Replication of plasmids derived from Shiga toxin-converting bacteriophages in starved *Escherichia coli*

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The pathogenicity of Shiga toxin-producing *Escherichia coli* (STEC) depends on the expression of stx genes that are located on lambdoid prophages. Effective toxin production occurs only after prophage induction, and one may presume that replication of the phage genome is important for an increase in the dosage of stx genes, positively influencing their expression. We investigated the replication of plasmids derived from Shiga toxin (Stx)-converting bacteriophages in starved *E. coli* cells, as starvation conditions may be common in the intestine of infected humans. We found that, unlike plasmids derived from bacteriophage λ, the Shiga toxin phage-derived replicons did not replicate in amino acid-starved relA+ and relA− cells (showing the stringent and relaxed responses to starvation, respectively). The presence of the stable fraction of the replication initiator O protein was detected in all tested replicons. However, while ppGpp, the stringent response effector, inhibited the activities of the λ PR promoter and its homologues from Shiga toxin-converting bacteriophages, these promoters, except for λ PR, were only weakly stimulated by the DksA protein. We suggest that this less efficient (relative to λ) positive regulation of transcription responsible for transcriptional activation of the origin contributes to the inhibition of DNA replication initiation of Shiga toxin-converting bacteriophages in starved host cells, even in the absence of ppGpp (as in starved relA− hosts). Possible clinical implications of these results are discussed.

INTRODUCTION

When infecting the human intestine, Shiga toxin-producing *Escherichia coli* (STEC) strains usually cause bloody diarrhoea (Nataro & Kaper, 1998; Besser et al., 1999). This applies mostly to a subset of STEC, called enterohaemorrhagic *E. coli* (EHEC), which are particularly effective in colonization of the human intestine; nevertheless, because of the subject of this study (see below), in this paper we will use the broader designation, STEC. Production of Shiga toxin results in serious changes in the host cell metabolism due to inhibition of protein synthesis, and these cause the symptoms mentioned above. Moreover, 15–20% of patients infected with STEC progress to haemorrhagic colitis and/or haemolytic uraemic syndrome, which is a dangerous disease, especially for children (Besser et al., 1999; Gyles, 2007; Serna & Boedeker, 2008). This figure may reach as much as 50%, if antibiotics are used for treatment of patients (Serna & Boedeker, 2008).

Genes encoding Shiga toxins (stx genes) are located on lambdoid prophages (called Shiga toxin-converting prophages), and without prophage induction, stx expression is mostly repressed. This is the reason why the use of antibiotics may worsen the symptoms in STEC-infected patients, as many antimicrobial agents cause lambdoid prophage induction. In fact, in most cases, the effective production of Shiga toxin requires prophage induction and its further lytic development, including replication of the phage genome (Schmidt, 2001; Wagner et al., 2001a, b, 2002; Herold et al., 2004; Waldor & Friedman, 2005; Loś et al., 2009, 2010). Importantly, Shiga toxin (Stx) phages that complete their lytic development may infect new hosts, including commensal strains of *E. coli*, which can lead to both an increase in the efficiency of Shiga toxin production in the human intestine and the spread of STEC strains (Gamage et al., 2004). Shiga toxin 1, unlike Shiga toxin 2, may be produced in response to low iron levels, particularly in phage H-19B (Weinstein et al., 1988), but without prophage induction the toxin is not transported outside the cell, as *E. coli* lacks an appropriate secretion system, which strengthens the requirement of STEC pathogenicity for prophage induction. Furthermore, effective expression of stx genes depends on their copy number in cells, indicating that the efficiency of phage DNA...
replication is also an important factor in STEC pathogenesis. Thus, understanding the specific conditions that cause induction of Shiga toxin-converting prophages and allow their replication in bacteria occurring in the human intestine is important.

Shiga toxin-converting phages belong to the lambdoid family of phages, of which bacteriophage λ is the best-investigated member (for reviews, see Ptashne, 2004; Węgrzyn & Węgrzyn, 2005). The efficiency of DNA replication of lambdoid phages can be studied by employing plasmids derived from these phages, which are simple replicons, while still having all the genes and regulatory sequences necessary for phage DNA replication (Taylor & Węgrzyn, 1995).

