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ORIGINAL ARTICLE

Control and benefits of CP4-57 prophage excision in *Escherichia coli* biofilms

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Earlier, we discovered that the global regulator, Hha, is related to cell death in biofilms and regulates cryptic prophage genes. Here, we show that Hha induces excision of prophages, CP4-57 and DLP12, by inducing excision genes and by reducing SsrA synthesis. SsrA is a tmRNA that is important for rescuing stalled ribosomes, contains an attachment site for CP4-57 and is shown here to be required for CP4-57 excision. These prophages impact biofilm development, as the deletion of 35 genes individually of prophages, CP4-57 and DLP12, increase biofilm formation up to 17-fold, and five genes decrease biofilm formation up to sixfold. In addition, CP4-57 excises during early biofilm development but not in planktonic cells, whereas DLP12 excision was detected at all the developmental stages for both biofilm and planktonic cells. CP4-57 excision leads to a chromosome region devoid of prophage and to the formation of a phage circle (which is lost). These results were corroborated by a whole-transcriptome analysis that showed that complete loss of CP4-57 activated the expression of the *flg, flh* and *fli* motility operons and repressed expression of key enzymes in the tricarboxylic acid cycle and of enzymes for lactate utilization. Prophage excision also results in the expression of cell lysis genes that reduce cell viability (for example, *alpA*, *intA* and *intD*). Hence, defective prophages are involved in host physiology through Hha and in biofilm formation by generating a diversified population with specialized functions in terms of motility and nutrient metabolism.
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Introduction

The battle between host and phage is epic, but may cease for the benefit of both. Lytic phages such as T4 invade, lyse their host and exit as victors. Temperate phages such as lambda decide when to integrate and when to lyse the host, and the integration/excision (Int/Xis) system is encoded by its own genes but works with a host-encoded integration factor (Cho *et al.*, 2002). Once integrated into the host, temperate phage and host reach an equilibrium when a balance between the costs and benefits to both is achieved (Chen *et al.*, 2005). For the host, integration of phages increases the ecological fitness of many bacteria; for example, integration prevents further phage invasion, increases serum resistance and helps the bacterium to invade or evade its host through toxins or adhesions (Chen *et al.*, 2005). For the phage, it benefits by passage to bacterial progeny during chromosome replication and leaves when the environment is not favorable.

Prophage-like elements and prophage remnants have been identified in almost all sequenced bacterial genomes (Canchaya *et al.*, 2003) and can constitute 10–20% of a bacterium's genome (Casjens, 2003). Attempts to identify prophage excision under various environments have shown very low frequencies of excision (Sozhamannan *et al.*, 2006), indicating a stable residence of prophage under most conditions. The widespread and relatively stable existence of these prophages in bacteria indicates that the battle between host and these phages has ceased under normal growth conditions.

Integration of prophage into the bacterium often causes coevolution of the host and phage (Canchaya *et al.*, 2003; de Mello Varani *et al.*, 2008). In this case, the prophages have capitulated to the host, and it is reasonable for the host to gain more control of the prophage by modulating the expression of its genes. There is evidence that the prophage accumulates major mutations rapidly by undergoing a complex decay process (Canchaya *et al.*, 2003; Casjens, 2003). These mutations render the prophages defective in functions that are detrimental to

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the host, whereas, at the same time, maintaining or enhancing the functions that are beneficial to the host (Canchaya *et al*., 2003; Casjens, 2003).

Phage-encoded integrase is among the most conserved prophage genes as shown by comparative genomics (Lindsey *et al*., 1989; Boyd and Brüssow, 2002). Integrase is required for both integration and excision of prophage (Cho *et al*., 2002). Excision of the phage from the host genome requires another protein called excisionase (Xis) (Cho *et al*., 2002), and excision allows the expression of phage-encoded lytic genes (Kirby *et al*., 1994; Casjens, 2003). In many cases, Xis is no longer functional (for example, DLP12 in *Escherichia coli* K-12 (Lindsey *et al*., 1989)), and its inactivation indicates a loss of self-control by the prophage. For example, there is evidence that the excision of lambda is stimulated by the host factor for inversion stimulation (Thompson *et al*., 1987).

Biofilm formation is arguably the dominant lifestyle for most bacteria (Watnick and Kolter, 2000; Webb *et al*., 2003a), wherein the most important actions for shaping bacteria and their phage take place, which make it the ideal growth state to study host–phage interactions. Moreover, the role of prophage in biofilms has been receiving increasing attention. We observed induction of several clusters of prophage genes, including CP4-57 and DLP12, in mature biofilms of *E. coli* K12 using whole-transcriptome analysis (Domka *et al*., 2007). Prophage genes are among the most highly induced genes during biofilm development in both Gram-negative *Pseudomonas aeruginosa* (Whiteley *et al*., 2001) and *E. coli* (Herzberg *et al*., 2006; Domka *et al*., 2006, 2007; Lee *et al*., 2007; García-Contreras *et al*., 2008) as well as in Gram-positive *Bacillus subtilis* (Stanley *et al*., 2003; Ren *et al*., 2004a). In addition to differential expression, prophage excision has been linked to both cell death and lysis for *P. aeruginosa* cells in biofilms, as filamentous-like prophage excision increases diversity in dispersing cells and impacts biofilm architecture (Bayles, 2003; Webb *et al*., 2003b). However, little is known about the regulation of filamentous prophage excision (Bayles, 2003; Webb *et al*., 2003b). Moreover, self-generated diversity, including generating small-colony variants (SCVs), is important in biofilm formation (Boles *et al*., 2004; Webb *et al*., 2004); yet, the genetic basis of the emergence of SCVs is largely unknown.

E. coli K-12 has served as a model organism in the field of biochemical genetics, molecular biology and biotechnology (Blattner *et al*., 1997). When isolated, *E. coli* K-12 carried the bacteriophage lambda plus the defective lambdoid prophages, CP4-57, DLP12, Rac and Qin, and the element, e14 (Blattner *et al*., 1997). Of these, CP4-57 (Kirby *et al*., 1994) and DLP12 (Lindsey *et al*., 1989) are two of the best-characterized cryptic prophages. CP4-57 is a putative defective prophage with 22 genes and 4 pseudogenes; however, it lacks both a signature capsid and assembly genes (http://www.ecogene.org/). DLP12 is a defective prophage with 22 genes and 10 pseudogenes, including both P22 and lambda phage modules (http://www.ecogene.org/). These two cryptic prophages have lost some functions essential for lytic growth but still retain some functional phage genes (Blattner *et al*., 1997), including *intA* and *alpA* of CP4-57 (Kirby *et al*., 1994), and *intD*, *ompT*, *ybcS* and *essD* of DLP12 (Lindsey *et al*., 1989; Srividhya and Krishnaswamy, 2007).

To understand how hosts control the gene expression of captured prophage, it is essential to identify the host factors that promote prophage excision. The chromosomal *hha* gene (*h*igh *h*emolysin *a*ctivity) of *E. coli* was first identified in a search for mutants that overproduced the toxin, α-hemolysin, from a plasmid-encoded hemolytic *hly* operon of *E. coli* strain 5K (Carmona *et al*., 1993). Hha is a small transcriptional regulator (8 kDa) that we discovered is induced in biofilms (Ren *et al*., 2004c) and that we found influences biofilm formation (González Barrios *et al*., 2006b); Hha overexpression reduces biofilm formation by increasing biofilm dispersal (García-Contreras *et al*., 2008). Rather than binding to specific DNA sequences, Hha exhibits nonspecific DNA binding (Nieto *et al*., 2000). We found that Hha binds *in vivo* to 15 genes or to intergenic regions of CP4-57 and DLP12 including regions close to the prophage attachment (*att*) sites and that Hha activates the prophage lytic genes *alpA*, *rzpD*, *appY* and *yfjZ*, as well as induces plaque formation and decreases cell viability (García-Contreras *et al*., 2008). Therefore, we hypothesized that Hha is involved in the regulation of prophage excision, including CP4-57 and DLP12, and that this excision would influence biofilm formation.

The aims of this study were to determine the functions of prophage in the life cycle of a biofilm, to test whether prophage excision occurs in biofilms and to explore the mechanism controlling prophage excision and gene expression. We also explore whether prophage excision is involved in population diversification in biofilms and address what are the benefits for bacteria that harbor prophages. Using *E. coli* as a reference system, we found that prophages, CP4-57 and DLP12, excise in biofilms, that Hha controls their excision and that excision of CP4-57 reduces cell fitness and affects biofilm formation.

