The lysis cassette of DLP12 defective prophage is regulated by RpoE

Karl-Gustav Rueggeberg,† Faustino A. Toba,† Jeremy G. Bird,‡§ Nathan Franck,¶ Mitchell G. Thompson¶¶ and Anthony G. Hay†,∥

Expression of the lysis cassette (essD, ybcT, rzpD/rzoD) from the defective lambdoid prophage at the 12th minute of Escherichia coli's genome (DLP12) is required in some strains for proper curli expression and biofilm formation. Regulating production of the lytic enzymes encoded by these genes is critical for maintaining cell wall integrity. In lambdoid phages, late-gene regulation is mediated by the vegetative sigma factor RpoD and the lambda antiterminator Q\(^\text{\textit{l}}\).

We previously demonstrated that DLP12 contains a Q-like protein (Q\(_{\text{DLP12}}\)) that positively regulates transcription of the lysis cassette, but the sigma factor responsible for this transcription initiation remained to be elucidated. In silico analysis of essD\(_{\text{p}}\) revealed the presence of a putative –35 and –10 sigma site recognized by the extracytoplasmic stress response sigma factor, RpoE. In this work, we report that RpoE overexpression promoted transcription from essD\(_{\text{p}}\) in vivo, and in vitro using purified RNAP. We demonstrate that the –35 region is important for RpoE binding in vitro and that this region is also important for Q\(_{\text{DLP12}}\)-mediated transcription of essD\(_{\text{p}}\) in vivo. A bacterial two-hybrid assay indicated that Q\(_{\text{DLP12}}\) and RpoE physically interact in vivo, consistent with what is seen for Q\(_{\text{l}}\) and RpoD.

We propose that RpoE regulates transcription of the DLP12 lysis genes through interaction with Q\(_{\text{DLP12}}\) and that proper expression is dependent on an intact –35 sigma region in essD\(_{\text{p}}\). This work provides evidence that the unique Q-dependent regulatory mechanism of lambdoid phages has been co-opted by E. coli harbouring defective DLP12 and has been integrated into the tightly controlled RpoE regulon.

INTRODUCTION

In nature, bacteria are frequently preyed upon by bacteriophages. However, this process can also allow bacteria to acquire beneficial genes that can facilitate their survival

†Present address: Department of Microbiology, University of Pennsylvania, Philadelphia, PA, USA.
‡Present address: Medical Instill Technologies Inc., New Milford, CT, USA.
§Present address: Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ, USA.
¶Present address: State University of New York Downstate Medical Center, Brooklyn, NY, USA.
¶¶Present address: Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA.

Abbreviations: Amp, ampicillin; Chl, chloramphenicol; HRP, horseradish peroxidase; Kan, kanamycin.

Three supplementary figures are available with the online Supplementary Material.

© 2015 The Authors
Printed in Great Britain
000115 DOI 10.1099/mic.0.000115

Microbiology (2015), 161, 1683–1693

The lysis cassette of DLP12 defective prophage is regulated by RpoE

Karl-Gustav Rueggeberg,† Faustino A. Toba,† Jeremy G. Bird,‡§ Nathan Franck,¶ Mitchell G. Thompson¶¶ and Anthony G. Hay†,∥

Expression of the lysis cassette (essD, ybcT, rzpD/rzoD) from the defective lambdoid prophage at the 12th minute of Escherichia coli’s genome (DLP12) is required in some strains for proper curli expression and biofilm formation. Regulating production of the lytic enzymes encoded by these genes is critical for maintaining cell wall integrity. In lambdoid phages, late-gene regulation is mediated by the vegetative sigma factor RpoD and the lambda antiterminator Q\(^\text{\textit{l}}\).

We previously demonstrated that DLP12 contains a Q-like protein (Q\(_{\text{DLP12}}\)) that positively regulates transcription of the lysis cassette, but the sigma factor responsible for this transcription initiation remained to be elucidated. In silico analysis of essD\(_{\text{p}}\) revealed the presence of a putative –35 and –10 sigma site recognized by the extracytoplasmic stress response sigma factor, RpoE. In this work, we report that RpoE overexpression promoted transcription from essD\(_{\text{p}}\) in vivo, and in vitro using purified RNAP. We demonstrate that the –35 region is important for RpoE binding in vitro and that this region is also important for Q\(_{\text{DLP12}}\)-mediated transcription of essD\(_{\text{p}}\) in vivo. A bacterial two-hybrid assay indicated that Q\(_{\text{DLP12}}\) and RpoE physically interact in vivo, consistent with what is seen for Q\(_{\text{l}}\) and RpoD.

We propose that RpoE regulates transcription of the DLP12 lysis genes through interaction with Q\(_{\text{DLP12}}\) and that proper expression is dependent on an intact –35 sigma region in essD\(_{\text{p}}\). This work provides evidence that the unique Q-dependent regulatory mechanism of lambdoid phages has been co-opted by E. coli harbouring defective DLP12 and has been integrated into the tightly controlled RpoE regulon.

INTRODUCTION

In nature, bacteria are frequently preyed upon by bacteriophages. However, this process can also allow bacteria to acquire beneficial genes that can facilitate their survival

†Present address: Department of Microbiology, University of Pennsylvania, Philadelphia, PA, USA.
‡Present address: Medical Instill Technologies Inc., New Milford, CT, USA.
§Present address: Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ, USA.
¶Present address: State University of New York Downstate Medical Center, Brooklyn, NY, USA.
¶¶Present address: Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA.

Abbreviations: Amp, ampicillin; Chl, chloramphenicol; HRP, horseradish peroxidase; Kan, kanamycin.

Three supplementary figures are available with the online Supplementary Material.

