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 *P_R* promoter and its homologues from Shiga toxin-converting bacteriophages, these promoters, except for λ *P_R*, 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; Loš *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

Abbreviation: STEC, Shiga toxin-producing *Escherichia coli*. 
replication is also an important factor in STEC pathogen-
esis. 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 \( \lambda \) is the best-
investigated member (for reviews, see Ptashne, 2004;
We˛grzyn & We˛grzyn, 2005). The efficiency of DNA
replication of lambdoid phages can be studied by employ-
ing plasmids derived from these phages, which are simple
replicons, while still having all the genes and regulatory
sequences necessary for phage DNA replication (Taylor &
We˛grzyn, 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 \( \text{ori} \), as transcription
starting from this promoter and proceeding through the
replication region leads to transcriptional activation of the
\( \text{ori} \), a process required even in the presence of all
proteins involved in \( \lambda \) DNA replication (Taylor &
We˛grzyn, 1995). Until recently, \( P_R \)-initiated transcriptional
activation of the \( \text{ori} \) 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 & We˛grzyn, 1995;
We˛grzyn & We˛grzyn, 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 \( \text{ori} \) 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
effect of stringent control. This nucleotide interacts with
RNA polymerase and transiently changes its properties
(Potrykus & Cashel, 2008; Szalewska-Pałasz, 2008). Recently,
another factor involved in the modulation of
ppGpp-mediated transcription regulation, the DksA pro-
tein, has been discovered (for a review, see Szalewska-
Pałasz 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 (Lyzien et al., 2009). In mutants defective in
the production of ppGpp (particularly \( \text{relA} \) 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
\( \text{ori} \) 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 differ-
ences that we discovered between the replication of
plasmids derived from \( \lambda \) and Shiga toxin-converting
bacteriophages, we studied the mechanisms of the various
responses of similar repliscons to starvation conditions.

**METHODS**

**Bacteria and growth conditions.** E. coli strains CF1648 (wild-type,
\( \text{relA}^+ \)) and CF1652 (\( \Delta \text{relA}^{L251:: \text{kan}} \)), described elsewhere (Xiao et al.,
1991), as well as a set of isogenic strains bearing a \( \text{lacZ} \) mutation and
combinations of \( \text{relA} \), \( \text{spoT} \) and \( \text{dksA} \) mutations (described by Lyzen et al.,
2009), were used. Bacteria were cultured in either LB medium
(Sambrook et al., 1989) or a minimal medium, MMGlu (Jasiecki &
We˛grzyn, 2003), at 37°C in shake flasks with agitation. Isoleucine
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 \( \lambda \) plasmid pCB104 (Boyd & Sherratt, 1995) and its \( \pi A66 
\)
derivative, pAW6 (We˛grzyn et al., 1996b), have already been
described. The construction of plasmids derived from Shiga toxin-
converting bacteriophages [originally named \( \phi 933W\Delta \text{tox} \),
\( \phi PT22\Delta \text{tox} \), \( \phi PT27\Delta \text{tox} \) and \( \phi PT32\Delta \text{tox} \) (Gamage et al., 2004);
Stx2φ-I (Watarai et al., 1998); StT2-8624, 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 \( P_R \) promoters from
plasmids pCB104, pRB64cmr and p933Wcmr were amplified using
following primers: PRCB104F (5’-TGC GGA TCC ATG CTC
TTG TGT TAA TG-3’), PRCB104R (5’-GCT AAG CTT TAG GCC
GAG ATC TTG AGC TG-3’), PRRB642F (5’-CAA GGA TCC CAG
ATT CGA TTT GCG AAT AT-3', PRR8624R (5'-GAA AAG CTT GCT AAT GCG GCG AGT CGC TT-3'), PR933WF (5'-CAT GGA TCC CAG CTT CTT TAC AGG CTT GC-3') and PR933WR (5'-TGC AAG CTT GCT AAC TCC ACA AGC CTT CG-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

Fig. 1. Genetic map of a lambdoid plasmid with a schematic of replication complex assembly (a) and the mechanism of inheritance of the stable λ replication complex (b). In (a), a fragment of the genetic map of a lambdoid phage bearing Shiga toxin-encoding genes is shown at the top, with functions encoded by particular genome fragments indicated (note that the phage λ genome does not contain the region responsible for encoding the toxin proteins). The replication region is enlarged below the map, with PR1 andIQ1 (a weak transcription terminator) regions shown, and the products of particular genes are indicated. A scheme for the assembly and activation of the replication complex is shown at the bottom of the panel. In (b), the inheritance of the stable λ replication complex is depicted schematically. The complex (small filled circle) is inherited after each round of plasmid (large circle) replication by one of two daughter plasmid molecules. On the second copy (chosen randomly), a new replication complex must be assembled. Both inherited and newly assembled replication complexes require transcription activation of the origin to efficiently initiate bidirectional plasmid DNA replication. The figure is based on the review of Wegrzyn & Wegrzyn (2002).
Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<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 pΔA66 mutation (causing the Arg→Gly amino acid substitution in the P protein)</td>
<td>Wegrzyńska (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 Pλ 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 Pλ 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 Pλ promoter sequence of bacteriophage 933W, inserted between BamHI and HindIII restriction sites</td>
<td>This work</td>
</tr>
</tbody>
</table>