Materials and methods

Bacterial strains, plasmids and growth conditions

The isogenic *E. coli* BW25113 strains and plasmids used in this study are listed in Table 1. For deleting and overexpressing genes, we used the Keio collection (Baba *et al*., 2006) and the ASKA library (Kitagawa *et al*., 2005). Experiments were conducted at 37 °C in either Luria-Bertani medium (LB) (Sambrook *et al*., 1989), LB containing 0.2% glucose (LB glu), M9 minimal medium with 0.2% casamino

Table 1 *E. coli* K-12 bacterial strains and plasmids used in this study

Strains and plasmids	*Genotype/relevant characteristics*	*Source*
Strains		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Baba *et al.* (2006)
BW25113 ΔCP4-57	BW25113 lacking chromosomal CP4-57 genes and circle	This study
BW25113 ΔCP4-57^{circle}	BW25113 ΔCP4-57 with phage circle CP4-57Δ*alpA* Ω Km^{R}	This study
BW25113 *ssrA*	BW25113 Δ*ssrA* Ω Km^{R}	This study
BW25113 *ssrA attL*$^{+}$	BW25113 Δ*ssrA* (partial deletion with *attL* intact) Ω Km^{R}	This study
BW25113 *hha*	BW25113 Δ*hha* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *alpA*	BW25113 Δ*alpA* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *intA*	BW25113 Δ*intA* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *intD*	BW25113 Δ*intD* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *appY*	BW25113 Δ*appY* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *borD*	BW25113 Δ*borD* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *essD*	BW25113 Δ*essD* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *ompT*	BW25113 Δ*ompT* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *rzpD*	BW25113 Δ*rzpD* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *ybcS*	BW25113 Δ*ybcS* Ω Km^{R}	Baba *et al.* (2006)
BW25113 *hha ssrA attL*$^{+}$	BW25113 Δ*hha* Δ*ssrA attL*$^{+}$Ω Km^{R}	This study
Plasmids		
pCA24N	Cm^{R}; *lacI*^{q}, pCA24N	Kitagawa *et al.* (2005)
pCA24N-*hha*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*hha*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*intA*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*intA*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*alpA*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*alpA*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*intD*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*intD*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*ypjF*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*ypjF*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*appY*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*appY*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*borD*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*borD*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*essD*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*essD*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*ompT*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*ompT*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*rzpD*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*rzpD*$^{+}$	Kitagawa *et al.* (2005)
pCA24N-*ybcS*	Cm^{R}; *lacI*^{q}, pCA24N P$_{T5\text{-}lac}$::*ybcS*$^{+}$	Kitagawa *et al.* (2005)

Km^{R} and Cm^{R} are kanamycin and chloramphenicol resistance, respectively. There are an additional 31 BW25113 deletion strains used for the biofilm studies (Figure 1) that are not listed here for brevity.

acids (M9C) (Rodriguez and Tait, 1983) or M9C with 0.1% lactate (M9C lactate). Kanamycin (50 μg ml^{-1}) was used for preculturing isogenic knockout mutants, and chloramphenicol (30 μg ml^{-1}) was used for maintaining pCA24N-based plasmids.

Construction of BW25113 ssrA*,* ssrA attL^{+} *and* hha ssrA attL^{+}

For mutant BW25113 *ssrA*, the whole region encoding the transcript of SsrA as well as its promoter region was deleted from the chromosome using a one-step inactivation procedure described previously (Baba *et al.*, 2006), with primers, delta *ssrA*-f and delta *ssrA*-r (Table 2), as previously designed, to remove *ssrA* from *E. coli* MG1655 (Christensen and Gerdes, 2003). For the construction of the partial SsrA mutant, BW25113 *ssrA attL*$^{+}$, primers, delta *ssrA*-f and delta *ssrA attL*$^{+}$-r (Table 2), were used to remove the promoter region as well as a part of the *ssrA* transcript; however, the *attL* site was not removed. The sequences near the deletions in these two mutants were verified by DNA sequencing from PCR fragments using primers flanking the upstream gene, *smpB* (delta *ssrA*-U), and the downstream gene, *intA* (delta *ssrA*-D) (Table 2), of *ssrA,* as described previously (Baba *et al.*, 2006). Double mutant BW25113 *hha ssrA attL*$^{+}$ was constructed as described previously through bacteriophage P1 transduction (Maeda *et al.*, 2008) to transfer the *hha* deletion from the Keio collection to *ssrA attL*$^{+}$.

Growth assays

The growth of the BW25113 wild-type strain and the CP4-57 deletion mutant was determined using turbidity measurements at 600 nm with LB, LB glu, M9C and M9C lactate. In addition, the toxicity of selected prophage proteins was investigated using pCA24N-based expression plasmids (Kitagawa *et al.*, 2005), with 1 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG) added at a turbidity of 0.5 at 600 nm (for RzpD, 2 mM IPTG was also added at a turbidity of 0.3, 0.8 and 1.8, at 600 nm). Cells were diluted by 10^{2}–10^{7}, using 10-fold serial dilution steps, into 0.85% NaCl solution and plated on LB agar including chloramphenicol to determine cell viability (Donegan *et al.*, 1991).

Crystal violet biofilm assay and CFU count of biofilm cells

Biofilm formation was assayed in 96-well polystyrene plates (Corning Costar, Cambridge, MA, USA), as described previously, with crystal violet staining (Pratt and Kolter, 1998). To remove growth effects, we normalized biofilm formation by dividing total biofilm by the maximal bacterial growth as mea-

Table 2 Nucleotide sequences and expected size of the primer sets used in this study

Primer name	*Primer sequence (listed 5′ to 3′)*	*Expected size (bp)*
Sequencing		
CP4-57f	AAGCACATAAACCTGGTTCAGCGAC	1219[a]
CP4-57r	TAGCTGGTGTGAACTGGTCGTTCTG	
DLP12f	CATAATGATCACCGACGTTGGTTTC	768[a]
DLP12r	GTATTGTCTAACTTTCTTGAGCAATCG	
CP4-57-Cf	AGTGGCTGTCTGGCAAATG	850[a]
CP4-57-Cr	TCCGATATTTACACTAGGAACAGC	
qPCR		
CP4-57-2f	AAGCATGTAGTACCGAGGATGTAGG	151
CP4-57-2r	TATGTCTCCTCACCGTCTGGTCGG	
DLP12-2f	CAAAAGCCATTGACTCAGCAAGG	175
DLP12-2r	ACGGATAAGACGGGCATAAATGA	
*purA*_f	GGGCCTGCTTATGAAGATAAAGT	209
*purA*_r	TCAACCACCATAGAAGTCAGGAT	
qRT-PCR		
alpA	GAACAATCGATCGGCCGTT	181
	CTCATCAATCTCGGCCTCA	
intA	TGAGATTAAGGCTCATACTCTGGTT	158
	TTCCGATATTTACACTAGGAACAGC	
intD	GTCTGAGTTGATACTGGCTTTGGTT	288
	AAAGCTAGGGGATTTACCTGATGTC	
ssrA	AAGAGTTCGAAGCGGGACTT	188
	TAACTTGCGGGTACGGGTAG	
rrsG	TATTGCACAATGGGCGCAAG	232
	ACTTAACAAACCGCTGCGT	
BW25113 ssrA mutant construction		
delta *ssrA*-f	CGAATAAAAATCAGGCTACATGGGTGCTAAATCTTTAACGATAACG	
	CCGTGTAGGCTGGAGCTGCTTC	
delta *ssrA*-r	CTTAGGACTTCATCGGATGACTCTGGTAATCACCGATGGAGAATTTTG	
	CATATGAATATCCTCCTTTAG	
delta *ssrA attL*$^+$-r	GCTTCCACGCGATCTCTTTTGGGTTTGACCTCTCTTGATCCCCGTCCTC	
	ATATGAATATCCTCCTTTAG	
delta *ssrA*-U	AAGAGTTCGAAGCGGGACTT	2274[b] or 2474[c]
delta *ssrA*-D	TTCCGTATCAGTTAACGGCTTGG	

f indicates forward primer and r indicates reverse primer.
[a]Size of amplified fragment upon deletion of prophage.
[b]Size of amplified fragment upon deletion of *ssrA*.
[c]Size of amplified fragment upon deletion of *ssrA attL*$^+$.

sured by turbidity at 620 nm for each strain. The total number of viable cells in a biofilm formed on 14 ml polystyrene tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) was determined by sonicating for 5 min at 22 W in an FS3 water bath sonicator (Fisher Scientific, Pittsburgh, PA, USA). The time for sonication was chosen to maximize the number of viable cells from the biofilm.

Quantitative real-time PCR

To determine the relative concentrations of specific DNA fragments, total DNA (50–200 ng) isolated using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) was used as the template for the quantitative real-time PCR (qPCR) using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA). The reaction and analysis were carried out by the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative amount of chromosomal DNA undergoing excision was calculated by comparing threshold cycle numbers of a target gene vs that of a reference gene (Pfaffl, 2001); the reference gene was adenylosuccinate synthase (*purA*). The number of chromosomes that are devoid of each prophage was quantified using primers flanking each prophage (primers CP4-57-2f/CP4-57-2r for CP4-57 and DLP12-2f/DLP12-2r for DLP12), which only give PCR products when the prophage is removed due to the size of the prophage. Genomic DNA from the ΔCP4-57 strain was used as a template positive control for testing primer-binding efficiencies. The binding efficiencies of the primers used here were tested with varying template concentrations to generate a standard curve, and their validity of application in the quantification was confirmed for CP4-57 and for DLP12. Primer sets used for the reference gene (*purA*), for the chromosome that undergoes excision of CP4-57 (CP4-57-2f and CP4-57-2r) and for excision of DLP12 (DLP12-2f and DLP12-2r), are indicated in Table 2.