© 2015 The Authors
Printed in Great Britain
000115 DOI 10.1099/mic.0.000115

Microbiology (2015), 161, 1683–1693

The lysis cassette of DLP12 defective prophage is regulated by RpoE

Karl-Gustav Rueggeberg,† Faustino A. Toba,† Jeremy G. Bird,‡§ Nathan Franck,¶ Mitchell G. Thompson¶¶ and Anthony G. Hay†,∥

Expression of the lysis cassette (essD, ybcT, rzpD/rzoD) from the defective lambdoid prophage at the 12th minute of Escherichia coli’s genome (DLP12) is required in some strains for proper curli expression and biofilm formation. Regulating production of the lytic enzymes encoded by these genes is critical for maintaining cell wall integrity. In lambdoid phages, late-gene regulation is mediated by the vegetative sigma factor RpoD and the lambda antiterminator Q\(^\text{\textit{l}}\).

We previously demonstrated that DLP12 contains a Q-like protein (Q\(_{\text{DLP12}}\)) that positively regulates transcription of the lysis cassette, but the sigma factor responsible for this transcription initiation remained to be elucidated. In silico analysis of essD\(_{\text{p}}\) revealed the presence of a putative –35 and –10 sigma site recognized by the extracytoplasmic stress response sigma factor, RpoE. In this work, we report that RpoE overexpression promoted transcription from essD\(_{\text{p}}\) in vivo, and in vitro using purified RNAP. We demonstrate that the –35 region is important for RpoE binding in vitro and that this region is also important for Q\(_{\text{DLP12}}\)-mediated transcription of essD\(_{\text{p}}\) in vivo. A bacterial two-hybrid assay indicated that Q\(_{\text{DLP12}}\) and RpoE physically interact in vivo, consistent with what is seen for Q\(_{\text{l}}\) and RpoD.

We propose that RpoE regulates transcription of the DLP12 lysis genes through interaction with Q\(_{\text{DLP12}}\) and that proper expression is dependent on an intact –35 sigma region in essD\(_{\text{p}}\). This work provides evidence that the unique Q-dependent regulatory mechanism of lambdoid phages has been co-opted by E. coli harbouring defective DLP12 and has been integrated into the tightly controlled RpoE regulon.

INTRODUCTION

In nature, bacteria are frequently preyed upon by bacteriophages. However, this process can also allow bacteria to acquire beneficial genes that can facilitate their survival

†Present address: Department of Microbiology, University of Pennsylvania, Philadelphia, PA, USA.
‡Present address: Medical Instill Technologies Inc., New Milford, CT, USA.
§Present address: Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ, USA.
¶Present address: State University of New York Downstate Medical Center, Brooklyn, NY, USA.
¶¶Present address: Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA.

Abbreviations: Amp, ampicillin; Chl, chloramphenicol; HRP, horseradish peroxidase; Kan, kanamycin.

Three supplementary figures are available with the online Supplementary Material.
and _Shewanella oneidensis_, or cryptic prophage elements, as has been reported for _E. coli_ (Binnenkade et al., 2014; Gödeke et al., 2011; Rice et al., 2009; Toba et al., 2011; Wang et al., 2010; Webb et al., 2004).

Many of the defective cryptic prophages of _E. coli_ are particularly interesting due to their attenuated ability to excise and enter the lytic phase of growth (Menouni et al., 2013; Wang et al., 2010). Over millions of years, the host has retained these phage remnants despite the apparent metabolic cost of maintaining the extra DNA (Casjens, 2003; Giovannoni et al., 2014; Lynch, 2006). Recently, it has been demonstrated that several defective phage genes play a role in _E. coli_ stress responses and biofilm formation (Toba et al., 2011; Wang et al., 2010). Of these cryptic phages, DLP12 (defective lambdoid prophage present at the 12th minute) is of particular interest due to its relatively intact 20 kb genome (Lindsey et al., 1989) and its functional lysis cassette. The enzymes encoded by the DLP12 lysis cassette are homologous to those of lambda (λ) phage that are required to disrupt the cell envelope and allow viral particles to escape the cell (Berry et al., 2008; Young et al., 2000).

We recently showed that expression of the DLP12 lysis genes (the holin S encoded by _essD_, the lysozyme R encoded by _ybcS_ and the spannin Rz/Rz1 encoded by _repDr/rozD_) is important for curli-mediated cell attachment and biofilm formation (Toba et al., 2011) and is dependent on a Q-like protein, Q<sup>DLP12</sup> (ybcQ) (Rueggeberg et al., 2013). Curli are pathogen-associated molecular patterns and have been associated with bacteremia in patients with urinary tract infections (Hung et al., 2014). Taken together, these results underscore the importance of prophage gene expression for curli production and virulence.

Phage λ regulates the expression of the lysis cassette through the production of Q<sup>λ</sup>, an antiterminator encoded by a gene located near the lysis cassette promoter, PR (Guo & Roberts, 2004). Q<sup>λ</sup> is necessary to extend transcripts produced from PR past the intrinsic terminator situated upstream of the lysis genes. Thus, expression of these potentially lethal genes is directly tied to the abundance of Q<sup>λ</sup> within the cell (Oppenheim et al., 2005; Salazar & Asenjo, 2007; Zhou et al., 2006). It is believed that phages have adopted this method of gene regulation as opposed to the conventional repression/derepression system used to dictate early phage gene expression due to its rapid response time (Casjens, 2003). Sequence alignment between Q<sup>DLP12</sup> and Q<sup>λ</sup> shows an 84 % sequence identity, which suggests that Q<sup>DLP12</sup> has a similar function to that of the canonical antiterminators.

In _E. coli_, expression of lambdoid phage genes has been reported to be under the control of the ubiquitous vegetative sigma factor RpoD or the stationary phase factor RpoS, with phage promoters containing readily identifiable RpoD or RpoS consensus sequences (Nakamura et al., 1979; Nickels et al., 2002; Roucourt & Lavigne, 2009). Computational analysis of the DLP12 lysis cassette promoter (_essDp_), however, predicted the presence of a −35 and −10 sigma binding region that is specific for RpoE, the extracellular stress sigma factor (Rhodius et al., 2006).