Starvation conditions are believed to be common in the human colon, and from the point of view of bacterial growth, ‘feast or famine’ situations are common in such an environment (Scheline, 1973; McBurney et al., 1987; Roediger, 1990, 1994). Therefore, we aimed to investigate the replication of plasmids derived from Shiga toxin-converting phages in starved E. coli cells. Such plasmids bear the replication region of the phage genome, which encompasses all genes and regulatory sequences required for the specific initiation of DNA replication from the unique site called origin or ori (Fig. 1). In plasmids derived from bacteriophage λ, and in homologous plasmids bearing replication regions of Shiga toxin-converting phages, the O and P genes encode an origin-binding replication initiator protein and a factor necessary to deliver the DnAB helicase to the replication start site, respectively (for a review, see Taylor & Węgrzyn, 1995). The activity of Pr (one of the early phase promoters) is necessary to initiate replication from ori, as transcription starting from this promoter and proceeding through the replication region leads to transcriptional activation of the origin, a process required even in the presence of all proteins involved in λ DNA replication (Taylor & Węgrzyn, 1995). Until recently, Pc-initiated transcriptional activation of the origin was believed to be required solely for transcription-caused changes in DNA topology, including changed superhelicity and partial unwinding of the DNA template, which should facilitate the formation and rearrangement of the replication initiation complex (for reviews and discussions, see Taylor & Węgrzyn, 1995; Węgrzyn & Węgrzyn, 2002, 2005). However, the results of very recent studies demonstrate that the O protein interacts directly with RNA polymerase (Szambowska et al., 2010), strongly suggesting that the mechanism of transcriptional activation of the origin is more complicated and perhaps includes coupling of transcription and replication machineries (factories).

Since amino acid starvation of bacteria is an example of extensively investigated famine conditions, in our studies we decided to use this form of starvation. Amino acid starvation of wild-type cells causes a rapid metabolic response, leading to the inhibition of transcription of a large number of genes, especially those encoding factors involved in translation, and to the stimulation of the transcription of some other genes. Such a response is called the stringent response (for a review, see Potrykus & Cashel, 2008). Guanosine tetraphosphate (ppGpp) is the main effector of stringent control. This nucleotide interacts with RNA polymerase and transiently changes its properties (Potrykus & Cashel, 2008; Szalewska-Pałaś, 2008). Recently, another factor involved in the modulation of ppGpp-mediated transcription regulation, the DksA protein, has been discovered (for a review, see Szalewska-Pałaś et al., 2007). This protein often cooperates with ppGpp; however, recent studies indicate that at some promoters, DksA and ppGpp may act independently and antagonistically (Lyzén et al., 2009). In mutants defective in the production of ppGpp (particularly relA mutants), transcription proceeds irrespective of the levels of available amino acids, which results in energetic exhaustion of cells in the absence of effective protein synthesis caused by a lack of substrates. Such a response to amino acid starvation is called the relaxed response (Potrykus & Cashel, 2008).

To learn about the efficiency of replication starting from ori located in the genomes of Shiga toxin-converting bacteriophages, we aimed to test the replication of plasmids derived from such phages (constructed and described previously by Nejman et al., 2009) under conditions effecting stringent and relaxed responses. Due to differences that we discovered between the replication of plasmids derived from λ and Shiga toxin-converting bacteriophages, we studied the mechanisms of the various responses of similar replicons to starvation conditions.

**METHODS**

**Bacteria and growth conditions.** E. coli strains CF1648 (wild-type, relA+), and CF1652 (ΔrelAE251::kan), described elsewhere (Xiao et al., 1991), as well as a set of isogenic strains bearing a lacZ mutation and combinations of relA spoT and dksA mutations (described by Lyžen et al., 2009), were used. Bacteria were cultured in either LB medium (Sambrook et al., 1989) or a minimal medium, M9 (Jasiecki & Węgrzyn, 2003), at 37 °C in shake flasks with agitation. Isolucine starvation was induced by the addition of l-valine to final concentration of 1 mg ml⁻¹.