Quantitative real-time reverse-transcription PCR
Different from the qPCR described above that determined the relative concentrations of specific DNA fragments, quantitative real-time reverse-transcription PCR (qRT-PCR) was used to quantify relative RNA concentrations using 50 ng as a template and included a complementary DNA synthesis step from RNA before the denaturation step. Primers for qRT-PCR for *alpA*, *intA*, *intD* and *ssrA* are listed in Table 2. The housekeeping gene, *rrsG* (16S rRNA gene), was used to normalize the gene expression data.

RNA isolation and whole-transcriptome studies
For the ΔCP4-57 vs BW25113 whole-transcriptome study, exponentially grown planktonic cells were collected when the cell density (turbidity at 600 nm) reached 0.5. Total RNA was isolated as described previously (Ren *et al.*, 2004c), using a bead beater (BioSpec, Bartlesville, OK, USA). The *E. coli* GeneChip Genome 2.0 array (Affymetrix, Santa Clara, CA, USA; P/N 4059655) was used, and cDNA synthesis, fragmentation and hybridizations were as described previously (Ren *et al.*, 2004b; González Barrios *et al.*, 2006a). Corroborating the deletion mutation, the microarray signals of the CP4-57 genes had very low signals. The gene expression data are accessible through GEO accession number GSE14472.

Motility assay
Cell motility was examined as described previously with low-salt (1% tryptone, 0.25% NaCl and 0.3% agar) (González Barrios *et al.*, 2006b) and high-salt (1% tryptone, 2.5% NaCl and 0.3% agar) (Muramoto *et al.*, 1995) media. Motility halos were quantified after 16 h using at least three plates for each condition and two independent cultures for each strain.

Results

Prophage genes affect E. coli *physiology*
To begin to discern the impact of prophage genes on *E. coli* physiology, we tested the effect of deleting each of the 40 genes of prophages, CP4-57 and DLP12, on biofilm formation. After 7 h, deleting *intA* (an integrase) of CP4-57 resulted in sixfold-less biofilm formation compared to the wild-type strain, whereas deleting the rest of the 17 genes increased biofilm formation by 2- to 12-fold (Figure 1a). Similar results were seen on deleting each DLP12 gene (Figure 1b). Hence, deleting many prophage genes affects biofilm formation in a complex manner.

Integrase overexpression leads to prophage excision
Given their large impact on biofilm formation, we tested whether the cryptic prophages, CP4-57 and DLP12, remain excision proficient using a PCR-based assay. The excision of cryptic prophage from the chromosome, using a site-specific recombination event between the *attL* and *attR* sites, should generate an extrachromosomal phage-like circle and completely remove the phage from the chromosome (Sozhamannan *et al.*, 2006). Primers flanking each prophage were designed to detect the presence of an empty site caused by prophage excision (Figure 2c shows CP4-57 primers, CP4-57f and CP4-57r). Due to the size of the prophage (22 kb) and the DNA amplification conditions, these primers do not amplify the intact region harboring prophage. After 12 h, overexpressing integrase from CP4-57 (IntA) using plasmid pCA24N-*intA* in an *intA* host led to the excision and loss of this prophage from planktonic cells, and overexpression of integrase from DLP12 (IntD) using plasmid pCA24N-*intD* in an *intD* host led to the excision of DLP12 from planktonic cells as seen by clear PCR products with expected sizes (Table 2). PCR products from the chromosomal template were sequenced to confirm the loss of each prophage. In addition, overexpressing AlpA, a key transcriptional regulator of IntA, led to CP4-57 excision from planktonic cells. This result is in agreement with an earlier study (Kirby *et al.*, 1994) in which AlpA expression increased IntA synthesis and led to the excision of this prophage. Overexpression via pCA24N of YpjF for CP4-57 and AppY, BorD, EssD, OmpT, RzpD and YbcS for DLP12 did not cause excision under these experimental conditions. The CP4-57 excision caused a loss of 22 030 bp (Figure 2) from the host chromosome, and DLP12 caused a loss of 21 302 bp. Therefore, both cryptic prophages retain the ability to excise from *E. coli* K-12. CP4-57 excision by AlpA and IntA overexpression was previously reported (Kirby *et al.*, 1994; Trempy *et al.*, 1994), but to our knowledge, this is the first report of DLP12 excision.

In addition, a quantification method (qPCR) using genomic DNA as templates was used to determine the fraction of cells that undergo excision upon overexpressing AlpA using primers CP4-57-2f and CP4-57-2r (Table 2), and upon overexpressing IntD using primers DLP12-2f and DLP12-2r (Table 2), which target each region devoid of prophage and by using the reference gene, *purA,* to quantify the number of all cells. In 12 h, overexpressing AlpA causes 18–32% of the planktonic cells to excise and lose CP4-57, and overexpressing IntD causes 44–87% of the cells to excise DLP12. Hence, the cryptic prophages, CP4-57 and DLP12, remain excision proficient, and the overexpression of integrase and its transcriptional activator leads to a large proportion of cells to excise prophage.

Prophage genes excise naturally in biofilms
Through a temporal study (Domka *et al.*, 2007), we found that AlpA was induced in biofilm cells but not in planktonic cells. Therefore, we tested whether prophage excision occurs naturally in the

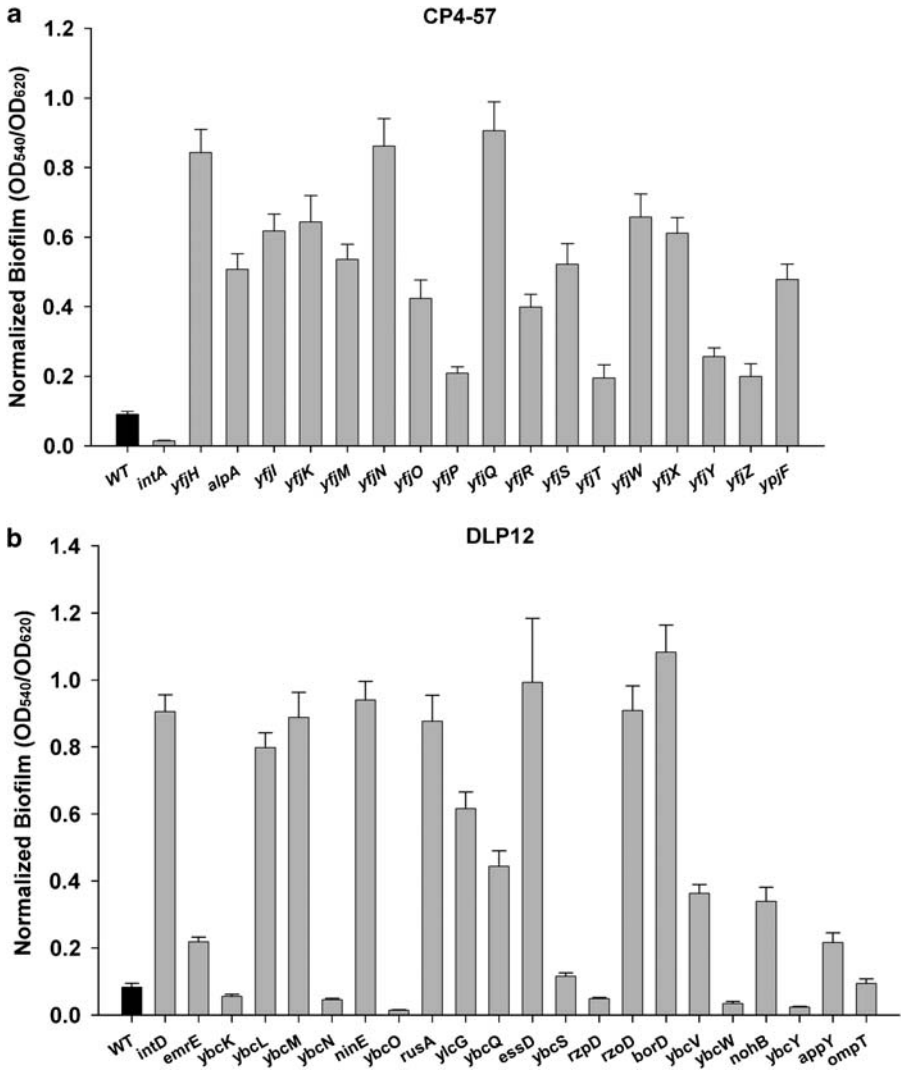

Figure 1 Biofilm formation of CP4-57 and DLP12 deletion mutants. Normalized biofilm formation (total biofilm/growth) after 7 h in Luria-Bertani (LB) medium at 37 °C in 96-well polystyrene plates after the deletion of each CP4-57 gene (**a**) and each DLP12 gene (**b**). Data are the average of 10 replicate wells from two independent cultures, and one standard deviation is shown. Genes are listed in the order of their position on the *Escherichia coli* chromosome.

wild-type strain. Biofilm and planktonic cells were collected after 4, 7, 15, 24, 48 and 72 h of incubation with 10 g glass wool. The fraction of cells that undergo excision was quantified by qPCR using genomic DNAs. The excision of CP4-57 was detected in biofilm cells from 4 to 72 h, whereas for planktonic cells, excision was only found after a long time (24, 48 and 72 h). The estimated fractions of cells undergoing CP4-57 excision were 1–2 per 10 000 for both biofilm and planktonic cells at 48 and 72 h. For DLP12, excision was consistently found from 4 to 72 h incubation times in both biofilm and planktonic cells. Hence, we discovered that the excision of CP4-57 and DLP12 occurs in the development of biofilms, and as CP4-57 is excised predominantly in biofilms at early stages, prophage excision may play a role in biofilm development.