In this work, we present evidence that the DLP12 lysis genes are controlled by RpoE, through direct binding to the −35 position of _essDp_. In addition, we present evidence that Q<sup>DLP12</sup> interacts with both RpoE and RNA polymerase (RNAP) β subunit in a manner analogous to that of Q<sup>λ</sup> with RpoD and β. Furthermore, we establish that Q<sup>DLP12</sup> remains stably bound to the RNAP core complex during transcription of the lysis cassette. Taken together these data demonstrate that _E. coli_ has co-opted the Q-based transcriptional regulator mechanism of DLP12 and domesticated it via integration into the RpoE regulon.

**METHODS**

**Bacterial strains and growth conditions.** _E. coli_ strain PHL628 is an MG1655 derivative with an _ompR234_ mutation (Vidal et al., 1998). _E. coli_ PHL628 Δ<sup>DLP12</sup> (628.1), a strain with _s_ _essD_ deleted from the chromosome (Toba et al., 2011), was utilized in cell count, fluorometry and chromatin pulldown assays unless otherwise stated, because Q overexpression is less toxic in this strain (data not shown). _E. coli_ strains were routinely grown in lysogeny broth (LB) supplemented with 50 μg kanamycin (Kan) ml<sup>−1</sup> at 37 °C overnight with shaking (150 r.p.m.), and subsequently diluted 1:10 into fresh medium and cultured at 30 °C for the experiments, unless otherwise stated. For promoter studies low salt LB (5 g NaCl l<sup>−1</sup>) was used. When required, plates and media were supplemented with ampicillin (Amp) (150 μg ml<sup>−1</sup>), chloramphenicol (Chl) (25 μg ml<sup>−1</sup>), tetracycline (10 μg ml<sup>−1</sup>) or spectinomycin (100 μg ml<sup>−1</sup>). For induction, medium was supplemented with 1 mM IPTG according to the experimental needs. OD<sub>600</sub> was measured using a µQuant spectrophotometer (Bio-Tek).

**Plasmid construction.** Plasmids were isolated using an alkaline lysis large scale plasmid prep protocol (Feliciello & Chinali, 1993). For RpoE overexpression, _rpoE_ was cloned into the expression vector pBAD18T to form pBAD18T:rpoE. In this plasmid, RpoE expression is under the control of an arabinose-inducible promoter (Sukchawalit et al., 1999). For _in vitro_ transcription reactions, the promoter proximal region of _essDp_ was cloned into pME50 (Amp<sup>+</sup>), upstream of the intrinsic terminator of _rpoC_ using the following primers: _essDp For2_ Upstream BamHI and _essDp Rev Short_ EcoRI. For fluorometry measurements, _pessDp-GFP_, a reporter plasmid containing the _essDp_ promoter, was utilized (Rueggeberg et al., 2013). The _promeP2-GFP_ plasmid was constructed by replacing _essDp_ with _rpoE_ promoter P2. Mutants of _pessDp-GFP_ with altered −10 or −35 RpoE binding regions were created with site-directed mutagenesis of _pessDp-GFP_ using primers listed in Table 1 and confirmed via sequencing. Briefly, inverse PCR was performed using one mutagenic forward primer and a common non-mutagenic reverse primer, such of _pessDp-GFP_ using primers listed in Table 1 and confirmed via sequencing. For _promeP2-GFP_ expression, _pOEX-Q-His_ was utilized (Rueggeberg et al., 2013).

**Promoter fusion studies.** Reporter plasmid _pessDp-GFP_ was used to transform each of the PHL628 strains and selected on low salt LB
Amp plates. Cells were allowed to grow with shaking (150 r.p.m.) in low salt LB at 30 °C. At the indicated time points, fluorescence was measured using a Bio-Tek Synergy HT spectrophotometer with a 485/20 excitation filter and a 530/25 emission filter, and normalized to the OD600 of the corresponding culture. Measurements were done in triplicate.

**In vitro transcription assay.** Reactions containing 2 nM linear template (PCR amplification of pM650-essDp with essDp For2 Upstream BamHI and essDp Rev Short EcoRI primers) and 20 nM RNAP (20 nM core reconstituted with 100 nM σ70 and σ5) were incubated in TB [20 mM Tris/HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT and 50 mM KCl], 0.1 mg BSA ml⁻¹ and 10 % glycerol (v/v), plus 200 μM ATP, GTP and CTP, and 50 μM UTP supplemented with 0.5 μCi [α-32P]UTP ml⁻¹ at 37 °C for 10 min to form the open complex. RNAP core was used with 6 × His-RpoE. Transcription was initiated by addition of MgCl2 to a final concentration of 5 mM and rifampicin to 10 μM. Aliquots were taken at the indicated times after initiation and added to 5 volumes cold 1.2 M NaCl, 300 mM NaCl, 250 mM imidazole pH 8.0) and rinsed via sonication. The lysate was centrifuged and the soluble fraction was mixed with 1.5 g (3 ml) Ni-IDA resin (PrepEase) precharged with Ni²⁺ and equilibrated with LEW buffer. The resin was then transferred to a new Falcon tube and washed with LEW buffer (10 resin volumes). The protein was eluted with elution buffer [50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole pH 8.0] in three fractions (3 resin volumes each). SDS-PAGE analysis showed sample purity >95 %. The protein was dialed overnight against storage buffer (20 mM Tris/HCl, 2 % glycerol (v/v), 500 mM NaCl, 1 mM EDTA, 1 mM DTT). The percentage of glycerol was increased to 50 % via dialysis and the His₆-RpoE fractions were stored at −20 °C. Core RNAP was purified as described by Hager et al. (1990). QDL12 was purified as described by Rueggeberg et al. (2013).