**Plasmids and gene fusions.** Plasmids are listed in Table 1. Wild-type λ plasmid pCB104 (Boyd & Sherratt, 1995) and its pA66 derivative, pAW6 (Węgrzyn et al., 1996b), have already been described. The construction of plasmids derived from Shiga toxin-converting bacteriophages [originally named φ933WΔtox, φPT22От, φPT27От and φPT32От (Gamage et al., 2004); Stx2Φ-I (Watarai et al., 1998); ST2-8624, isolated by Dr Gail Christie, Virginia Commonwealth University, VA, USA (Nejman et al., 2009) and mutants of these plasmids have been described previously (Nejman et al., 2009). For the construction of supercoiled templates for in vitro transcription, plasmid pTE103 (Elliott & Geiduschek, 1984) was used as vector. The sequences of Pr promoters from pCB104, pR8624cmr and pJ933cmr were amplified using following primers: PRCB104F (5’-TGC GGA TCC AAG CTG CTC TTG TGT TAA TG-3’), PRCB104R (5’-GAG ATC TTT AGC TG-3’), PRR8624F (5’-CAA GGA TCC CAG TTG-3’), PRR8624R (5’-CAA GGA TCC CAG TG-3’).
The obtained PCR fragments were cut with BamHI and HindIII, and ligated with a pTE103 plasmid digested by the same enzymes. All molecular cloning procedures were performed according to Sambrook & Russell (2001). All constructs were verified by DNA sequencing. The pTAC3734-based plasmid was used as a backbone for the cloning. The obtained constructs were transformed into Escherichia coli strain DH5α, and the transformed bacteria were grown on nutrient agar plates supplemented with ampicillin. The plasmids were extracted from the transformed bacteria and sequenced to confirm their identity.
Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>pCB104gly</td>
<td>As pCB104 but bearing a mutation causing the Ser282→Gly amino acid substitution in the O protein</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pCB104thr</td>
<td>As pCB104 but bearing a mutation causing the Ala20→Thr amino acid substitution in the P protein</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pCB104glythr</td>
<td>As pCB104 but bearing mutations causing the Ser282→Gly and the Ala20→Thr amino acid substitutions in the O and P proteins, respectively</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pAW6</td>
<td>As pCB104 but bearing the αA66 mutation (causing the Arg→Gly amino acid substitution in the P protein)</td>
<td>Węgrzyn et al. (1996b)</td>
</tr>
<tr>
<td>pR8624cmr</td>
<td>Wild-type plasmid carrying the replication region of bacteriophage ST2-8624 and a chloramphenicol-resistance gene</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pR8624cmrgly</td>
<td>As pR8624cmr but bearing a mutation causing an amino acid change in the O protein, corresponding to the Ser282→Gly substitution in the O protein of bacteriophage λ</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pR8624cmrthr</td>
<td>As pR8624cmr but bearing a mutation causing an amino acid change in the P protein, corresponding to the Ala20→Thr substitution in the P protein of bacteriophage λ</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pR8624cmrglythr</td>
<td>As pR8624cmr but bearing mutations causing amino acid changes in the O and P proteins, corresponding to the Ser282→Gly and Ala20→Thr substitutions in the O and P proteins of bacteriophage λ, respectively</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>p27cmr</td>
<td>Wild-type plasmid carrying the replication region of bacteriophage φPT27Δtox and a chloramphenicol-resistance gene</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>p27cmrgly</td>
<td>As p27cmr but bearing a mutation causing an amino acid change in the O protein, corresponding to the Ser282→Gly substitution in the O protein of bacteriophage λ</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>p27cmrthr</td>
<td>As p27cmr but bearing a mutation causing an amino acid change in the P protein, corresponding to the Ala20→Thr substitution in the P protein of bacteriophage λ</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>p27cmrglythr</td>
<td>As p27cmr but bearing mutations causing amino acid changes in the O and P proteins, corresponding to the Ser282→Gly and Ala20→Thr substitutions in the O and P proteins of bacteriophage λ, respectively</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pRstx2cmr</td>
<td>Wild-type plasmid carrying the replication region of bacteriophage Stx2Φ-I and a chloramphenicol-resistance gene</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>p933Wcmr</td>
<td>Wild-type plasmid carrying the replication region of bacteriophage 933W and a chloramphenicol-resistance gene</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>p32cmr</td>
<td>Wild-type plasmid carrying the replication region of bacteriophage φPT32Δtox and a chloramphenicol-resistance gene</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>p22cmr</td>
<td>Wild-type plasmid carrying the replication region of bacteriophage φPT22Δtox and a chloramphenicol-resistance gene</td>
<td>Nejman et al. (2009)</td>
</tr>
<tr>
<td>pTE103</td>
<td>Plasmid vector containing an ampicillin-resistance gene and a multiple cloning site from pUC8, placed upstream of the bacteriophage T7 transcriptional terminator</td>
<td>Elliott &amp; Geiduschek (1984)</td>
</tr>
<tr>
<td>pTEPRCB104</td>
<td>As pTE103 but bearing the P λ promoter sequence of bacteriophage λ, inserted between BamHI and HindIII restriction sites</td>
<td>This work</td>
</tr>
<tr>
<td>pTEPRR8624</td>
<td>As pTE103 but bearing the P λ promoter sequence of bacteriophage ST2-8624, inserted between BamHI and HindIII restriction sites</td>
<td>This work</td>
</tr>
<tr>
<td>pTEPR933W</td>
<td>As pTE103 but bearing the P λ promoter sequence of bacteriophage 933W, inserted between BamHI and HindIII restriction sites</td>
<td>This work</td>
</tr>
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</table>

Fusion of the λ P  λ promoter (bearing a 207 bp DNA fragment, corresponding to nucleotide positions from −73 to +135 relative to the transcription start site) with the lacZ gene has already been described (Łyżen et al., 2009), and analogous fusions bearing the P  λ promoter region from phages ST2-8624 and 933W were constructed using the primers described above. All fusions were verified by DNA sequencing.