Hha causes prophage excision

Earlier, we found that Hha is related to CP4-57 and DLP12, in that 8 CP4-57 genes and 10 DLP12 genes are differentially expressed in biofilms relative to planktonic cells for the *hha* mutant, and Hha also binds CP4-57 and DLP12 *in vivo* as well as to regions flanking these prophages (García-Contreras *et al.*, 2008). Therefore we tested the role of Hha in prophage excision. In planktonic cells, Hha overexpression in an *hha* mutant using pCA24N-*hha* caused at least a 2048-fold increase in CP4-57 excision when compared with the *hha* strain with an empty vector in LB medium after 15 h. The chromosome devoid of CP4-57 caused by Hha overexpression was present at low frequencies, ranging from 1 to 2 excisions per 10 000 planktonic cells. Similar results were obtained in biofilm and planktonic cells in LB glu medium after 15 h,

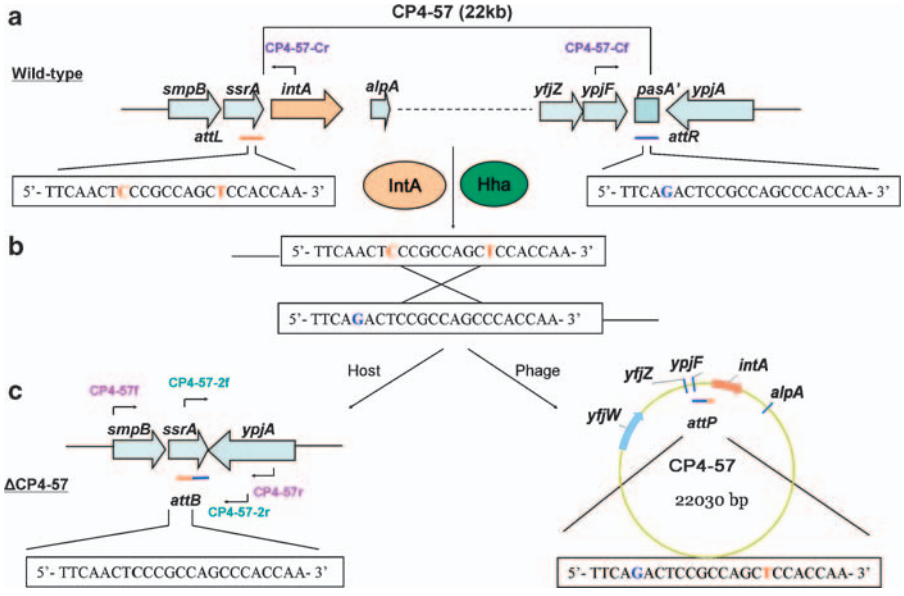

Figure 2 Schematic of CP4-57 excision. The position and unique sequences of the two attachment sites in the wild-type strain are indicated. The left-hand attachment site (*attL*) is an orange bar and the right-hand attachment site (*attR*) is a blue bar (**a**). During excision, a crossover occurs between these two *att* sites after the host protein, Hha, induces excision (**b**). After excision, a new strain, ΔCP4-57^{circle}, is formed with CP4-57 removed from the chromosome and excised CP4-57 circularized to form a phage-like circle. The *attB* site is retained in the bacterium and *attP* is retained in the phage-like circle (**c**). The position of the primers is indicated. To form a strain that completely lacks the CP4-57 prophage, named ΔCP4-57, AlpA (alternative Lon protease), a transcriptional regulator of *intA*, whose overexpression is known to induce CP4-57 excision (Kirby *et al.*, 1994), was overexpressed in an *alpA* deletion background with plasmid pCA24N-*alpA* using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h during exponential growth to induce CP4-57 excision. Cells that underwent excision were screened by PCR for the presence of PCR products indicating the removal of the prophage. Upon removal of CP4-57, PCR using forward primer (CP4-57f) from the upstream gene, *smpB,* and reverse primer (CP4-57r) from the downstream gene (*ypjA*) generates a 1.2 kb excision-specific fragment (**c**). Genomic DNA was used as a template for PCR. Initially, we found that excised CP4-57 forms a phage circle by using a PCR-based assay, and this circle is retained in the cell, named ΔCP4-57^{circle}. Primers, CP4-57-Cf inside *ypjF* and CP4-57-Cr, inside *intA* inside of CP4-57 facing outward on the chromosome (**a**) amplify a PCR product only when a DNA circle is formed at the *attP* site (**c**). The expected PCR product (850 bp) was obtained and was sequenced to confirm the circularization of CP4-57. To obtain ΔCP4-57 that lacks the phage circle, cells were further cultured in Luria-Bertani (LB) medium for 24 h and screened for the loss of the kanamycin-resistance gene that replaced *alpA* inside CP4-57 (Baba *et al.*, 2006). The kanamycin-sensitive strain was purified, then cultured in LB medium at 25 °C for 24 h to remove pCA24N-*alpA* by screening for the loss of chloramphenicol resistance. The complete deletion of the CP4-57 prophage in ΔCP4-57 was verified by PCR using primer sets, CP4-57f and CP4-57r (Table 2), by DNA sequencing (GenBank accession number FJ619521), as well as by the whole-transcriptome study of ΔCP4-57 vs the wild-type strain.

wherein we measured 1.5–1.9 excisions per 10 000 cells. For DLP12, we measured 0.5–1.8 excisions per 10 000 cells under the conditions of Hha overexpression described above for both biofilm and planktonic cells. Hence, Hha promotes CP4-57 and DLP12 excision. Corroborating the Hha overexpression results, there was no detectable CP4-57 or DLP12 excision in an *hha* deletion background for both biofilm and planktonic cells.

Deletion of Hha represses prophage gene expression

qRT-PCR was performed first to test the effect of deleting *hha* on the expression of prophage genes that are related to excision in biofilm cells. Compared with the wild-type strain, deleting *hha* repressed the transcription of *alpA* of CP4-57 (−4.00 ± 0.04-fold), of *intA* of CP4-57 (−2.29 ± 0.04-fold) and of *intD* of DLP12 (−1.52 ± 0.03-fold), after 15 h of incubation in LB glu medium. These results indicate that the host protein, Hha, induces prophage-excision genes.

Excision of CP4-57 and SsrA

SsrA, also known as tmRNA or 10Sa RNA (Dulebohn *et al.*, 2007), serves as an attachment site for CP4-57. It has been proposed that the use of attachment sites at the 3′-end of tRNAs or tmRNAs ensures the evolutionary stability of the phage (Hou, 1999). Therefore, we investigated whether CP4-57 excision causes a change in SsrA by comparing the sequences near the *att* site before and after CP4-57 excision. DNA sequencing showed that there is a one-base difference between the *att* site before prophage insertion (*attB*) (Figure 2c) and the *att* site after prophage insertion (*attL*) (Figure 2a). Hence, CP4-57 excision leads to a one-base change (deletion of a 'T') at the 3′-end of the mature SsrA, in agreement with that which was predicted previously (Kirby *et al.*, 1994). This indicates that a crossover occurs between *attL* and *attR* upon excision, as shown in Figure 2b (between position 8 and position 17 at *attL*). The additional base T at the 3′-end of SsrA was also found in *E. coli* strains that do not harbor CP4-57, including SMS-3-5, HS,

IAI1 and W3110, as well as in *Shigella boydii* CDC 3083-94. These results indicate that CP4-57 excision causes a change in the *ssrA* gene.

We also tested whether the deletion of CP4-57 altered SsrA transcription, using qRT-PCR, and found that the transcription of SsrA in the ΔCP4-57 strain was similar to that of the wild-type strain in both biofilm cells (fold change 0.96 ± 0.09) and in planktonic cells (fold change 1.14 ± 0.04) after 7 h incubation in LB medium. In addition, a whole-transcriptome study (BW25113 ΔCP4-57 vs BW25113) using exponentially growing planktonic cells also showed that there was no change in SsrA transcription. Taken together, these results demonstrate that excision of the downstream prophage does not alter SsrA transcription even though the prophage utilizes SsrA for attachment.