Electrophoretic mobility shift assay of RpoE binding. A 151 bp dsDNA probe encompassing nucleotides −86 to +65 containing the putative RpoE binding region in essDp was made by PCR and gel purified. The probe was then radiolabeled using [γ-32P]ATP and T4PNK enzyme (Promega). His₆-RpoE in storage buffer was added to 2.5 ng purified probe (≈ 5000 c.p.m.) and 100-fold excess of herring sperm DNA (competitor DNA) in a 25 μl binding reaction; the final buffer was 20 mM Tris/HCl (pH 8.0), 25 mM KCl, 12 % glycerol (v/v), 1 mM DTT, 0.1 mM EDTA and 40 μg BSA ml⁻¹. The samples were mixed, incubated at room temperature for 25 min and briefly stored on ice. A 3–8 % gradient polyacrylamide gel (0.5 × TAE (20 mM Tris/acetate pH 8.5, 1 mM EDTA) running buffer was pre-chilled and the putative RpoE binding region in essDp with 0.5 mM ATP, GTP and CTP, and [α-32P]UTP μl⁻¹ on ice. A 25–85 % gradient polyacrylamide gel in 0.5 M UTP supplemented with 0.5 μCi [α-32P]UTP μl⁻¹ at 37 °C for 10 min to form the open complex. RNAP core was used with 6 × His-RpoE. Transcription was initiated by addition of MgCl2 to a final concentration of 5 mM and rifampicin to 10 μM. Aliquots were taken at the indicated times after initiation and added to 5 volumes cold 1.2 M NaCl, 300 mM NaCl, 250 mM imidazole pH 8.0) and rinsed via sonication. The lysate was centrifuged and the soluble fraction was mixed with 1.5 g (3 ml) Ni-IDA resin (PrepEase) precharged with Ni²⁺ and equilibrated with LEW buffer. The resin was then transferred to a new Falcon tube and washed with LEW buffer (10 resin volumes). The protein was eluted with elution buffer [50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole pH 8.0] in three fractions (3 resin volumes each). SDS-PAGE analysis showed sample purity >95 %. The protein was dialed overnight against storage buffer (20 mM Tris/HCl, 2 % glycerol (v/v), 500 mM NaCl, 1 mM EDTA, 1 mM DTT). The percentage of glycerol was increased to 50 % via dialysis and the His₆-RpoE fractions were stored at −20 °C. Core RNAP was purified as described by Hager et al. (1990). QDL12 was purified as described by Rueggeberg et al. (2013).

<table>
<thead>
<tr>
<th>Table 1. Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer name</td>
</tr>
<tr>
<td>--</td>
</tr>
<tr>
<td>−35-2 For</td>
</tr>
<tr>
<td>−35-3 For</td>
</tr>
<tr>
<td>−35-4 For</td>
</tr>
<tr>
<td>−35 Rev</td>
</tr>
<tr>
<td>−10-1 For</td>
</tr>
<tr>
<td>−10 Rev</td>
</tr>
<tr>
<td>essDp For2 Upstream BamHI</td>
</tr>
<tr>
<td>essDp Rev Short EcoRI</td>
</tr>
<tr>
<td>rpoEP2 For Kpd</td>
</tr>
<tr>
<td>rpoEP2 Rev Xbal</td>
</tr>
</tbody>
</table>

RpoE, core RNAP and QDL12 overexpression and purification. His₆-RpoE was expressed and purified as described elsewhere (Rouvière et al., 1995) with certain modifications. Briefly, pPER76 was electroporated into BL21(DE3)pLysS) to create the expression strain. A 1 l culture of this expression strain was grown in LB with shaking at 30 °C. When the OD600 reached 0.7, IPTG (1 mM) was added to the culture. Cells were harvested 3 h post-induction, pelleted, resuspended in lysis–equilibration–wash (LEW) buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) and lysed via sonication. The lysate was centrifuged and the soluble fraction was mixed with 1.5 g (3 ml) Ni-IDA resin (PrepEase) precharged with Ni²⁺ and equilibrated with LEW buffer. The resin was then transferred to a new Falcon tube and washed with LEW buffer (10 resin volumes). The protein was eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole pH 8.0) in three fractions (3 resin volumes each). SDS-PAGE analysis showed sample purity >95 %. The protein was dialed overnight against storage buffer (20 mM Tris/HCl, 2 % glycerol (v/v), 500 mM NaCl, 1 mM EDTA, 1 mM DTT). The percentage of glycerol was increased to 50 % via dialysis and the His₆-RpoE fractions were stored at −20 °C. Core RNAP was purified as described by Hager et al. (1990). QDL12 was purified as described by Rueggeberg et al. (2013).
vector pBR332, and contains a cassette for Amp resistance (Dove & Hochschild, 2004). It encodes the N-terminal domain of the z subunit of bacterial RNAP whose C-terminus is fused to the same truncated version (residues 831 to 1057) of the β subunit of RNAP found in pACζl β-831–1057, and is also under the control of the lacUV5 promoter. Different portions of rpoE were cloned into the Npf and BamHI cloning sites of pBRβ σ70 D581G to create RpoE fusion protein constructs. All pBR derivative plasmids created for this system were cloned in the same manner as pAC derivative plasmids, except that Amp was utilized instead of Chl for selection purposes.

Bait and prey plasmids (Table 2) were used to transform E. coli BN469, a lac− strain specifically designed for bacterial two-hybrid systems. It harbours an F′ plasmid bearing an artificial test promoter that contains a binding site for λcl (λ operator) and the lac core promoter, p lacOR2-62, linked to lacZ. It also encodes a marker for Kan resistance (50 μg ml⁻¹) (Dove & Hochschild, 2004; Nickels, 2009). Plasmid combinations were used to simultaneously transform the reporter strain through electroporation (Table 3). Successful transformants were simultaneously selected for with Kan-(reporter strain specific), Amp-(selects for pBR derivative plasmids) and Chl-(selects for pAC derivative plasmids) supplemented LB. The presence of all plasmids except for empty vector plasmids (pBRz and pACζl) was confirmed through PCRs utilizing a vector-specific forward primer and an insert-specific reverse primer, and verified by sequencing.