Estimation of efficiency of plasmid DNA replication. The replication of plasmid DNA in host cells was investigated as described previously (Herman et al., 1994; Szalewska-Palasz et al., 1994). Briefly, a known number of bacterial cells (5 × 10^7) was withdrawn at indicated times. At a given time point, isoleucine starvation was induced by the addition of L-valine to a final concentration of 1 mg ml⁻¹. Samples of bacterial cultures were centrifuged (5 min, 2000 g, 4 °C) and pellets were frozen in liquid nitrogen and kept at −70 °C. After thawing, plasmid DNA was isolated by alkaline lysis (Sambrook & Russell, 2001). Following plasmid linearization with a restriction endonuclease, DNA was subjected to agarose gel electrophoresis. After staining with ethidium bromide, the intensities of plasmid bands, which corresponded to the relative amounts of plasmid DNA, were measured by densitometry using the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories) and Quantity One (version 4.5.2) software. Densitometric analysis of DNA bands was performed with
respect to a known amount of a linear form of a plasmid of similar size, separated on the same gel.

**Measurement of the stability of replication initiator proteins.** Bacteria bearing a λ plasmid or plasmids derived from Shiga toxin-converting bacteriophages (Table 1) were grown in MG/MGlu medium to OD_{600} 0.2. At time 0, tetracycline was added to a final concentration of 200 μg ml^{-1} to inhibit protein synthesis. Isolecine starvation was induced by the addition of l-Valine (to a final concentration of 1 mg ml^{-1}) together with tetracycline. Samples of the cultures (5 ml) were withdrawn at the indicated times and transferred immediately to an ice bath. Then, bacteria were sedimented (5 min, 2000 g, 4 °C) and the pellet was immediately frozen in liquid nitrogen. After thawing, the bacteria were suspended in Lysis Buffer [50 mM Tris-HCl, pH 8.0, 2 % SDS, 1 % β-mercaptoethanol, 10 % (v/v) glycerol, 12.5 mM EDTA and 0.02 % bromophenol blue] with protease inhibitors, and transferred to a boiling water bath for 5 min. The cell lysates were centrifuged (1 min, 3000 g, 4 °C), and subjected to 11 % SDS-PAGE. Subsequently, proteins were transferred to a PVDF membrane for 90 min in Transfer Buffer (25 mM Tris, 250 mM glycin, 0.1 % SDS, 20 % methanol) at 90 mA. The λ O protein and its homologues encoded by plasmids derived from Shiga toxin-converting bacteriophages (there are high levels of homology between all these proteins, as reported by Nejman et al., 2009) were detected by Western blotting with overnight incubation in Blocking Buffer [PBS, pH 7.4 (0.137 mM NaCl, 2.7 mM KCl, 10 mM NaH_{2}PO_{4}·7H_{2}O, 1.47 mM KH_{2}PO_{4}), with 0.1 % Tween 20 and 5 % low-fat milk] overnight incubation in anti-λ O serum diluted 1:2000 in Blocking Buffer, and 1.5 h incubation with goat anti-rabbit [horseradish peroxidase (HRP)-conjugated] IgG diluted 1:4000 in the above-mentioned buffer. The blots were developed in a solution of enhanced chemiluminescence (ECL) reagents for detecting HRP. The proteins were visualized using a Fluor-S MultiImager (Bio-Rad Laboratories), and the relative amounts of these proteins were estimated by densitometry using Quantity One (version 4.5.2) software. In preliminary experiments, we found that the specificity of the anti-λ O serum for each tested O homologue (from each tested phage) was similar, as was the expression level of each tested O homologue (data not shown).

**In vitro transcription.** Supercoiled DNA template was obtained by isolation of plasmids (pTEPRCB104, pTEPRR8624, pTEPR933W) from E. coli strain CF1648 and their purification by ultracentrifugation in a CsCl-ethidium bromide density gradient (Sambrook & Russell, 2001). The in vitro transcription reaction was performed in a total volume of 20 μl in transcription buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl_{2}, 10 mM β-mercaptoethanol, 10 μg BSA ml^{-1}, 140 mM KCl). Supercoiled DNA template (10 μM), 10 μg amounts of ppGpp and/or DksA, 25 nM RNA polymerase holoenzyme (Epicenter Technologies) and nucleotides [final concentrations: 150 μM CTP and GTP, 1 mM ATP, 15 μM UTP and 1 μCi (3.7×10^{4} Bq) [α-^{32}P]UTP (3000 Ci mmol^{-1}) (Hartmann Analytic)] were included in the reaction mixture. After addition of RNA polymerase, the samples were incubated at 37 °C for 10 min. The reactions were started by the addition of nucleotides, and the mixtures were incubated at 37 °C for 12 min. Following the addition of heparin (to a final concentration of 100 μg ml^{-1}), the samples were incubated at 37 °C for 5 min. The reactions were terminated by the addition of 5 μl stop buffer [150 mM EDTA, 1.05 M NaCl, 7 M urea, 10 % (v/v) glycerol, 0.0375 % xylene cyanol, 0.0375 % bromophenol blue]. The samples were separated by electrophoresis in a 4 % Tris-buffered EDTA (TBE)-buffered polyacrylamide gel containing 7 μM urea at 30 mA. The gel was dried, and RNA bands were visualized using a Molecular Imager FX (Bio-Rad Laboratories) and quantified by densitometry using Quantity One (version 4.5.2) software.