Hha represses SsrA transcription, and SsrA is required for CP4-57 excision

Although not essential in *E. coli*, SsrA activity is essential for bacterial growth under adverse conditions because of its function in *trans* translation (Hong *et al.*, 2007). We previously indicated that SsrA may be under the control of Hha, and both are involved in the activation of prophage and cell lysis (García-Contreras *et al.*, 2008). Here, qRT-PCR using RNA samples extracted from biofilm cells after 15 h of incubation in LB glu medium showed that overexpressing Hha repressed SsrA transcription by −3.0 ± 0.5-fold. This result is consistent with our earlier whole-transcriptome analysis, under the same experimental conditions, wherein SsrA transcription was repressed by −3.9-fold (García-Contreras *et al.*, 2008). Corroborating this result, qRT-PCR using RNA samples from the *hha* deletion isogenic mutant and from the wild-type strain also showed that *ssrA* transcription is induced (2.1 ± 0.1-fold) upon deleting *hha*. Taken together, these three independent results show Hha negatively regulates *ssrA* transcription.

The transcribed region of SsrA overlaps with the CP4-57 prophage genes, in that the 3′-end of the mature *ssrA* transcript includes the attachment CP4-57 site (*attL*) (Figure 2). To study the effect of SsrA on CP4-57, we constructed two *ssrA* deletion mutants in which the promoter and the entire or a part of the mature transcript of SsrA were deleted from the chromosome of BW25113. Mutant *ssrA* no longer has an *attL* site, whereas mutant *ssrA attL*$^+$ retains an intact *attL* site. When AlpA was overexpressed by plasmid pCA24N in the *ssrA* deletion strain, due to the absence of *attL* required for excision, no CP4-57 excision was detected by qPCR in LB medium after 15 h. Moreover, in the SsrA deletion mutant that contains the *attL* site (*ssrA attL*$^+$), CP4-57 excision was not detected by qPCR when AlpA was overexpressed in LB medium after 15 h that leads to an at least 70 000-fold repression of excision. Therefore, both functional SsrA and its *attL* site are necessary for CP4-57 excision, and the role of SsrA in CP4-57 excision is more than serving as an attachment site, but the exact role of SsrA in the Hha induction of CP4-57 excision remains to be fully elucidated.

The effect of Hha and SsrA on biofilm was also investigated. The deletion of Hha increased early biofilm formation by 7 ± 1-fold, whereas the partial deletion of SsrA (*ssrA attL*$^+$) decreased early biofilm formation by 2.1 ± 0.1-fold at 7 h in LB medium. Moreover, the deletion of both Hha and SsrA decreased early biofilm formation by the same amount as that by the deletion of SsrA. Therefore, deletion of host factors that control prophage excision affect biofilm formation at early stages in a complex manner. As expected, we also found that the strain, *ssrA attL*$^+$, was more sensitive to the toxicity caused by overexpressing Hha, as SsrA is required for growth under stressful conditions (Hong *et al.*, 2007).

Deletion of complete CP4-57 reduces cell growth

To study the effect of CP4-57 on *E. coli* gene expression and physiology, we constructed a new isogenic strain, BW25113 ΔCP4-57, in which the whole prophage (22 030 bp) was removed (including the excised phage circle) in a way that is equivalent to natural excision (Figure 2). To study the effects of the removal of CP4-57 on host fitness, we tested the growth of ΔCP4-57 in different media. Compared with the isogenic wild-type strain, ΔCP4-57 grew more slowly in both nutrient-rich and nutrient-poor media. The specific growth rate (h^{-1}) was 1.40 ± 0.01 vs 1.67 ± 0.01 in LB (Figure 3a) and 1.51 ± 0.01 vs 1.71 ± 0.02 in LB glu (Figure 3b) for ΔCP4-57 and the wild-type strain, respectively. Even larger differences in the specific growth rate were observed between these two strains in minimal medium; the specific growth rate (h^{-1}) was 0.24 ± 0.01 vs 0.70 ± 0.01 in M9 minimal medium (M9C) (Figure 3c) and 0.27 ± 0.06 vs 0.93 ± 0.02 in M9C with 0.1% lactate (M9C lactate) (Figure 3d) for ΔCP4-57 and the wild-type strain, respectively. Hence, growth inhibition in ΔCP4-57 was shown in a nutrient-dependent manner, indicating an active and beneficial role of prophage in host physiology especially in nutrient-poor conditions.

Removal of CP4-57 affects biofilm formation

As with deleting each gene of CP4-57 (Figure 1), deleting all of CP4-57 affected biofilm formation. Deleting CP4-57 increased early biofilm formation up to 4.6 ± 0.2-fold at 7 h and increased dispersal as there was a 2.3 ± 0.1-fold-less biofilm at later stages (33 h) in 96-well polystyrene plates (Figure 4a). Corroborating these results, the total number of viable cells in the biofilm formed on polystyrene tubes increased slightly for ΔCP4-57 at early stages (1.7 ± 0.2-fold after 15 h, Figure 4b) and decreased at

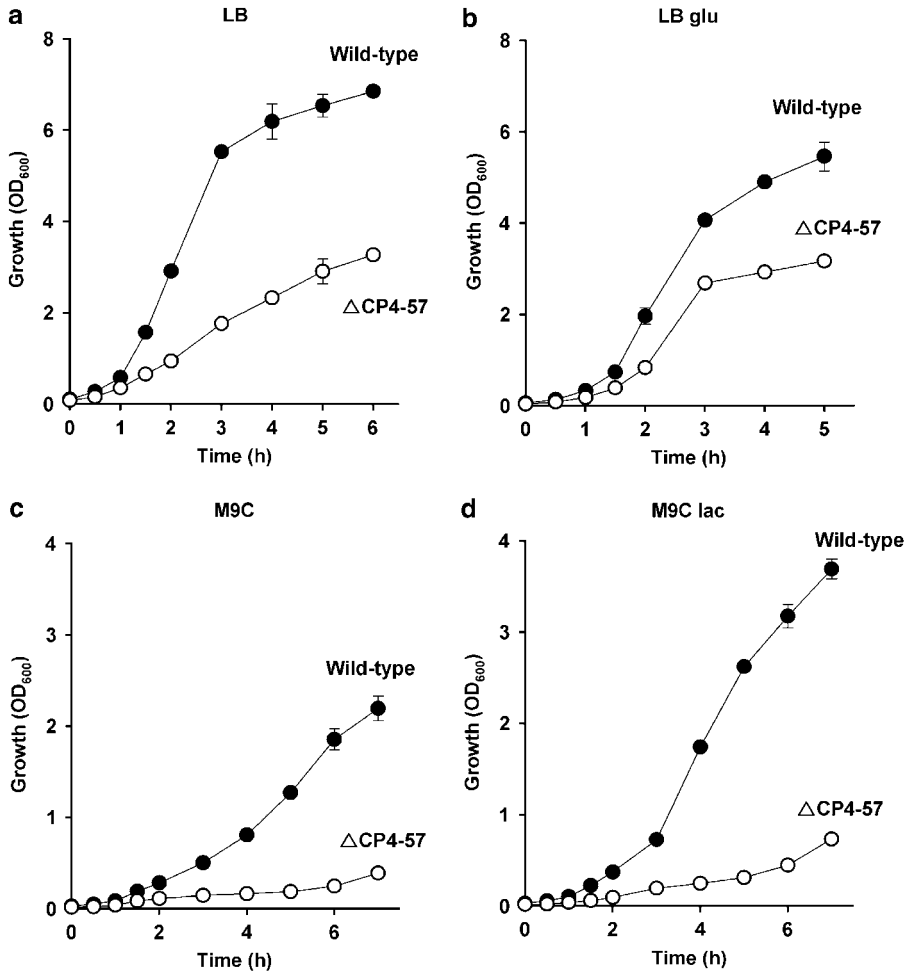

Figure 3 Growth of ΔCP4-57 in different media. Growth in Luria-Bertani (LB) (**a**), LB containing 0.2% glucose (LB glu) (**b**), M9 minimal medium with 0.2% casamino acids (M9C) (**c**) and M9C with 0.1% lactate (M9C lactate) (**d**) media, at 37 °C. Data are from two independent cultures, and one standard deviation is shown.

later stages (2.8 ± 0.3-fold after 36 h, Figure 4b). For the planktonic cells of both the wild-type strain and ΔCP4-57, there was no significant change in turbidity after 40 h of incubation and no change in the number of viable cells; hence, there was no apparent cell lysis or change in growth that led to these differences in biofilm formation. Therefore, prophage CP4-57 affects biofilm formations in a temporal manner, and the genes of CP4-57 primarily serve to reduce early biofilm formation.

Deleting CP4-57 induces motility through flagella and represses carbohydrate metabolism

A whole-transcriptome study was performed to characterize the effect of removing CP4-57 from *E. coli* and to explore the effects of CP4-57 on host physiology at the transcriptional level. In exponentially growing planktonic wild-type cells, 25 prophage genes had signals higher than 500, the average signal (Supplementary Table S1). Hence, prophage genes actively participate in host physiology even in planktonic cells.