Estimation of efficiency of plasmid DNA replication. The replication of plasmid DNA in host cells was investigated as described previously (Herman et al., 1994; Szałewska-Pałasz et al., 1994). Briefly, a known number of bacterial cells (5 × 10^8) 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, corresponding to the Ser282→Gly and Ala20→Thr substitutions in the O and P proteins, respectively, 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 fusion of the λ Pλ 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 (Łyzien et al., 2009), and analogous fusions bearing the Pλ promoter region from phages ST2-8624 and 933W were constructed using the primers described above. All fusions were verified by DNA sequencing.
Measurement of the stability of replication initiator proteins. Bacteria bearing a \( \lambda \) plasmid or plasmids derived from Shiga toxin-converting bacteriophages (Table 1) were grown in MMGl medium to \( \text{OD}_{600} \approx 0.2 \). At time 0, tetracycline was added to a final concentration of 200 \( \mu \text{g} \text{ml}^{-1} \) to inhibit protein synthesis. Isolecine starvation was induced by the addition of l-valine (to a final concentration of 1 mg \( \text{ml}^{-1} \)) 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 6.8, 2 % SDS, 1 % \( \beta \)-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 glycine, 0.1 % SDS, 20 % methanol) at 90 mA. The \( \lambda \) 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 Na₂HPO₄·7H₂O, 1.47 mM KH₂PO₄), with 0.1 % Tween 20 and 5 % low-fat milk] overnight incubation in anti-\( \lambda \)-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 Multimager (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-\( \lambda \)-O serum for each tested O homologue (from each tested plasmid) 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, pTEPRRR8624, 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 \( \mu \)l in transcription buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl₂, 10 mM \( \beta \)-mercaptoethanol, 10 \( \mu \)M BSA \( \text{ml}^{-1} \), 140 mM KCl). Supercoiled DNA template (10 \( \mu \)M), 10 \( \mu \)g DNA polymerase (Epicentre Technologies) and nucleotides [final concentrations: 150 \( \mu \)M CTP and GTP, 1 mM ATP, 15 \( \mu \)M UTP and 1 \( \mu \)M (3.7 \( \times 10^{6} \) Bq) \( \text{[\text{32P]UTP}} \) (3000 Ci mmol \(^{-1}\) ) (Hartmann Analytic)] were included in the reaction mixture. After addition of RNA polymerase, the incubation mixtures 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 \( \mu \)g \( \text{ml}^{-1} \)), samples were incubated at 37 °C for 5 min. The reactions were terminated by the addition of 5 \( \mu \)l 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 M 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 \( \beta \)-galactosidase activity in cells.** The activity of \( \beta \)-galactosidase in *E. coli* cells was measured according to Zhang & Bremer (1995). Since multicopy 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 (Lyžen et al., 2009).

**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 \( \lambda \) (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 P₆ promoter and the resultant low efficiency of transcriptional activation of oriₗ (Szalewska-ŁaPaś 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 oriₗ is efficient (Wegrzyn et al., 1992, 1995, 1996a; Szalewska-ŁaPaś 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 \( \lambda \) plasmid replication (Herman et al., 1994; Szalewska-ŁaPaś 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 \( \lambda \) plasmid (pCB104) and its mutant bearing a \( \pi \) mutation in the *P₆* gene (pAW6) were used. Note that the \( \pi \) mutation was originally defined as a mutation causing a suppression of inhibition of \( \lambda \) phage development in *E. coli* mutants called groP (for growth of phage), and was mapped in *dnaB, dnaJ, dnaK* or *grpE*, encoding either a helicase or molecular chaperones necessary for rearrangement of the \( \lambda \) replication complex (see Taylor & Wegrzyn, 1995 and references therein).
Subsequent studies have indicated that the \( \pi \) 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 \( \lambda \) plasmid, and despite a high level of homology between the \( \lambda \) replication region and corresponding regions of genomes from Shiga toxin-converting phages (Nejman et al., 2009), plasmids derived from phages Stx2Φ-I, ST2-8624, 933W, \( \varphi PT22\Delta tox \), \( \varphi PT27\Delta tox \) and \( \varphi PT32\Delta tox \) (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 \( \lambda \), ST2-8624 and \( \varphi PT27\Delta tox \). There is a Leu37Ile substitution in the O protein of phage \( \varphi PT27\Delta tox \) that is present in all other lambdoid phages tested in this work, except those encoded by \( \lambda \) and ST2-...
Fig. 3. Replication of various lambdoid plasmids estimated by measurement of relative plasmid content at various times, in isoleucine-starved *E. coli* relA+ (open symbols) and relA− (closed symbols) bacteria. The ‘gly’ and ‘thr’ abbreviations in the names of plasmids indicate mutations causing a Ser282Gly substitution in the \( \lambda \) O protein and an Ala20Thr substitution in the \( \lambda \) 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\(^{-1}\). Results shown are mean ± SD from three experiments.
Homologous proteins encoded by phages Stx2Φ-1, 933W, φPT22Δtox and φPT32Δtox have two important amino acid substitutions: Ser282Gly in the O protein and Ala20Thr in the P protein, numbered according to the coordinates for the λ phage (the nucleotide change that causes the substitution in the P protein is, however, not a π type mutation) (Nejman et al., 2009). Although the biochemical properties of these variants of the O and P proteins are not known, the substitutions have been found previously to be able to suppress the DnaA dependence of lambdoid plasmid replication (Nejman et al., 2009), suggesting that they weaken the requirement for transcriptional activation of the origin, as DnaA is a stimulator of transcription from the $P_R$ promoter (Szalewska-Pałasz et al., 1998). Therefore, to determine whether the mutations able to influence the replication initiation requirements influence the response to starvation conditions of replicons derived from both λ and Shiga toxin-converting phages, we studied the replication of plasmids bearing the above-described mutations in amino acid-starved rel$A^+$ (wild-type, CF1648) and rel$A^-$ (ΔrelA251::kan, CF1652) strains.