**Measurement of β-galactosidase activity in cells.** The activity of β-galactosidase in E. coli cells was measured according to Zhang & Bremer (1995). Since multiplicity fusions were employed, the obtained values were normalized with respect to the amount of plasmid DNA (to minimize any effects of potential differences in lacZ gene dosage if different plasmid copy numbers occurred in various strains), as described previously (Lyzen et al., 2009).

**Efficiency of transformation.** The calcium chloride method of transformation of E. coli cells with supercoiled plasmid DNA (Sambrook et al., 1989) was used. Transformation efficiency was calculated in transformants with respect to one microgram of plasmid DNA.

**RESULTS AND DISCUSSION**

**Inhibition of replication of plasmids derived from Shiga toxin-converting phages in amino acid-starved cells during both the stringent and the relaxed response**

As reported previously, the replication of plasmids derived from bacteriophage λ (see Table 1 for their characteristics) is inhibited in amino acid-starved wild-type (relA^{+}) E. coli cells, i.e. during the stringent response, due to ppGpp-mediated impairment of activity of the P_{E} promoter and the resultant low efficiency of transcriptional activation of ori_{l} (Szalewska-Pałasz et al., 1994). When ppGpp cannot be produced in starved relA mutants, i.e. during the relaxed response, these plasmids can still replicate, despite an amino acid deprivation-caused lack of production of new proteins, including an unstable O replication initiator protein, as this protein is stabilized in the replication complex that is inherited by one of two daughter copies after each replication round, and can function during the next replication event, provided that the transcriptional activation of ori_{l} is efficient (Wegrzyn et al., 1992, 1995, 1996a; Szalewska-Pałasz et al., 1994; for a review, see Wegrzyn & Wegrzyn, 2001; for a scheme, see Fig. 1). Although the spoT gene product is responsible for some ppGpp synthesis under standard growth conditions and during carbon starvation, this SpoT-mediated ppGpp production is not effective in amino acid-starved cells; thus, under the latter conditions, the effects observed in a single relA mutant and a double relA spoT mutant are similar (Potrykus & Cashel, 2008). This applies also for effects on λ plasmid replication (Herman et al., 1994; Szalewska-Pałasz et al., 1994, 1998). Therefore, the relA mutant was used in experiments described in this subsection.

The phenomena described above were evident in the control experiments (Fig. 2) performed in this study, in which wild-type λ plasmid (pCB104) and its mutant bearing a π mutation in the P gene (pAW6) were used. Note that the π mutation was originally defined as a mutation causing a suppression of inhibition of λ phage development in E. coli mutants called groP (for growth of phage), and was mapped in dnaB, dnaI, dnaK or grpE, encoding either a helicase or molecular chaperones necessary for rearrangement of the λ replication complex (see Taylor & Wegrzyn, 1995 and references therein).
Subsequent studies have indicated that the π mutation results in less efficient interactions between the P protein and the DnaB helicase (Konieczny & Marszałek, 1995). These two proteins are members of the stable and heritable replication complex (Potrykus et al., 2002). In the experiments depicted in Fig. 2, the amount of plasmid DNA in cells was monitored over time using samples of equal bacterial cell mass. Since amino acid starvation causes inhibition of bacterial growth in both stringent and relaxed strains, an increase in the amount of plasmid DNA indicates ongoing plasmid replication, while a constant or decreased DNA level (due to incomplete growth inhibition) is characteristic of an inhibition of plasmid replication. Unlike the wild-type λ plasmid, and despite a high level of homology between the λ replication region and corresponding regions of genomes from Shiga toxin-converting phages (Nejman et al., 2009), plasmids derived from phages Stx2Φ-1, ST2-8624, 933W, φPT22Δtox, φPT27Δtox and φPT32Δtox (Table 1) did not replicate efficiently during either the stringent or the relaxed response (Fig. 2). Since SpoT activity is not sufficient to produce ppGpp during amino acid starvation of relA mutants (see preceding paragraph), we conclude that replication of these plasmids is inhibited by starvation conditions per se, rather than by ppGpp.