Upon deleting the CP4-57 prophage, 36 flagella-related genes were induced, and these were the most upregulated genes (Table 3). Flagella affects cell motility, which is critical for early attachment for biofilm development (Pratt and Kolter, 1998), and it affects mature biofilm architecture in *E. coli* (Wood *et al.*, 2006). Corroborating this increase in transcription of the flagellar loci, motility assays showed clearly that deletion of CP4-57 induced motility 8 ± 1-fold (halo diameter for ΔCP4-57 is 5.8 ± 0.6 cm) compared with that of the wild-type strain (halo diameter for wild type is 0.8 ± 0.1 cm) on a low-salt motility agar plate (Figure 5a). Furthermore, the wild-type strain did not show any motility in high-salt motility plates, whereas ΔCP4-57 formed halos (Figure 5b). Salt concentrations (NaCl) affect cell motility by affecting the rotation rate of the flagella motor (Muramoto *et al.*, 1995). This result provides another line of evidence that CP4-57 prophage is involved in the development of biofilms through flagella synthesis and rotation.

The loss of CP4-57 repressed a group of genes involved in carbohydrate metabolism (for example,

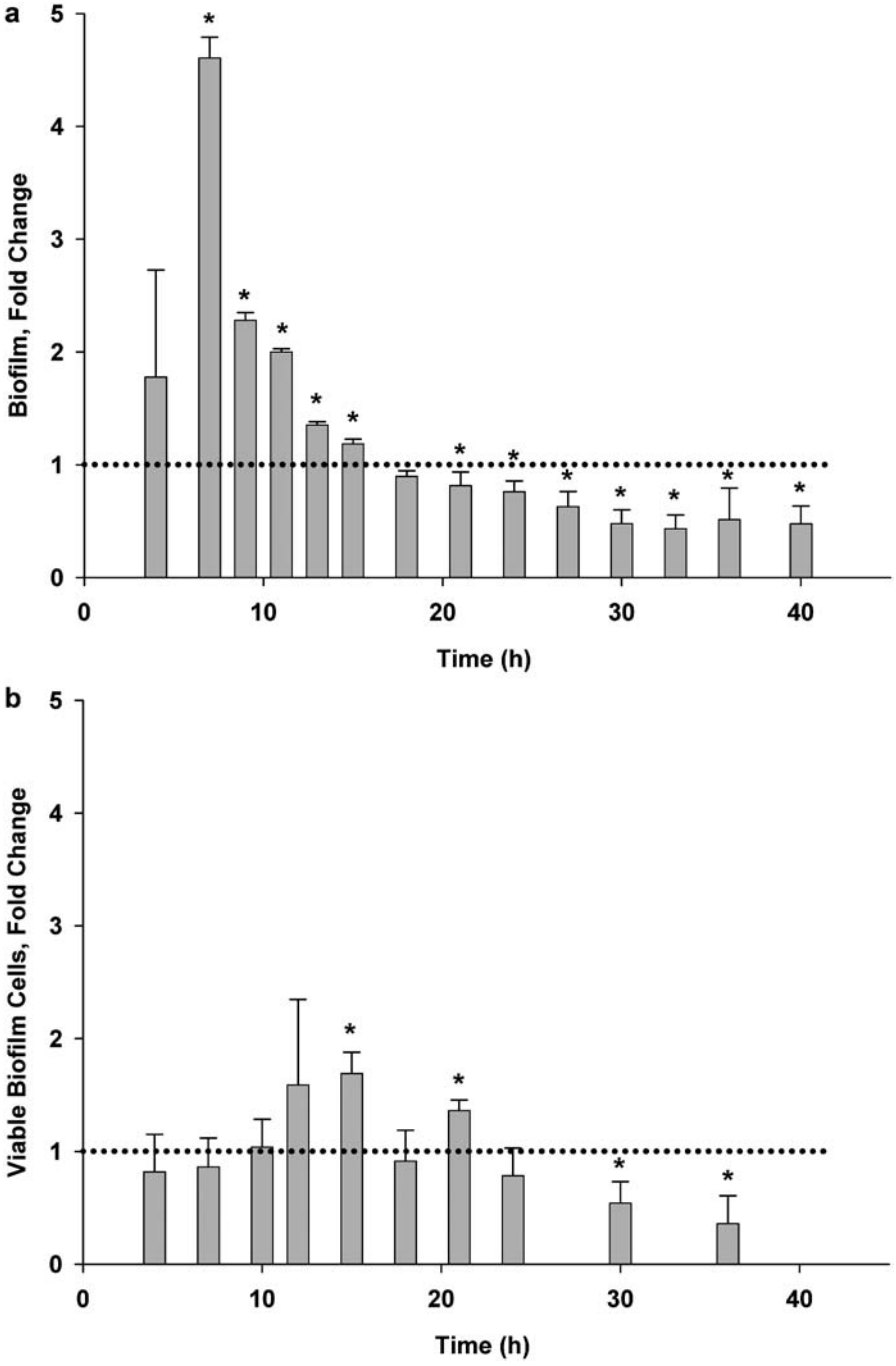

Figure 4 Biofilm formation of ΔCP4-57. (**a**) The fold change in normalized biofilm formation in 96-well polystyrene plates in Luria-Bertani (LB) medium at 37 °C was determined for ΔCP4-57 vs for the wild-type strain. Data are the average of 12 replicate wells from four independent cultures, and one standard deviation is shown. (**b**) Fold change for the total number of viable biofilm cells (CFU per ml) in 14 ml polystyrene tubes in LB medium at 37 °C was determined for ΔCP4-57 vs for the wild-type strain. Data are the average of two replicate tubes from two independent cultures, and one standard deviation is shown. Time point when $P<0.05$ (Student's *t*-test) is marked with asterisk.

lldPRD, *sdhCDAB*, *sucABCD* and *aceAB*), and amino-acid metabolism (for example, *dadAX*, *putAP*, *tnaBL* and *trpAB*) (Table 3). Succinyl-CoA synthetase (encoded by *sucABCD*), succinate dehydrogenase (encoded by *sdhABCD*) and malate synthetase (encoded by *aceB*) are key enzymes in the tricarboxylic acid (TCA) cycle, and a repression of the TCA cycle reduces energy production. These results could explain the reduced growth rate of ΔCP4-57 in different media (Figure 3). In addition, *lldP* and *lldR*, encoding for a lactate utilization system, were also two of the three genes found most differentially expressed in a lambda lysogen vs nonlysogenic *E. coli* (Osterhout *et al.*, 2007), although the changes were relatively small. The loss of CP4-57 also affected the two terminal

Table 3 List of differentially expressed genes in exponentially grown, planktonic cells of BW25113 ΔCP4-57 vs wild-type strain in LB medium at 37 °C

Group and gene	*b number*	*Fold change*	*Description*
Flagella related			
flgA	b1072	2.5	Assembly of basal-body periplasmic P ring
flgB	b1073	2.5	Cell-proximal portion of basal-body rod
flgC	b1074	2.6	Cell-proximal portion of basal-body rod
flgD	b1075	2.6	Initiation of hook assembly
flgE	b1076	2.1	Hook protein
flgF	b1077	3.7	Cell-proximal portion of basal-body rod
flgG	b1078	2.5	Cell-distal portion of basal-body rod
flgH	b1079	2.1	Membrane L (lipopolysaccharide layer) ring protein
flgI	b1080	2.0	Basal body P-ring flagellar protein
flgJ	b1081	2.0	Motor activity
flgK	b1082	2.0	Motor activity
flgL	b1083	1.6	Hook-filament junction protein
flgM	b1071	2.0	Anti-sigma 28 (FliA) factor, regulator of FlhD
flgN	b1070	1.9	Initiation of flagellar filament assembly
flhA	b1879	1.6	Possible export of flagellar proteins
flhB	b1880	1.9	Putative part of export apparatus for flagellar proteins
flhC	b1891	1.7	Flagellar transcriptional activator
flhD	b1892	1.9	Flagellar transcriptional activator
flhE	b1878	1.5	Function unknown
fliA	b1922	3.0	Regulation of flagellar operons
fliC	b1923	1.6	Flagellin, filament structural protein
fliD	b1924	1.5	Filament capping protein, enables filament assembly
fliE	b1937	2.3	Basal-body component, possibly at (MS-ring)-rod junction
fliF	b1938	2.5	Basal-body component
fliG	b1939	2.3	Rotor protein for flagellar motor switching and energizing
fliH	b1940	2.3	Negative regulator of FliI ATPase activity
fliI	b1941	2.1	Flagellum-specific ATP synthase
fliJ	b1942	2.1	Flagellin export apparatus soluble chaperone
fliK	b1943	1.9	Flagellar hook-length control protein
fliL	b1944	2.5	Affects rotational direction of flagella during chemotaxis
fliM	b1945	2.5	Switch and energizing, enabling rotation and determining its direction
fliN	b1946	2.5	Switch and energizing, enabling rotation and determining its direction
fliO	b1947	1.9	Flagellum organization and biogenesis
fliP	b1948	1.7	Protein secretion
fliQ	b1949	1.6	Protein secretion
fliZ	b1921	2.8	DNA integration
Small RNA			
rydB	b4430	2.1	Novel sRNA, function unknown
Electron transport			
cydA	b0733	1.9	Cytochrome *d* terminal oxidase subunit I
cydB	b0734	1.9	Cytochrome *d* terminal oxidase subunit II
cyoB	b0431	−1.6	Cytochrome *o* ubiquinol oxidase subunit I
cyoC	b0430	−1.7	Cytochrome *o* ubiquinol oxidase subunit III
cyoD	b0429	−1.7	Cytochrome *o* ubiquinol oxidase subunit IV
cyoE	b0428	−1.6	Protoheme IX farnesyltransferase
betA	b0311	−1.7	Choline dehydrogenase
betB	b0312	−1.7	Betaine aldehyde dehydrogenase, NAD dependent
betI	b0313	−1.5	Choline sensing
Amino-acid metabolism			
tdcA	b3118	1.9	Transcription activator of threonine dehydratase
tdcB	b3117	1.9	Threonine dehydratase
tdcC	b3116	1.5	Threonine dehydratase
dadA	b1189	−2.1	D-amino acid dehydrogenase subunit
dadX	b1190	−2.5	Alanine racemase 2, catabolic
putA	b1014	−2.3	Proline dehydrogenase, P5C dehydrogenase
putP	b1015	−2.8	Major sodium/proline symporter
tnaB	b3709	−1.5	Low-affinity tryptophan permease
tnaL	b3707	−1.5	Tryptophanase leader peptide
trpA	b1260	−1.6	Tryptophan synthase, α-protein
trpB	b1261	−1.6	Tryptophan synthase, β-protein