**Table 2. Plasmid constructs**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACζl β-831–1057</td>
<td>Positive control bait plasmid and fusion plasmid (bait) construction template plasmid; encodes ζl fused via three alanine residues to a truncated RNAP β subunit (residues 831–1057)</td>
<td>Deighan et al. (2008)</td>
</tr>
<tr>
<td>pACζlI</td>
<td>Empty vector bait plasmid; encodes ζl only</td>
<td>Deighan et al. (2008)</td>
</tr>
<tr>
<td>pBRz β-831–1057</td>
<td>Fusion plasmid (prey) construction template plasmid; encodes the N-terminal domain of the z subunit of RNAP (residues 1–248) fused to a truncated RNAP β subunit (residues 831–1057)</td>
<td>Deighan et al. (2008)</td>
</tr>
<tr>
<td>pBRz σ70 D581G</td>
<td>Prey plasmid; encodes the N-terminal domain of the z subunit of RNAP (residues 1–248) directly fused to domain 4 of σ70 (residues 528–613) carrying the D581G substitution (increased affinity to the β regin of RNAP)</td>
<td>Deighan et al. (2008)</td>
</tr>
<tr>
<td>pBRz</td>
<td>Empty vector bait plasmid; encodes the N-terminal domain of the z subunit of RNAP (residues 1–248)</td>
<td>Deighan et al. (2008)</td>
</tr>
<tr>
<td>pBRz σ6</td>
<td>Prey plasmid; encodes the N-terminal domain of the z subunit of RNAP (residues 1–248) fused via three alanine residues to the N-terminus of full-length σ6</td>
<td>This work</td>
</tr>
<tr>
<td>pACζl cl-ybcQ</td>
<td>Bait plasmid; encodes ζl fused via three alanine residues to full-length YbcQ</td>
<td>This work</td>
</tr>
<tr>
<td>pBRz σ6 (3.0–4.2)</td>
<td>Prey plasmid; encodes the N-terminal domain of the z subunit of RNAP (residues 1–248) fused via three alanine residues to the N-terminus regions 3.0 to 4.2 of σ6 (residues 94–191)</td>
<td>This work</td>
</tr>
<tr>
<td>pBRz σ6 (4.2)</td>
<td>Prey plasmid; encodes the N-terminal domain of the z subunit of RNAP (residues 1–248) fused via three alanine residues to the N-terminus region 4.2 of σ6 (residues 155–191)</td>
<td>This work</td>
</tr>
<tr>
<td>pBRz σ6 (1.2–2.4)</td>
<td>Prey plasmid; encodes the N-terminal domain of the z subunit of RNAP (residues 1–248) fused via three alanine residues to the N-terminus regions 1.2 to 2.4 of σ6 (residues 1–93)</td>
<td>This work</td>
</tr>
<tr>
<td>pPER76-rpoE-His</td>
<td>RpoE overexpression plasmid for RpoE purification</td>
<td>Ann Hochschild* This work</td>
</tr>
<tr>
<td>pBAD18T-rpoE</td>
<td>RpoE expression plasmid for in vivo induction of essDp-GFP</td>
<td>Rueggeberg et al. (2013) This work</td>
</tr>
<tr>
<td>pessDp-GFP</td>
<td>GFP reporter plasmid containing the essD promoter</td>
<td>Rueggeberg et al. (2013) This work</td>
</tr>
<tr>
<td>pOFX-Q-His</td>
<td>GFP reporter plasmid containing the rpoE promoter 2</td>
<td>Rueggeberg et al. (2013) This work</td>
</tr>
<tr>
<td>proEP2GFP</td>
<td>Q&lt;sup&gt;3&lt;/sup&gt;D&lt;sup&gt;12&lt;/sup&gt; overexpression plasmid under control of lacZ promoter</td>
<td>Rueggeberg et al. (2013) This work</td>
</tr>
</tbody>
</table>

*Harvard Medical School, Boston, USA.
Table 3. Bacterial two-hybrid assay plasmid combinations

<table>
<thead>
<tr>
<th>Plasmid combination</th>
<th>Abbreviation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAC2cl-β 831–1057 + pBRz σ70 D581G</td>
<td>β (831–1057) + σ70 D581G</td>
<td>Positive control, test for a known interaction (strong) between this portion of the β subunit of RNAP and domain 4 of σ70 (residues 528–613) carrying the D581G substitution</td>
</tr>
<tr>
<td>pAC2l-β ybcQ + pBRz σE</td>
<td>ybcQ + σE</td>
<td>Tests for an interaction between σE and YbcQ</td>
</tr>
<tr>
<td>pAC2l-β ybcQ + pBRz σE (1.2–2.4)</td>
<td>ybcQ + σE (1.2–2.4)</td>
<td>Tests for an interaction between σE and the 1.2 to 2.4 regions of σE</td>
</tr>
<tr>
<td>pAC2l-β ybcQ + pBRz σE (3.0–4.2)</td>
<td>ybcQ + σE (3.0–4.2)</td>
<td>Tests for an interaction between σE and the 3.0 to 4.2 regions of σE</td>
</tr>
<tr>
<td>pAC2l-β ybcQ + pBRz σE (4.2)</td>
<td>ybcQ + σE (4.2)</td>
<td>Tests for an interaction between σE and the 4.2 region of σE</td>
</tr>
<tr>
<td>pAC2l-β D581G</td>
<td>ybcQ + σ70 D581G</td>
<td>Tests for an interaction between domain 4 of σ70 (residues 528–613) carrying the D581G substitution (increased affinity to the β region of RNAP) and YbcQ</td>
</tr>
<tr>
<td>pAC2cl + pBRz</td>
<td>λcl only + z only</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
<tr>
<td>pAC2cl-β 831–1057 + pBRz</td>
<td>β (831–1057) + z only</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
<tr>
<td>pAC2cl + pBRz σ70 D581G</td>
<td>λcl only + σ70 D581G</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
<tr>
<td>pAC2cl + pBRz ybcQ + pBRz</td>
<td>ybcQ + z only</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
<tr>
<td>pAC2cl + pBRz σE</td>
<td>λcl only + σE</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
<tr>
<td>pAC2cl + pBRz σE (3.0–4.2)</td>
<td>λcl only + σE (3.0–4.2)</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
<tr>
<td>pAC2cl + pBRz σE (4.2)</td>
<td>λcl only + σE (4.2)</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
<tr>
<td>pAC2cl + pBRz σE (1.2–2.4)</td>
<td>λcl only + σE (1.2–2.4)</td>
<td>Negative control (no expected interaction between protein products)</td>
</tr>
</tbody>
</table>

0.1 % SDS, 1 % Tween 20, pH 2.2) and then reprobed with monoclonal anti-RpoB antibody (Neoclone) 1:5000, followed by incubation with goat anti-mouse HRP as described above. Quantification of the immunoblot was performed using ImageJ.