On the basis of DNA sequence analysis, one can predict almost identical amino acid sequences of O and P proteins of phages λ, ST2-8624 and φPT27Δtox. There is a Leu37Ile substitution in the O protein of phage φPT27Δtox that is present in all other lambdoid phages tested in this work, except those encoded by λ and ST2-

![Figure 2](http://mic.sgmjournals.org) 225
Fig. 3. Replication of various lambdoid plasmids estimated by measurement of relative plasmid content at various times, in isoleucine-starved *E. coli* relA<sup>+</sup> (open symbols) and relA<sup>−</sup> (closed symbols) bacteria. The ‘gly’ and ‘thr’ abbreviations in the names of plasmids indicate mutations causing a Ser282Gly substitution in the λ O protein and an Ala20Thr substitution in the λ P protein (or corresponding substitutions in proteins encoded by other lambdoid phages), respectively. Isoleucine starvation was induced at time 1 h by addition of L-valine to the minimal medium to a final concentration of 1 mg ml<sup>−1</sup>. Results shown are mean ± so from three experiments.
suppress the starvation-caused inhibition of replication of these plasmids.

Stability of the initiator protein encoded by Shiga toxin-converting bacteriophages

When occurring in a free form in the cell, the O initiator protein of phage λ is highly unstable, with a half-life between 1 and 2 min (Węgrzyn et al., 1992). Stabilization of this protein in the heritable replication complex, protecting it against proteases in other components of the complex, is necessary for λ DNA replication in the absence of protein synthesis, such as during amino acid starvation (Węgrzyn et al., 1992, 1996a; Szalewska-Pałasz et al., 1994) (for a scheme, see Fig. 1).

Looking for the reason for the inhibition of the replication of plasmids derived from Shiga toxin-converting phages in amino acid-starved cells, we tested the occurrence of the stable fraction of the O protein in bacteria bearing these plasmids. In preliminary experiments, we found that anti-O protein polyclonal antibodies could efficiently recognize homologues of the protein from all tested lambdoid phages (Fig. 4 and data not shown), thus allowing us to test the stability of all these proteins. When O protein decay was tested under conditions of translation inhibition, an initial rapid decrease in the amount of the protein indicated proteolysis of its free form, probably by the ClpXP protease (Węgrzyn et al., 1995 and references therein), while the occurrence of a constant amount of O protein, visible at later times of the experiment, represented its stable fraction, included in the heritable replication complex (Węgrzyn et al., 1992, 1995, 1996a; Potrykus et al., 2002). We found the presence of such a stable fraction of the O protein in cells bearing all tested plasmids, irrespective of the type of the parental phage and the presence or absence of the Ser282Gly substitution in the O protein (Figs 4 and 5). These results indicate that protection of the O replication initiator protein from proteolysis is a common feature of all tested lambdoid phages, and that inhibition of replication of plasmids derived from Shiga toxin-converting phages during amino acid starvation is not caused by a lack of the heritable replication complex.

Fig. 4. Evidence for suitability of anti-λO antibodies to detect the O protein encoded by Shiga toxin-converting bacteriophage φPT27Δtox, and an example of the determination of the stability of the O protein. Bacterial strains (relA⁺ or relA⁻) bearing either no plasmid (−) or one of the plasmids derived from lambdoid bacteriophages (pCB104 or p27cmgly) were used for determination of O protein stability, employing anti-λO antibodies, as described in Methods.
Fig. 5. Stability of the O protein in relA⁺ (left panels) and relA⁻ (right panels) E. coli cells bearing the plasmids indicated in each panel. The bacteria were either starved for isoleucine (open symbols) or not starved (closed symbols). Results shown are mean ± SD from three experiments.
Regulation of the activity of \( P_R \) promoters of lambdoid phages by ppGpp and DksA

Inhibition of \( \lambda \)-derived plasmid replication during the stringent response depends on ppGpp-mediated impairment of the \( P_R \) promoter activity (Szalewska-Pałasz et al., 1994). Recently, it has been found that another factor, DksA, is involved in the stringent response (for reviews, see Szalewska-Pałasz et al., 2007; Potrykus & Cashel, 2008). The DksA protein influences transcription from \( P_R \) (Lyżen et al., 2009). Unlike many other promoters for which ppGpp and DksA act synergistically and cooperatively, \( P_R \) is inhibited by ppGpp and stimulated by DksA (Lyżen et al., 2009). Therefore, we tested the effects of ppGpp and DksA on the activities of \( P_R \) promoters from Shiga toxin-converting phages. Since the \( P_R \) sequences of phages Stx2\( \Phi \)-I, 933W, \( \varphi \)PT22\( \Delta \)tox, \( \varphi \)PT27\( \Delta \)tox and \( \varphi \)PT32\( \Delta \)tox are identical, though different from those of phages \( \lambda \) and ST2-8624 (Nejman et al., 2009), we used constructs bearing \( P_R \) sequences of \( \lambda \), 933W and ST2-8624 in our experiments.