Table 3 Continued

Group and gene	*b number*	*Fold change*	*Description*
Carbohydrate metabolism			
lldP	b3603	−3.0	L-lactate permease
lldR	b3604	−2.5	Transcriptional regulator
lldD	b3605	−2.3	L-lactate dehydrogenase
sdhA	b0723	−1.9	Succinate dehydrogenase, flavoprotein subunit
sdhB	b0724	−1.9	Succinate dehydrogenase, iron sulfur protein
sdhC	b0721	−1.7	Succinate dehydrogenase, cytochrome b556
sdhD	b0722	−1.7	Succinate dehydrogenase, hydrophobic subunit
sucA	b0726	−1.9	Succinate dehydrogenase (decarboxylase component)
sucB	b0727	−1.6	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex
sucC	b0728	−1.9	Succinyl-CoA synthetase, β-subunit
sucD	b0729	−1.7	Succinyl-CoA synthetase, α-subunit
aceA	b4015	−1.7	Isocitrate lyase
aceB	b4014	−1.5	Malate synthetase A
mglA	b2149	−1.6	Alactoside transport and galactose taxis
mglC	b2148	−1.7	Alactoside transport and galactose taxis
gltA	b0720	−2.1	Citrate synthetase
fumA	b1612	−1.6	Fumarate hydratase class I
fumC	b1611	−1.7	SoxRS regulon, fumarate hydratase class II
acs	b4069	−1.7	Acetyl-CoA synthetase
aldA	b1415	−1.7	Aldehyde dehydrogenase, NAD linked
maeB	b2463	−1.9	NADP-dependent malic enzyme; NADP-ME
Unknown			
yecR	b1904	1.9	Function unknown
ydcI	b1422	−1.7	Function unknown

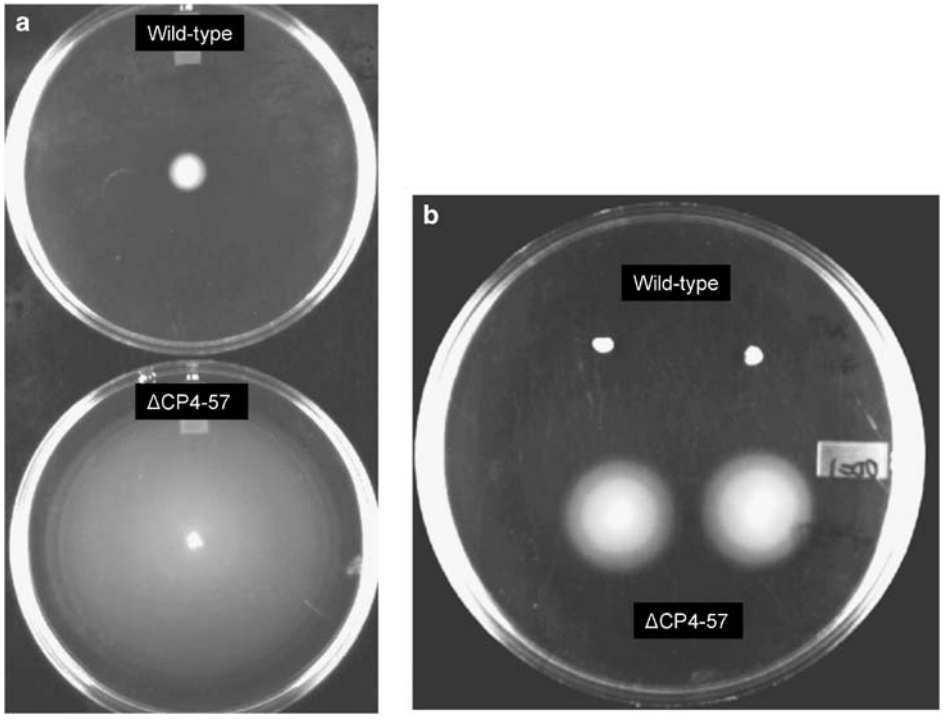

Figure 5 Motility of ΔCP4-57. Swimming motility of ΔCP4-57 in low-salt motility agar plates (**a**) and high-salt motility agar plates (**b**). One microliter of overnight culture was inoculated onto motility agar plates that were photographed after incubation for 16 h at 37 °C. Two independent cultures were used and only image is shown in panel a.

oxidases for the *E. coli* respiratory chain by activating genes involved in cytochrome *d* terminal oxidase *cydAB* (Table 3) and by repressing genes involved in cytochrome *o* terminal oxidase *cyoBCDE* (Table 3); these changes are usually associated with anaerobic growth (Iuchi *et al*., 1990). Growth in minimal medium M9C with lactate (Figure 3d) and without lactate (Figure 3c) showed that ΔCP4-57

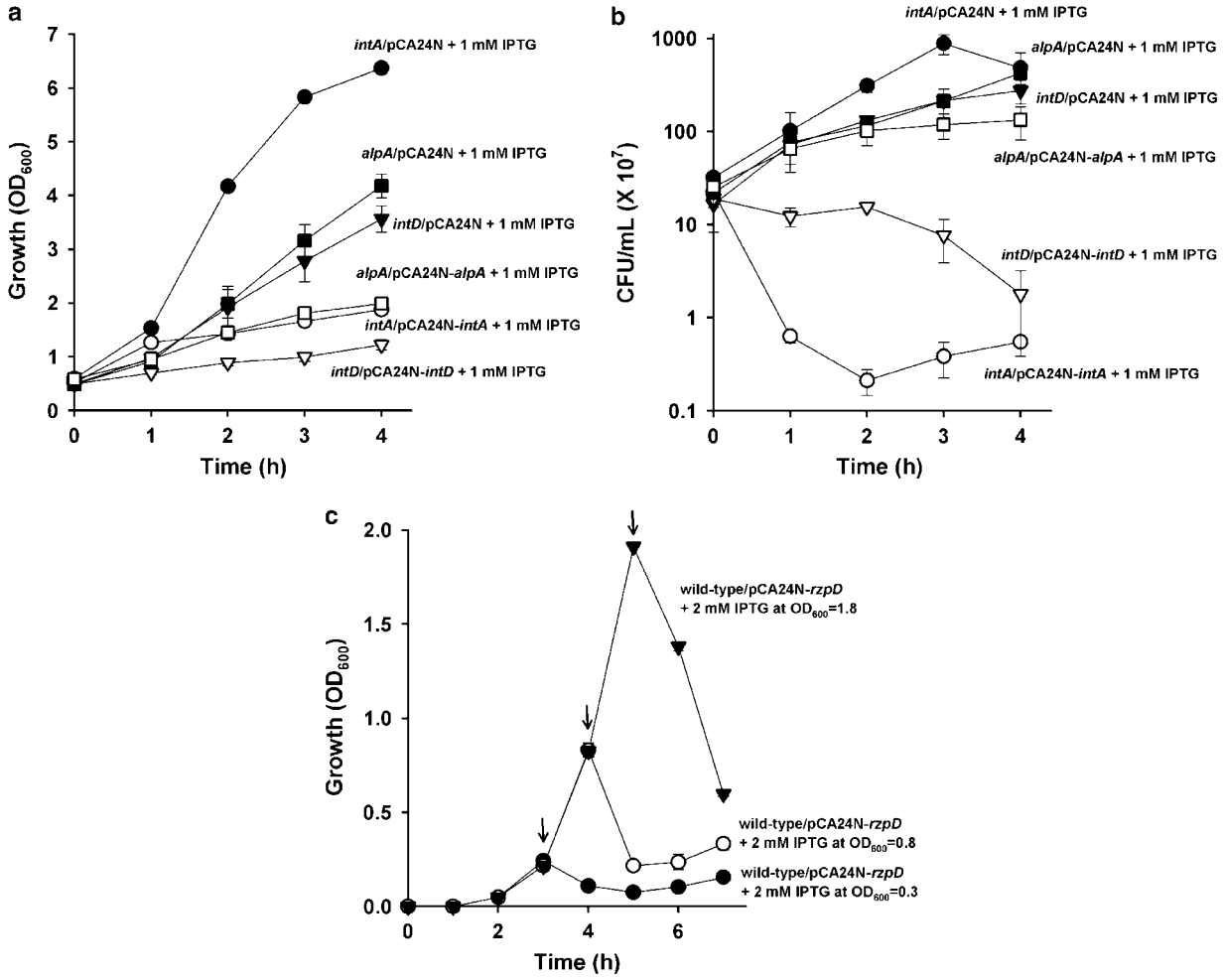

Figure 6 Toxicity of prophage genes. Growth (**a**) and cell viability (CFU per ml) (**b**) after overexpressing IntA, AlpA and IntD by pCA24N-*intA*, pCA24N-*alpA* and pCA24N-*intD* in Luria-Bertani (LB) medium with 1 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG) at a turbidity of 0.5 at 600 nm. Empty pCA24N vector of each strain with 1 mM IPTG served as a negative control. Growth for overexpression of RzpD by adding 2 mM IPTG at a turbidity of 0.3, 0.5 and 1.8 at 600 nm (**c**). Arrows indicate the time points for adding 2 mM IPTG. Data are from two independent cultures, and one standard deviation is shown.

