman and Weisberg 2004; Gottesman and Yarmolinsky 1968). This difference in genome topology would allow promoters of abortive lysogens to read into

attB80 phage sequences whereas in normal lysogens these promoters would read into the host genome sequences flanking the prophage. Hence phage genes that are not expressed in normal lysogens (e.g., those for head and tail morphogenesis) could be expressed in abortive lysogens and inhibit phage production. Whatever the explanation, the fact that lack of functional *attB80* site inhibits phage production should lessen spread of the phage.

When site specific recombination between the phage and bacterial *att* sites cannot occur, the resulting unstable lysogens are unable to pass a test for normal lysogeny (Gottesman and Yarmolinsky 1968; Shimada et al. 1972). Indeed, neither strain SW113 nor strain SW118 showed growth beyond the cross-streak, indicating that these strains could not be stably lysogenized by Φ 80 (Figure 3B). The lack of lysogen formation was verified by PCR amplification across the *attB80* site using primers Lys check F/Lys check R. The presence of an amplified band would indicate that the site was intact (with the exception of a kanamycin marker utilized to generate mutants) whereas integration of the large phage genome (46,150 bp) would result in the lack of a band (Figure 3C) since PCR polymerases lack the necessary processivity.

We also constructed two additional strains (Table 1). In strain SW114 the *yciI* ORF was completely deleted leaving only the stop codon (*yciI::Kan*) whereas in strain SW115 the entire genomic segment from the 5'-end of *yciI* to the 3'-end of *tonB* (*yciI-tonB::Kan*) was deleted (Figure 2A). The kanamycin resistance cassette of strain SW115 is flanked by a pair of FRT sites and thus can be easily removed by expressing the FLP recombinase encoded by plasmid pCP20 (Datsenko and Wanner 2000). However, we left the kanamycin cassette in place to provide a selectable marker to facilitate transfer of the deletion allele to other strains by phage P1 transduction or conjugation.

Note that the *yciI-tonB* deletion (Figure 2A) of strain SW115 resulted in the loss of both the chromosomal attachment site (*attB80*) and the TonB Φ 80 outer membrane receptor. Hence, this strain can neither be infected by Φ 80 (Figure 3D) nor efficiently form Φ 80 lysogens. The presence of both mutations may seem redundant. However, note that Φ 80 genomes can enter strain SW114 by means that do not require the TonB receptor, such as by phage P1 transduction with a phage stock grown on a Φ 80 lysogen. Phage P1 readily packages prophage λ and Φ 80 genomes together with the flanking bacterial chromosomal segments (Cronan 2013; Franklin et al. 1965; Matsushiro 1963). Upon introduction of the Φ 80 genomes by P1 transduction, the Φ 80 genome can be inserted into the *E. coli* chromosome either via the standard lysogenic pathway or by homologous recombination using the flanking host sequences. It should be noted that, like phage λ , Φ 80 can insert into secondary attachment sites (Kholodii and Mindlin 1984). However, integration into secondary attachment sites occurs at very low frequency ($\sim 10^{-6}$ of the normal frequency of lysogeny). Moreover, excision of Φ 80 from the secondary sites is much less efficient ($10^{-4} - 10^{-5}$) than excision from *attB80* (Kholodii and Mindlin 1984). When combined, the low frequencies of each event argue that the secondary attachment sites are not a complicating factor. In conclusion the *yciI-tonB::kan* deletion of strain SW115 can be readily moved to other *E. coli* strains by transduction or conjugation to prevent propagation and spreading of the robust phage Φ 80 in the laboratory or industrial environment.

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Figure 1. Plaque formation by phage $\Phi 80$.

Exponentially growing cultures of *E. coli* strains BW25113 (wild type), SW103 *fhuA* and SW116 *tonB* strains were infected with a phage $\Phi 80$ stock and plated on LB agar. Strains SW103 and SW116 could not be infected by $\Phi 80$ as shown by the lack of clearing. In contrast the strain BW25113 plate showed almost confluent plaques (roughly 10^5 plaques) resulting in a clearing of the bacterial lawn relative to those of the plates with strains SW103 and SW116. The four discontinuities seen in *fhuA* plate are bubbles in the top agar.

Representations of (A) the genetic manipulations performed in strain BW25113. (B) The phage $\Phi 80$ bacterial attachment site (*attB80*) and phage attachment site (*attP80*) with homology indicated in capital letters and core region marked by arrows. (C) Nucleotide sequences of the *attB80* mutant strains. The nucleotide sequence of the core region was changed in the *attB80** strain SW113 while preserving the amino acid sequence of YciI whereas in the *yciI* strain SW118 the entire *att* site core region was deleted.

Figure 3. Tests of lysis, lysogeny and phage production.

