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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; Łoś *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 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 P_R (one of the early phage promoters) is necessary to initiate replication from *ori*λ, 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, P_R-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-Pałasz, 2008). Recently, another factor involved in the modulation of ppGpp-mediated transcription regulation, the DksA protein, 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 (Łyżeń *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*s 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 (Δ*relA251*::*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 Łyżeń *et al.*, 2009), were used. Bacteria were cultured in either LB medium (Sambrook *et al.*, 1989) or a minimal medium, MMGlu (Jasiecki & Węgrzyn, 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 λ plasmid pCB104 (Boyd & Sherratt, 1995) and its πA66 derivative, pAW6 (Węgrzyn *et al.*, 1996b), have already been described. The construction of plasmids derived from Shiga toxin-converting bacteriophages [originally named φ933WΔtox, φPT22Δtox, φPT27Δtox and φPT32Δtox (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 P_R promoters from plasmids pCB104, pR8624cmr and p933Wcmr were amplified using following primers: PRCB104F (5′-TGC GGA TCC AAG CTG CTC TTG TGT TAA TG-3′), PRCB104R (5′-GCT AAG CTT TAC GCC GAG ATC TTT AGC TG-3′), PRR8624F (5′-CAA GGA TCC CAG

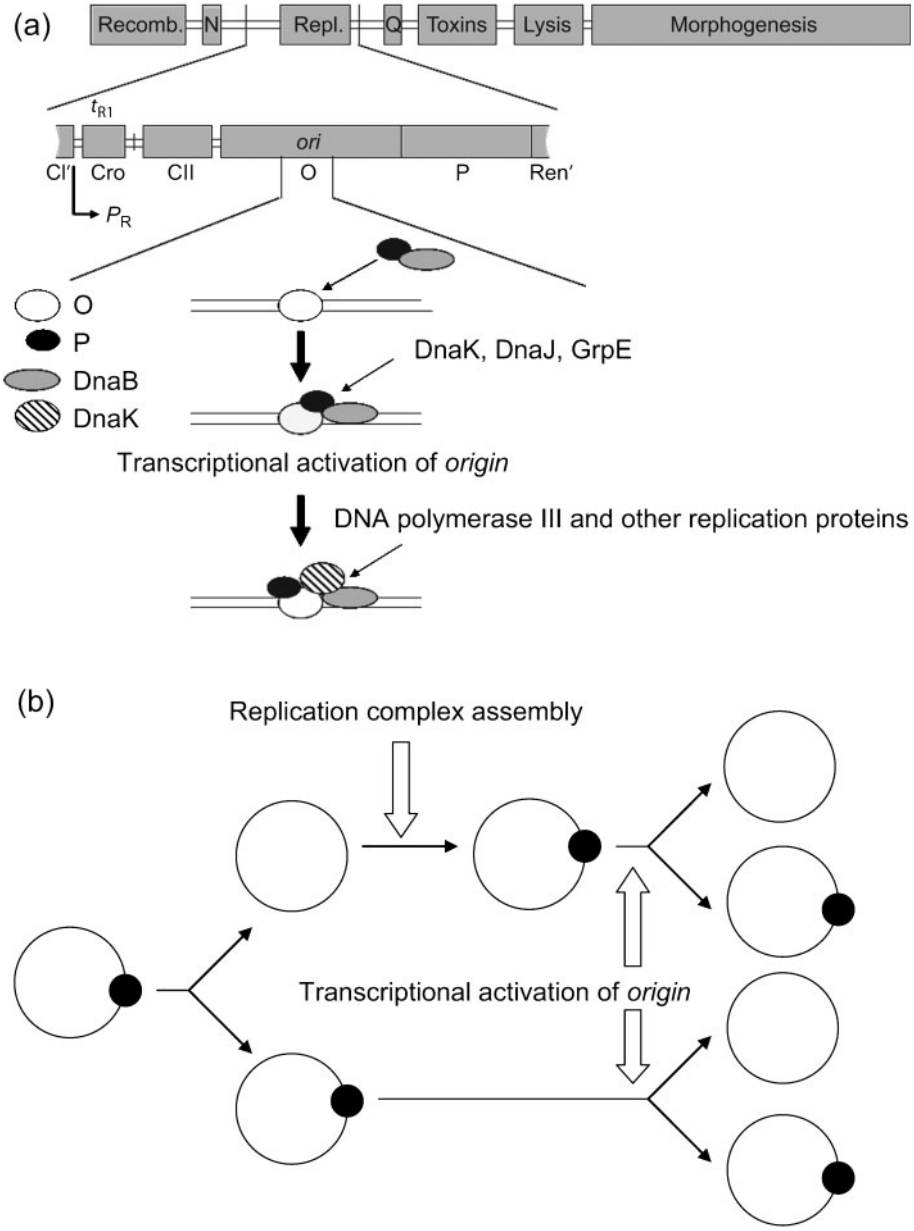

Fig. 1. Genetic map of a lambdoid plasmid with a schematic of replication complex assembly (a) and the mechanism of inheritance of the stable λ replication complex (b). In (a), a fragment of the genetic map of a lambdoid phage bearing Shiga toxin-encoding genes is shown at the top, with functions encoded 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 P_R and t_{R1} (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 Węgrzyn & Węgrzyn (2002).