Chromatin pulldown assay. Overnight cultures (200 ml) of PHL628.1 pOFX-Q-His were prepared as in the Q-His pulldown assay, except immediately following cross-linking and quenching pelleted cells were washed twice in 200 ml cold PBS (pH 7.5) then resuspended in denaturing lysis buffer. Q-His-associated DNA remained bound to Q throughout the pulldown of the protein, allowing for assessment of bound DNA in downstream quantitative PCRs. The DNA was sheared by sonication to a mean size of ~500 bp. Following centrifugation at 12 000 g, at 4 ºC for 10 min, a subsample of the lysate was retained as an input control and the remainder was loaded onto a nickel-IDA column (PrepEase) and purified as described above. The eluted Q-His/DNA complex was boiled for 10 min to reverse cross-links, and the free DNA was purified using Zippy Spin columns and eluted in water. RNaseA was added (100 μg ml⁻¹ final concentration) and incubated for 90 min at 42 ºC. Multiplex PCR was employed to quantify the relative enrichment at essDp both proximal to (immediately following) the transcription start site and within the DLP12 lysis genes. These amplicons were normalized to that of the 16S rRNA gene. For primers see Table 1. PCRs were performed with 25 amplification cycles. PCR products were run on 1 % agarose gel, stained with ethidium bromide and bands quantified using ImageJ software.

Statistical analyses. The statistical differences between treatments were determined by calculating the P values derived from a two-tailed Student’s t-test using Microsoft Excel.

RESULTS

In vivo RpoE overexpression stimulates transcription from essDp

An in silico search for sigma factor consensus binding sites within the essD promoter revealed the presence of a potential RpoE −10 and −35 site (Rhodius et al., 2006). To test whether RpoE regulates transcription from essDp, RpoE was overexpressed in an E. coli essDp-GFP reporter strain. There was a sevenfold increase in essDp-GFP fluorescence, indicating that RpoE was involved in controlling expression of the DLP12 lysis cassette (Fig. 1). We verified functional RpoE expression from this overexpression construct in an rpoE-promoter-GFP reporter strain (rpoEP2) and found
that RpoE induction led to a greater than 10-fold increase in GFP (Fig. S1, available in the online Supplementary Material).

RpoE directly stimulates transcription from essDp

To determine whether the in vivo RpoE-induced transcription from essDp was a direct result of RpoE interacting with essDp and RNAP, we performed a single round of transcription assays and found that full-length essDp mRNA was produced over a 5 min period only when RpoE was present (Fig. 2). These results reveal that RpoE targets RNAP to transcribe directly from essDp. The functional activity of purified RpoE was confirmed with a positive control in which a known RpoE target promoter (rpoEP2) was substituted for essDp in the transcription reaction. The control reaction produced an rpoEP2 transcript of appropriate length, validating the functionality of the RpoE protein (Fig. 2).

RpoE binding at essDp and Q-mediated essDp transcription is dependent on the −35 region

Although it was apparent that RpoE was directly targeting RNAP to essDp, we were uncertain as to whether the predicted −10 and −35 regions of essDp were the true RpoE binding sites required for this process. To test this, we mutagenized the −10 and −35 regions at the most conserved residues and assayed for changes in RpoE binding at essDp. Shift assays of RpoE binding to WT essDp probe revealed a concentration-dependent increase in bound probe upon addition of RpoE (Fig. 3a). We failed to see any difference in RpoE binding affinity to the −10 region mutant probe, which suggests that the exact −10 sequence is not a requirement for RpoE binding in this case. In contrast, when we tested RpoE binding to the −35 region mutant probes, RpoE could not bind effectively, thus demonstrating that the predicted −35 region (Rhodius et al., 2006) was critical for RpoE binding of essDp (Fig. 3b).

In order to investigate the physiological ramifications of these −35 region mutations, we tested each −35 region mutant promoter for in vivo transcriptional activity under Q overexpression conditions. The −10 and −35 region mutant versions of the essDp-GFP reporter were used for this purpose. The −10 mutant reporter had no defect in essDp transcription, but instead had a 50 % increase in GFP transcription upon Q overexpression (Fig. 4). This effect seems to be independent of RpoE binding as there was no defect observed in the gel shift assay. It is possible that the increased in vivo expression of the −10 mutant may be attributed to other factors such as

![Image](https://example.com/image.png)

**Fig. 1.** RpoE overexpression causes an increase in transcription from essDp. Fluorescence from pessDp-GFP was quantified to assess the effects of RpoE overexpression on essDp transcription over a 12 h time-course following arabinose addition (0.2 %). Overnight cultures were diluted in fresh LB and allowed to grow at 30 °C in the presence (■) or absence (♦) of arabinose-induced RpoE overexpression. Data are the means± so from three independent experiments (*P<0.05).

![Image](https://example.com/image.png)

**Fig. 2.** Purified RpoE allows RNAP to transcribe essDp in vitro. RpoE (100 nM) was mixed with 20 nM core RNAP to reconstitute holoenzyme and incubated with essDp template at 37 °C prior to transcription initiation. Reactions were sampled over a 5 min time-course (lanes 1–5). rpoEP2 control: positive RpoE control containing RNAP holoenzyme incubated with rpoEP2 promoter template and allowed to transcribe for 5 min. The arrow indicates the mRNA transcript location. No transcript was detected in the absence of RpoE after 5 min of transcription (Control). Experiments were performed independently three times (the image shown is from one representative replicate).
altered Q binding or facilitated RNAP core melting of the promoter DNA prior to initiation. In contrast, all −35 mutant reporters exhibited a threefold to fourfold defect in GFP expression when Q was overexpressed (Fig. 4), underscoring the link between the RpoE binding defect seen for the −35 region mutants in the gel shift assay and their attenuated in vivo essDp-GFP expression. Together, these results strongly suggest that Q-mediated essDp upregulation is dependent on proper RpoE binding to the −35 region of the lysis cassette promoter.