In control experiments with multiple rounds of \textit{in vitro} transcription from the \( \lambda \) \( P_R \) promoter, we confirmed previous results from single-round transcription studies (Lyżen et al., 2009) that indicated that ppGpp inhibits, while DksA stimulates, the activity of the \( P_R \) promoter (Fig. 6). When both these compounds were present in the reaction mixture, the final effect depended on their concentrations and ppGpp/DksA ratios (Fig. 6). However, when the activities of the \( P_R \) promoters from ST2-8624 or 933W were studied, only a weak stimulation of transcription by DksA could be observed, while ppGpp-mediated transcription inhibition was similar to that found in \( \lambda \) \( P_R \).

The results of the \textit{in vitro} studies were corroborated by \textit{in vivo} experiments, in which the activities of \( P_R \) promoters

![Fig. 6. In vitro transcription from the \( P_R \) promoter of phage \( \lambda \) (panels ‘Lambda’), or a corresponding promoter from the ST2-8624 and 933W phages in the presence of ppGpp and/or DksA. Results of experiments with various concentrations of ppGpp (■), DksA (300 nM) together with various concentrations of ppGpp (□), various concentrations of DksA (●), and ppGpp (300 \( \mu \)M) together with various concentrations of DksA (○), are presented. Results shown are mean ± SD from three experiments. Lower panels indicate examples of detection of the reaction products.](http://mic.sgmjournals.org)
were estimated by measurement of β-galactosidase activity in bacteria bearing fusions of the tested promoters with the lacZ gene. Unlike λ \( P_R \), significantly less pronounced effects of the lack of DksA were observed at the corresponding promoters of ST2-8624 and 933W (Fig. 7). Interestingly, \( P_R \) promoters from phages ST2-8624 and 933W appeared to be weaker than that of phage λ. That is, in wild-type cells, the activity of the λ \( P_R \) promoter (3772 ± 350 Miller units) was about twofold higher than that of \( P_R \) from 933W (1903 ± 407 Miller units), and about threefold higher than that of \( P_R \) from ST2-8624 (1179 ± 89 Miller units).

It has been demonstrated previously that due to a strong impairment of synthesis of the O protein in amino acid-starved cells bearing a lambdoid plasmid (Węgrzyn et al., 1995, 1996a; Potrykus et al., 2002), the only plausible explanation of the replication of such a replicon under these conditions is that the function of the heritable replication complex is employed. Moreover, such a complex still requires transcriptional activation of the origin (for reviews, see Węgrzyn & Węgrzyn, 2001, 2002, 2005). Therefore, based on the results shown in Figs 6 and 7, we speculate that effective DksA-mediated stimulation of

**Fig. 7.** Estimation of activities of \( P_R \) promoters from lambdoid phages (λ, ST2-8624 and 933W) in ppGpp-null (relA spoT) and dksA mutants by measurement of β-galactosidase activities in cells bearing corresponding fusions of the tested promoters with lacZ. The relative values were normalized to the activities measured in samples from wild-type hosts, which were 3772 ± 350, 1179 ± 89 and 1903 ± 407 Miller units for \( P_R \) from λ, ST2-8624 and 933W, respectively (these values correspond to the value of 100% for the wild-type column in each panel). Results shown are mean ± SD from three experiments. All differences between wild-type and mutant hosts, except for \( P_R \) from 933W in the dksA ppGpp-null strain, proved to be statistically significant (see symbols above each mutant strain column). Other statistically significant differences, between pairs of mutant hosts, are shown by links between corresponding columns. Abbreviations for \( t \) test results: *, \( 0.01 < P < 0.05 \); **, \( 0.001 < P < 0.01 \); ***, \( P < 0.001 \).
transcription from the P promoter contributes to lambdoid plasmid replication. In fact, an impairment of transformation of dksA mutants by λ plasmids has already been reported (Łyzen et al., 2009), which may support this suggestion. Moreover, we found that the Ser282Gly substitution in the O protein and the Ala20Thr substitution in the P protein, which weaken a requirement for transcriptional activation of ori, can either totally (Ser282Gly in O or a double mutant) or partially (Ala20Thr in P) suppress this transformation defect (Table 2). Importantly, transformation of the dksA mutant by plasmids derived from Shiga toxin-converting phages was either severely impaired (plasmids derived from bacteriophages ST2-8624 and φPT27Δtox) or totally inhibited (plasmids derived from bacteriophages Stx2Φ-I, 933W, φPT22Δtox and φPT32Δtox) (Table 2). The substitutions in the O and P proteins could improve only weakly the transformation efficiency of the dksA mutant by ST2-8624- and φPT27Δtox-derived plasmids (Table 2).

One should also take into consideration the fact that unlike the λ genome, which contains four iterons (sequences to which the O replication initiator binds) in the ori region, the corresponding regions of lambdoid phages encoding Shiga toxins, studied in this work, contain six iterons (Łyzen et al., 2009). Therefore, it is likely that the interactions of the replication complexes of these bacteriophages with origin sequences are stronger than those occurring at ori. One possibility is that these differences result in inter- or intramolecular handcuffing at the O protein–iteron level. If this is the case, a more efficient (relative to λ) transcriptional activation of the origin might be required for effective initiation of Shiga toxin phage DNA replication. This might be achieved under favourable growth conditions (such as in a nutrient medium), but not in amino acid-starved cells.