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 *Bam*HI and *Hin*dIII, 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

Table 1. Plasmids used in this study

Plasmid	Characteristics	Reference or source
pCB104	Wild-type λ plasmid carrying the replication region of bacteriophage λ and a chloramphenicol-resistance gene	Boyd & Sherratt (1995)
pCB104gly	As pCB104 but bearing a mutation causing the Ser282→Gly amino acid substitution in the O protein	Nejman *et al.* (2009)
pCB104thr	As pCB104 but bearing a mutation causing the Ala20→Thr amino acid substitution in the P protein	Nejman *et al.* (2009)
pCB104glythr	As pCB104 but bearing mutations causing the Ser282→Gly and the Ala20→Thr amino acid substitutions in the O and P proteins, respectively	Nejman *et al.* (2009)
pAW6	As pCB104 but bearing the πA66 mutation (causing the Arg→Gly amino acid substitution in the P protein)	Węgrzyn *et al.* (1996b)
pR8624cmr	Wild-type plasmid carrying the replication region of bacteriophage ST2-8624 and a chloramphenicol-resistance gene	Nejman *et al.* (2009)
pR8624cmrgly	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	Nejman *et al.* (2009)
pR8624cmrthr	As pR8624cmr but bearing a mutation causing an amino acid change of the P protein, corresponding to the Ala20→Thr substitution in the P protein of bacteriophage λ	Nejman *et al.* (2009)
pR8624cmrglythr	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	Nejman *et al.* (2009)
p27cmr	Wild-type plasmid carrying the replication region of bacteriophage φPT27Δtox and a chloramphenicol-resistance gene	Nejman *et al.* (2009)
p27cmrgly	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	Nejman *et al.* (2009)
p27cmrthr	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	Nejman *et al.* (2009)
p27cmrglythr	As p27cmr but bearing mutations causing amino acid changes in the O and P proteins, corresponding to the Ser282→Gly and the Ala20→Thr substitutions in the O and P proteins of bacteriophage λ, respectively	Nejman *et al.* (2009)
pRstx2cmr	Wild-type plasmid carrying the replication region of bacteriophage Stx2Φ-I and a chloramphenicol-resistance gene	Nejman *et al.* (2009)
p933Wcmr	Wild-type plasmid carrying the replication region of bacteriophage 933W and a chloramphenicol-resistance gene	Nejman *et al.* (2009)
p32cmr	Wild-type plasmid carrying the replication region of bacteriophage φPT32Δtox and a chloramphenicol-resistance gene	Nejman *et al.* (2009)
p22cmr	Wild-type plasmid carrying the replication region of bacteriophage φPT22Δtox and a chloramphenicol-resistance gene	Nejman *et al.* (2009)
pTE103	Plasmid vector containing an ampicillin-resistance gene and a multiple cloning site from pUC8, placed upstream of the bacteriophage T7 transcriptional terminator	Elliott & Geiduschek (1984)
pTEPRCB104	As pTE103 but bearing the P_R promoter sequence of bacteriophage λ, inserted between *Bam*HI and *Hin*dIII restriction sites	This work
pTEPRR8624	As pTE103 but bearing the P_R promoter sequence of bacteriophage ST2-8624, inserted between *Bam*HI and *Hin*dIII restriction sites	This work
pTEPR933W	As pTE103 but bearing the P_R promoter sequence of bacteriophage 933W, inserted between *Bam*HI and *Hin*dIII restriction sites	This work

fusion of the λ P_R 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 (Łyżeń *et al.*, 2009), and analogous fusions bearing the P_R 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-Pałasz *et al.*, 1994). Briefly, a known number of bacterial cells (5×10^9) 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 MMGlu 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. Isoleucine 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 6.8, 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 glycine, 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 $Na_2HPO_4.7H_2O$, 1.47 mM KH_2PO_4), with 0.1 % Tween 20 and 5 % low-fat milk] overnight incubation in anti-λO 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-λO 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 nM KCl). Supercoiled DNA template (10 nM), indicated 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}), 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 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 β-galactosidase activity in cells. The activity of β-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 (Łyżeń *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_R promoter and the resultant low efficiency of transcriptional activation of *oriλ* (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 *oriλ* is efficient (Węgrzyn *et al.*, 1992, 1995, 1996a; Szalewska-Pałasz *et al.*, 1994; for a review, see Węgrzyn & Węgrzyn, 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 *gro*wth of *p*hage), and was mapped in *dnaB*, *dnaJ*, *dnaK* or *grpE*, encoding either a helicase or molecular chaperones necessary for rearrangement of the λ replication complex (see Taylor & Węgrzyn, 1995 and references therein).