Fig. 3. Purified RpoE binds essDp proximal to the transcription start site in vitro. (a) Gel shift analysis of RpoE binding to essDp DNA (1.02 nM). RpoE was present in lanes 1–7 at 0, 0.17, 0.35, 0.52, 0.7, 1.4 and 2.1 μM, respectively. Lane 8 contained 2.1 μM RpoE plus an eightfold excess of unlabelled probe. Estimated Ki for RpoE binding: 0.92 μM. (b) Gel shift analysis of RpoE binding to mutated −10 and −35 regions of essDp DNA (1.02 nM). −35 mutants showed significantly reduced RpoE binding when compared to WT and −10.1 mutant. Gel bands were quantified using ImageJ software. Experiments were performed independently three times (the images shown are from one representative replicate). (c) essDp region depicting putative −35 and −10 regions (underlined) that were mutated at the most highly conserved residues based on the consensus sequence for RpoE promoters (Rhodius et al., 2006). The underlined guanine represents the putative transcription start site.

Fig. 4. Q-mediated upregulation of the lysis cassette is dependent on the RpoE −35 promoter region of essDp. Fluorescence from pessDp and −10/−35 mutant versions (p10.1, p35.2–35.4) was quantified to assess the effects of mutating conserved −35 and −10 residues on essDp transcription 12 h after IPTG addition. Overnight cultures were diluted in fresh LB and allowed to grow at 30 °C in the presence (black bars) or absence (white bars) of 1 mM IPTG. Fluorescence was measured using a Bio-Tek Synergy HT spectrophotometer and normalized to OD600. Data are the means ± SD from three independent experiments. All mutants (including p10.1) were statistically different from the control when Q was overexpressed (*P<0.05).

RpoE regions 1.2–2.2 and 3.0–4.2 interact with Q in vivo

In phage λ, Q binds to the lysis cassette promoter region adjacent to DNA-bound RpoD, and through a direct interaction with RpoD, causes RpoD displacement and subsequent transcription elongation (Nickels et al., 2002). Furthermore, in a previous report, we showed that QDLP12 also binds to essDp (Rueggeberg et al., 2013). Based on similarities between late phage gene expression in phage λ and DLP12, it is possible that Q and RpoE were physically interacting with one another. To test this hypothesis we employed a bacterial two-hybrid assay where QDLP12 was fused to λcl and RpoE or its subunits (1.2–2.4, 3.0–4.2 or 4.2) were fused to zCTD of RpoD (Fig. 5b). Co-expression of fusions to Q and the entirety of RpoE was toxic. Co-expression of Q and either RpoE domains 1.2–2.4 or 3.0–4.2 showed twofold and fourfold increases in activity over background, respectively (Fig. 5a). These data suggest that QDLP12 interacts with RpoE subunit 3.0–4.2 and, to a lesser degree, 1.2–2.4, in vivo.

To confirm the in vivo interactions observed between QDLP12 and RpoE in the two-hybrid assay, we utilized an affinity pulldown assay to determine whether QDLP12 and RpoE copurify. However, nano liquid chromatography-mass spectrometry (nanoLC-MS) failed to identify RpoE as a component of the protein pulldown fraction (data not shown). This was not entirely surprising given the Q5
QDLP12 and RNAP, but that QDLP12 would continue to interact with the β subunit of RNAP as transcription progressed.

Q is complexed with the β subunit of RNAP

In canonical phage λ, Q becomes stably bound to core RNAP during transcription of the lysis genes through specific interaction with the β subunit (Vorobiev et al., 2014). We suspected that QDLP12 was potentially acting through a similar mechanism to facilitate transcription of the DLP12 lysis genes. To investigate this possibility, we cross-linked intracellular proteins and performed an affinity pulldown of Q-His. Western blotting of the lysate from the pulldown assay with anti-His and anti-βE subunit (Vorobiev et al., 2014) captured the QDLP12-specific bands. The cross-linked fraction showed a high molecular mass complex band and a QDLP12-specific band when probed with the anti-His antibody. In contrast, the boiled fraction in which the cross-links were reversed had very little signal from the high molecular mass fraction, although it still retained the QDLP12-specific band. This suggests that QDLP12 is interacting with other proteins in a high molecular mass complex. In addition, when the same blot was stripped and reprobed with anti-β antibody, two notable bands appear: a β-specific band and a band of high molecular mass at the same location as the anti-β band. As before, the high molecular mass band was only present in the cross-linked sample, while the sample in which the cross-links were reversed contained only a β-specific band at the predicted molecular mass (Fig. S2). In addition, analysis with nanoLC-MS confirmed the presence of β subunit in the Q pulldown (data not shown). Taken together these data suggest that QDLP12 and β are present in the same protein complex; however, similar results

![Fig. 5. RpoE directly interacts with Q in vivo. (a) Reporter strains containing various combinations of QDLP12 (YbcQ), RpoE (σE), σE subunit 1.2–2.4, σE subunit 3.0–4.2 and σE subunit 4.2 fused to either λcl or α subunit of RNAP (see chart) were tested for β-galactosidase expression in a bacterial two-hybrid assay. Skull and crosses indicate lethal plasmid combination. Cell density was recorded, and then β-galactosidase activity was measured as described in Methods. Relative β-galactosidase activity is proportional to interaction strength between the two proteins being tested. Data are the means ± SD from three independent experiments (*P<0.05). (b) Schematic of RpoE and its domains fused to α subunit used in the bacterial two-hybrid assay.](image-url)
from a reciprocal pulldown assay are still needed to strengthen this conclusion.