### Implications for management of STEC infections

As described in the Introduction, the pathogenicity of Shiga toxin-producing bacteria depends on the induction of Shiga toxin-converting prophages. Since in bacterial cells, the level of gene dosage is often directly proportional to the efficiency of gene expression, it is likely that the kinetics of replication of phage DNA significantly influence stx expression. Obviously, effective stx transcription is crucial for high-level production of the toxin. Accordingly, any factors that impair these processes (prophage induction, phage DNA replication and stx transcription) may be considered as potential anti-STEC drugs, and any cellular or phage factors that stimulate these processes can be potential targets for such drugs. This is especially important in the light of the fact that some antibiotics cause both lambdoid prophage induction and indirect stimulation of stx gene expression (Matsushiro et al., 1999; Kimmitt et al., 2000; Serna & Boedeker, 2008; Łos et al., 2009, 2010), and this has resulted in the recommendation to avoid antibiotic treatment of patients infected with STEC.

The results presented here indicate that DNA replication starting from origins for DNA replication of Shiga toxin-converting bacteriophages is impaired in starved E. coli cells, and that both ppGpp-mediated inhibition of the Pλ promoter activity and its insufficient stimulation by the DksA protein may contribute significantly to this regulation. These results might also explain, at least partially, why the development of lambdoid phages is slower and less efficient in slowly growing host cells, in which concentrations of ppGpp are relatively high, and almost completely inhibited in starved bacteria, as reported previously (Łos et al., 2007, 2009, 2010).

In the light of our results, one might speculate that conditions causing starvation of bacteria in the human intestine would be favourable to the management of STEC infections. Importantly, there are still opposing recommendations for the treatment of patients with acute diarrhoea, a symptom which is also characteristic of STEC infections. Namely, one approach favours either reducing oral intake or even fasting during illness, while another approach recommends continued feeding (see, for example, Brown, 1994; Grimwood & Forbes, 2009; Koletzko & Osterrieder, 2009). The data presented in this

### Table 2. Efficiency of transformation of E. coli wild-type and dksA strains with various plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>dksA transformation rate (e.o.t.(_{dksA})/e.o.t.wt)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCB104</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td>pCB104gly</td>
<td>0.99 ± 0.17</td>
</tr>
<tr>
<td>pCB104thr</td>
<td>0.67 ± 0.11</td>
</tr>
<tr>
<td>pCB104glythr</td>
<td>0.90 ± 0.04</td>
</tr>
<tr>
<td>prR8624cmr</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>prR8624cmrgly</td>
<td>0.21 ± 0.01†</td>
</tr>
<tr>
<td>prR8624cmtthr</td>
<td>0.13 ± 0.02†</td>
</tr>
<tr>
<td>prR8624cmrglythr</td>
<td>0.65 ± 0.10†</td>
</tr>
<tr>
<td>p27cmr</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>p27cmrgly</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>p27cmrthr</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>p27cmrglythr</td>
<td>0.25 ± 0.01†</td>
</tr>
<tr>
<td>pRstx2cmr</td>
<td>&lt;0.01‡</td>
</tr>
<tr>
<td>p933Wcmr</td>
<td>&lt;0.01‡</td>
</tr>
<tr>
<td>p32cmr</td>
<td>&lt;0.01‡</td>
</tr>
<tr>
<td>p22cmr</td>
<td>&lt;0.01‡</td>
</tr>
</tbody>
</table>

*The dksA transformation rate was calculated as a ratio: efficiency of transformation (e.o.t.) of the dksA mutant (e.o.t.\(_{dksA}\)) divided by the e.o.t. of the wild-type strain (e.o.t.wt). The measured values of e.o.t.wt were between 1.0 × 10\(^{5}\) and 1.7 × 10\(^{4}\) transformants per microgram of DNA of various plasmids. Results shown are mean ± sd from three independent experiments.
†Only very small colonies, visible after 48 h incubation of plates (in contrast to the standard 24 h incubation), were reproducibly obtained.
‡No transformants were obtained in experiments with the dksA mutant.

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report may support the fasting strategy in the case of STEC infection.

Finally, our studies confirmed that $P_R$ promoter function is crucial for the replication of lambdoid phages, irrespective of environmental conditions. The activity of this promoter is regulated in a complicated manner, with many factors influencing the process (Szalewska-Pałasz et al., 2007; Łyżen et al., 2009). Therefore, one might suppose that any factors specifically impairing the actions of positive regulators of this promoter, or enhancing its negative regulators, could be considered as potential anti-STECS therapeutics.

ACKNOWLEDGEMENTS

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