was unable to utilize lactate as well as the wild-type strain. The specific growth rate increased from 0.70 ± 0.01 to 0.93 ± 0.02 with the addition of 0.1% lactate for the wild type, whereas for ΔCP4-57, growth did not change appreciably (0.24 ± 0.01 vs 0.27 ± 0.06). Hence, the lactate utilization system seems to play an important role in host–phage interactions for both CP4-57 and lambda. Overall, the whole-transcriptome analysis showed that the loss of CP4-57 affected genes involved in metabolism and biofilm formation, especially motility, indicating that CP4-57, although defective, is still involved in host physiology and affects biofilm formation.

Excised CP4-57 circularizes

The excised CP4-57 prophage was found to form a circle, as confirmed by a PCR-based assay, followed by sequencing (Figure 2). In addition, there was an indication that the prophage genes were expressed while being a part of this phage circle after excision, as kanamycin resistance encoded within CP4-57 (*alpA* gene) was retained in the cell, which is distinct from the ΔCP4-57 strain that does not have circularized CP4-57; hence, we named this strain ΔCP4-57$^{\text{circle}}$ (Table 2). However, in agreement with that which has been reported previously (Kirby *et al.*, 1994), the circularized prophage was not maintained in the manner of a stable plasmid, probably due to the loss of its ability to complete a full phage replication cycle once excised from the host (Casjens, 2003), as, after 24 h of growth in fresh LB medium, more than 90% of the cells no longer had the phage circle.

Overexpression of IntA, IntD, AlpA and RzpD leads to cell death and lysis

IntA of CP4-57 and IntD of DLP12 remain functional as integrases, as shown by their ability to cause prophage excision as described earlier in this study.

To explore whether these integrases are involved in cell death, we determined growth and cell viability by overexpressing IntA and IntD as well as AlpA (transcriptional activator for IntA). Growth and cell viability were significantly repressed after induction of *intA* and *intD*, and a slight decrease was seen for *alpA* (Figures 6a and b). Moreover, the overexpression of *rzpD* (a putative murein endopeptidase of DLP12) dramatically induced cell lysis (Figure 6c). Therefore, IntA, IntD, AlpA and RzpD are capable of causing cell death and cell lysis.

Discussion

Although prophages are abundant and widespread in bacteria, their roles in host physiology remain largely unexplored. We present the following lines of evidence to show that CP4-57 affects host *E. coli* physiology: (1) deleting each prophage gene and deleting all of CP4-57 affect biofilm formation in a temporal manner (Figures 1a, 4a and b); (2) prophage genes are actively expressed in exponentially growing planktonic cells, as shown by a whole-transcriptome study (Supplementary Table S1); (3) deleting CP4-57 decreases the specific growth rate and final cell density, especially in minimal medium (Figure 3); (4) deleting CP4-57 dramatically increases cell motility (Figure 5) as corroborated by induction of 36 flagella genes (Table 3); and (5) overexpression of prophage genes (IntA and AlpA) causes cell death (Figure 6). Taken together, these results show clearly that CP4-57 prophage participates in *E. coli* physiology including biofilm development.

Here we also show that prophage excision is a natural process during *E. coli* biofilm development. CP4-57 and DLP12 remain excision proficient, and the overexpression of integrase (IntA and IntD) as well as the activator of integrase (AlpA) leads to a large fraction of cells (18–87%) that undergo excision. A low fraction of cells (around the order of 1 per 10 000) undergo excision of CP4-57 and DLP12 in wild-type biofilms, and at early developmental stages (4–15 h), the excision of CP4-57 was only detected in biofilm cells but not in planktonic cells. This is consistent with our earlier study (Domka *et al*., 2007) that during the development of *E. coli* biofilms, AlpA is induced consistently from 4 to 24 h up to 11-fold (24 vs 15 h), and this induction should lead to CP4-57 excision.

We conclude from this study that the process of prophage excision is beneficial to *E. coli* biofilm development, at least for prophage CP4-57. The short-term growth of *P. aeruginosa* in biofilms generated extensive genetic diversity through a *recA*-dependent recombination inside the community, and this self-generated diversity produces 'insurance effects' to increase the ability of cells to withstand various environmental stresses (Boles *et al*., 2004). The excision of CP4-57 from *E. coli* leads to the formation of a prophage-deficient strain and a phage-like circle that expresses some functional phage genes. The resulting prophage-deficient strain was shown to process specialized functions in biofilms, including enhanced motility, reduced growth and increased biofilm dispersal. Flagella motility plays an important role for early attachment to various surfaces (Pratt and Kolter, 1998) as well as in dispersal (Wood *et al*., 2006). Here we show that as early as at 4 h, CP4-57 excision occurs only in biofilm cells of the wild-type strain. When CP4-57 is removed, swimming motility increases through induction of flagella synthesis, and this result is consistent with the observation that ΔCP4-57 formed biofilm more quickly than did the wild-type strain at early stages, and is consistent with the increased dispersal (Figures 4a and b). This also provides an advantage, at least in part, for a wild-type strain to undergo prophage excision during the development of *E. coli* biofilms. The influence of CP4-57 on motility is a novel role for prophage. Moreover, the small proportion of cells that undergo prophage excision helps to reduce the overall requirement of nutrients by generating slow-growing cells (for example, ΔCP4-57) or by killing a fraction of cells through the expression of prophage genes (for example, *alpA* and *intA*). Hence, for *E. coli*, prophage excision is involved in the population diversification inside the biofilm community, which has gained recognition as an important component in other bacteria, including *P. aeruginosa* (Webb *et al*., 2003b; Boles *et al*., 2004; Rice *et al*., 2009). One could imagine circumstances in which the presence of bacteria with differing motilities or divergent nutritional requirements would have advantages in the face of the adverse conditions faced in biofilms (Boles *et al*., 2004). It seems that ΔCP4-57 displays a behavioral strategy that can enhance fitness in social groups despite being costly to itself in terms of growth (Crespi, 2001; Velicer and Yu, 2003).

The DNA-binding protein, Hha, is related to prophage gene expression, as we reported previously (García-Contreras *et al*., 2008), and Hha is a global regulator that has an important function in regulating horizontally transferred elements including pathogenicity islands (Vivero *et al*., 2008). Hha has been related to cell death by its binding to rare codon tRNAs to inhibit translation and by activating proteases (Lon, ClpP and ClpX) (García-Contreras *et al*., 2008). We show here that Hha promotes prophage excision directly by activating prophage genes. The lines of evidence that indicate Hha promotes excision are the following: (1) Hha induces the expression of *intA* and *alpA*, which are known to induce excision and lead to cell death and (2) Hha causes prophage excision (shown by overexpressing Hha and deleting *hha*). Moreover, Hha repressed the *trans*-translation process by repressing SsrA, which facilitates the release of stalled ribosomes from damaged mRNAs and

targets the aberrant protein for degradation (Hong *et al.*, 2007). In this study, we found that Hha represses the transcription of SsrA, which in turn enhances the toxicity of Hha and which indicates that Hha and SsrA are intertwined as they regulate cell death.

Comparing ΔCP4-57 with wild-type strains shows clearly that a complex coevolution event has occurred between the host and the phage for the wild-type strain. A whole-transcriptome study shows that one of the single most important phenotypes for biofilms, flagella synthesis, is induced and the most important metabolic pathway, the TCA cycle, is repressed when CP4-57 is removed. Both actions affect cell physiology and use many genes across the *E. coli* genome. In addition, the slow growth and increased motility of ΔCP4-57 could be a trade-off under energetically limiting conditions during the coevolution of chromosomal genes (Pfeiffer *et al.*, 2001) because both cell growth and flagella biogenesis/rotation are energetically costly (Tian *et al.*, 2008). Since Hha induces expression of prophage genes and promotes excision, prophages have become a part of the regulatory circuitry of the *E. coli* host. We hypothesize that *E. coli* takes control of prophage and its excision, and uses these foreign genes to benefit itself for the biofilm mode of life.

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