We found that the Ser282Gly substitution in the O protein, but not the Ala20Thr substitution in the P protein, allowed the λ plasmid to replicate not only during the relaxed response but also in amino acid-starved wild-type cells (Fig. 3). This strengthened the assumption that in the presence of such a form of the O protein, less efficient (due to ppGpp-mediated negative regulation of the $P_R$ promoter) transcriptional activation of ori$\iota$ is still sufficient to support plasmid replication initiation. Interestingly, the double mutant, encoding the Ser282Gly O protein and the Ala20Thr P protein, could not replicate efficiently during either the stringent or the relaxed response (Fig. 3). However, neither Ser282Gly in the O protein nor Ala20Thr in the P protein could allow replication of plasmids derived from Shiga toxin-converting bacteriophages in amino acid-starved rel$A^+$ and rel$A^-$ bacteria (Fig. 3). These results demonstrate that a decreased requirement for transcriptional activation of the origin is not sufficient to 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](image-url)

**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 (rel$A^+$ or rel$A^-$) 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 $P_R$ (Łyzień 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 (Łyzień 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 Stx2Φ-I, 933W, $\varphi$PT22Δtox, $\varphi$PT27Δtox and $\varphi$PT32Δtox 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 in vitro transcription from the $\lambda$ $P_R$ promoter, we confirmed previous results from single-round transcription studies (Łyzień 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 in vitro studies were corroborated by in vivo experiments, in which the activities of $P_R$ promoters

![Fig. 6](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 Pr, significantly less pronounced effects of the lack of DksA were observed at the corresponding promoters of ST2-8624 and 933W (Fig. 7). Interestingly, Pr 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 Pr promoter (3772 ± 350 Miller units) was about twofold higher than that of Pr from 933W (1903 ± 407 Miller units), and about threefold higher than that of Pr 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 (We˛grzyn 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 We˛grzyn & We˛grzyn, 2001, 2002, 2005). Therefore, based on the results shown in Figs 6 and 7, we speculate that effective DksA-mediated stimulation of

![Graphs](https://via.placeholder.com/150)

Fig. 7. Estimation of activities of Pr 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 Pr 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 Pr 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 \( P_{\beta} \) promoter contributes to lambdoid plasmid replication. In fact, an impairment of transformation of \( dksA \) mutants by \( \lambda \) plasmids has already been reported (Łyz˙en´l 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 substitution in the O protein and the Ala20Thr substitution. Moreover, we found that the Ser282Gly substitution 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 \( \phi PT27\)tox) or totally inhibited (plasmids derived from bacteriophages STx20-I, 933W, \( \phi PT22\)tox and \( \phi PT32\)tox) (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 \( \phi PT27\)tox-derived plasmids (Table 2).

One should also take into consideration the fact that unlike the \( \lambda \) genome, which contains four iterons (sequences to which the O replication initiator bind) in the \( ori_{\lambda} \) region, the corresponding regions of lambdoid plasmids encoding Shiga toxins, studied in this work, contain six iterons (Nejman et al., 2009). Therefore, it is likely that the interactions of the replication complexes of these bacteriophages with \( ori \) sequences are stronger than those occurring at \( ori_{\lambda} \). 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 \( \lambda \)) transcriptional activation of the \( ori \) 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-STE C 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 & Boe deker, 2008; Loš 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 \( P_{\beta} \) 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 plasmids 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 (Loš 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>pr8624cmr</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>pr8624cmrgly</td>
<td>0.21±0.01†</td>
</tr>
<tr>
<td>pr8624cmrthr</td>
<td>0.13±0.02†</td>
</tr>
<tr>
<td>pr8624cmrglythr</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 1.0×10⁸ and 1.7×10⁸ transformants per microlitre 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.
report may support the fasting strategy in the case of STEC infection.

Finally, our studies confirmed that $P_k$ 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; Łyżeni 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-STEC therapeutics.

ACKNOWLEDGEMENTS

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