A) Lysogenic strains were tested by streaking exponentially growing cultures across a thick streak of a 10^9 pfu/ml phage stock. Lysogens survive due to their inability to be superinfected whereas non-lysogens are killed by phage infection. B) Phage production was tested by inoculation of early exponential cultures of the strains with 10^4 pfu/ml of $\Phi 80$ phage followed by growth for 4 h at 30°C . After chloroform treatment to kill and lyse any remaining cells (Arber et al. 1983) phage titers were obtained by spotting $10\ \mu\text{l}$ of ten-fold dilutions of each phage lysate on top agar containing exponentially growing cells of susceptible strain BW25113 followed by incubation of the plates at 30°C . Clear zones denote confluent lysis whereas discrete plaques were formed on the last two dilutions of the *attB80* strain and the mutant *attB80** strain. C) PCR amplification across the bacterial attachment site to verify lack of phage lysogeny. The larger bands in the mutant strains relative to the wild type band are due to insertion of the kanamycin resistance determinant. D) Plaque formation by phage $\Phi 80$ on plates seeded with exponential cultures of *E. coli* strains BW25113 (wild type), JW1243 (*yciI756::Kan*, the Keio collection strain) and SW115 (*yciI-tonB::Kan*) were infected with a phage $\Phi 80$ stock and plated on LB agar. Strain SW115 could not be infected by $\Phi 80$ as shown by the lack of clearing. In contrast, the plates seeded with strains BW25113 and JW1243 formed roughly 10^5 plaques whereas the plate seeded with the SW115 *yciI-tonB* strain formed no plaques. Symbols: M denotes the 1kb Plus DNA ladder from New England Biolabs and WT denotes wild type *E. coli* BW25113.

Table 1.

Bacterial Strains, Plasmids and Oligonucleotides

Bacterial Strains		
Strains	Relevant Genotype	Source
BW25113	<i>lacI</i> <i>rrnBT14</i> <i>lacZ</i> <i>WJ16</i> <i>hsdR514</i> <i>araBADAH33</i> <i>rhaBADLD78</i> <i>rph-1</i>	CGSC 7636
BW25113::Φ80	Φ80 lysogen	A. Kuzminov
JW1243	BW25113 <i>yciI</i> 759::Kan	CGSC 9122
SW103	BW25113 <i>fhuA</i> ::Kan	This work
SW113	BW25113 <i>attB80</i> ::Kan	This work
SW114	BW25113 <i>yciI</i> ::Kan	This work
SW115	BW25113 <i>yciI-tonB</i> ::Kan	This work
SW116	BW25113 <i>tonB</i> ::Kan	This work
SW118	BW25113 <i>attB80</i> ::Kan	This work
Plasmids		
Plasmids	Relevant Characteristics	Source
pKD46	Amp ^R <i>ori</i> (Ts), Arabinose-inducible expression of λ-Red genes	Datsenko and Wanner, 2000
pKD4	Amp ^R <i>pir</i> -dependent provides FRT-KanR-FRT	Datsenko and Wanner, 2000
Oligonucleotide primers		
Oligonucleotides	Sequence	
del <i>tonA</i> UP	ATCATTCTCGTTTACGTTATCATTCACTTTACATCAGAGATATACCAATGGTGTAGGCTGGAGCTGCTTCG	
del <i>tonA</i> DN	GCACGGAAATCCGTGCCCCAAAAGAGAAATTAGAAACGGAAGGTTGCGGTCATATGAATATCCTCCTTAGT	
del <i>tonB</i> UP	ATTTAAAATCGAGACCTGGTTTTTCTACTGAAATGATTATGACTTCAATG CATATGAATATCCTCCTTAGT	
del <i>tonB</i> DN	CTGTTGAGTAATAGTCAAAGCCTCCGGTCGGAGGCTTTTGACTTTCTGCGTGTAGGCTGGAGCTGCTTCG	
del <i>yciI</i> UP	CCCTTTCAAATAACGTACTTTACAACCTTTCCCGAACAAGGAGTTGTGCCCCGTGTAGGCTGGAGCTGCTTCG	
del <i>yciI</i> DN	AAACAAGAACACGGTTGCAAAAACCGTGCCCTTAAATATTGAATCTCTATCATATGAATATCCTCCTTAGT	
del <i>att</i> UP	ATGC TGACCCGTAC GTAGCGGCAG GAGTCTATGA ACACGTTTCAGTGTGAGTGTAGGCTGGAGCTGCTTCG	
mut <i>att</i> UP	ATGCTGACCCGTACGTAGCGGCAGGAGTCTATGAACACGTTTCAGTGAAGCCTTTCAAAAAGGTCTTTTGAGTGTAGGCTGGAGCTGCTTCG	
Lys check F	CTTTATGCATGGCCTTAACAAAGAC	
Lys check R	TATTATCTGCTTGTGGTGGTGAATG	