**Q is enriched at essDp and the downstream lysis genes**

Since Q³ becomes stably incorporated into the RNAP holoenzyme during transcription of the lysis genes (Deighan & Hochschild, 2007), we hypothesized that Q^{DLP12} would function similarly. We performed an affinity chromatkin pulldown assay against Q-His^{DLP12} and purified the cross-linked DNA from bacteria with or without Q-His^{DLP12} overexpression. PCR analysis of Q-associated DNA indicated a 3.5-fold increase in essDp proximal to the transcription start site and a 4-fold enrichment downstream of the start site (Fig. 3). This suggests that Q is present in the transcription complex throughout expression of the DLP12 lysis genes; however, further evidence is required to confirm this.

**DISCUSSION**

Our work demonstrates that the alternative sigma factor RpoE is a positive regulator of the DLP12 lysis genes (Fig. 1). In λ phage, Q³ is stably incorporated into the RNAP holoenzyme through multiple interactions with the core subunits as well as specific regions of RpoD (Deighan et al., 2008; Deighan & Hochschild, 2007; Nickels et al., 2002). These interactions with RpoD are believed to destabilize the RpoD-core RNAP complex, which results in the release of RpoD from the complex and subsequent transcription elongation, as the Q-bound core RNAP complex moves past the terminator, thereby continuing transcription. In λ phage, RpoD is recruited to PR for transcription of the lysis genes, but in DLP12 our data suggest that RpoE performs the analogous function. This was confirmed via an in vitro transcription assay which demonstrated that purified RpoE was sufficient to permit core RNAP to transcribe the essDp template (Fig. 2).

We confirmed that a putative RpoE −35 region within essDp is essential for proper expression of the lysis genes (Fig. 3), but found that the mutations in specific residues in the −10 region were not sufficient to block RpoE-mediated transcription from the promoter. Q^{DLP12} overexpression in −35 mutants resulted in reduced lysis cassette expression levels, suggesting that excess Q^{DLP12} was not able to compensate for poor RpoE binding (Fig. 4). Taken together, it seems that Q^{DLP12}-mediated essDp regulation is dependent on proper RpoE binding to the −35 region of the lysis cassette promoter.

Based on those published observations about Q³ and our data, we postulated that RpoE was interacting with Q^{DLP12} in a manner similar to that of Q³ and RpoD. As expected, the bacterial two-hybrid assay revealed a significant interaction between RpoE and Q^{DLP12} in vivo (Fig. 5). Furthermore, the β subunit of RNAP co-purified with Q-His^{DLP12} when the latter was pulled down from cross-linked cell lysates (Fig. S2), strongly suggesting that the two transcription factors are functioning in concert to transcribe the DLP12 lysis genes. Amplification of the DNA recovered from the same pulldown assay further suggests that Q^{DLP12} remains stably bound to RNAP core during transcription of the lysis genes and that this interaction is not just occurring at the promoter (Fig. S3).

These data make a compelling case for co-option and domestication of the native lambdoid regulatory mechanism by E. coli and adds to a growing list of sigma factors (RpoD and RpoS) known to promote the transcription of lambdoid lysis cassettes (Nakamura et al., 1979; Nickels et al., 2002; Roucourt & Lavigne, 2009).

Prophage genes have been shown to impact bacterial physiology in various ways, including altering growth rate (Wang et al., 2010), evading host immune responses (Barondess & Beckwith, 1995; Vaca Pacheco et al., 1997), altering endogenous mutation rates (Chikova & Schaper, 2006; Pal et al., 2007), increasing resistance to antibiotics and biocides (Wang et al., 2010), and altering biofilm formation (Rice et al., 2009; Wang et al., 2010; Webb et al., 2004). A recent study also revealed that a holin-like gene, chW, and a putative endopeptidase gene, chX, were required for secretion of three chitinase virulence factors in the opportunistic Gram-negative pathogen Serratia marcescens (Hamilton et al., 2014). While biofilm formation has been shown to be affected by the production of functional filamentous prophage Pfi and P4 in P. aeruginosa (Rice et al., 2009; Webb et al., 2004), the mechanisms whereby defective prophages affect biofilm formation in E. coli are not well understood (Wang et al., 2010).

We previously showed that the putative antiterminator Q^{DLP12} was important for expression of the DLP12 lysis genes and biofilm formation in a curli-overexpressing E. coli strain (Rueggeberg et al., 2013). However, the exact mechanism through which the lysis cassette was expressed remained to be elucidated. In this work, we provide both in vivo and in vitro evidence that the alternative sigma factor RpoE targets the −35 region of essDp and works with Q^{DLP12} and the β subunit of RNAP to transcribe the lysis genes. This suggests that E. coli has not only co-opted this important phage regulatory mechanism, but it has also domesticated it by placing it under the control of the tightly regulated sigma factor RpoE. Although the evolutionary driving force behind this process remains unclear, there exists a distinct possibility that RpoE functions as a link between extracytoplasmic stress and DLP12-mediated biofilm formation. Through upregulation of RpoE in response to such stresses, E. coli might increase expression of the lysis cassette, and in turn enhance biofilm formation, which would be expected to result in increased survival. This level of domestication contributes to the evidence that defective prophage, which were long considered genetic baggage (Casjens, 2003), are important for host fitness (Wang et al. 2010).
**ACKNOWLEDGEMENTS**

Thanks to Jeffrey Roberts for graciously providing the resources and knowledge to perform the in vitro transcription assay, Ann Hochschuld for providing the pPER76-rpoE-His overexpression construct for RpoE purification and the vectors to construct the two-hybrid system, and Hanh Nguyen for technical assistance. This research was funded in part by a Cornell Provosts Diversity Fellowship.

**REFERENCES**


development in *Escherichia coli* by controlling expression of the DLP12 lysis cassette. *Microbiology* 159, 691–700.


Edited by: A. Van Vliet