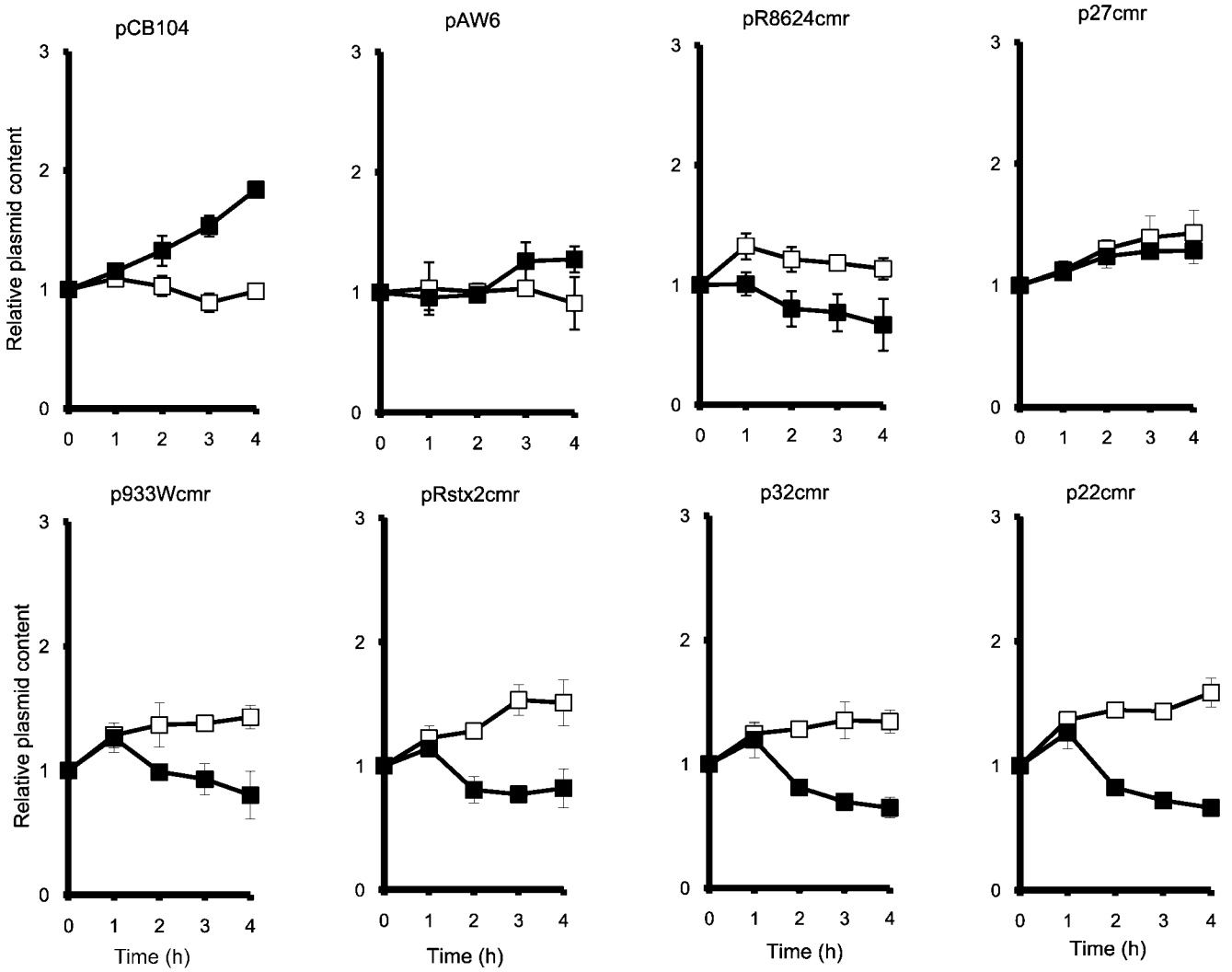

Fig. 2. Replication of various lambdoid plasmids estimated by measurement of relative plasmid content at various times, in isoleucine-starved *E. coli relA*$^+$ (open symbols) and *relA*$^-$ (closed symbols) bacteria. Isoleucine starvation was induced at time 1 h by addition of L-valine to the minimal medium to a final concentration of 1 mg ml^{-1}. Results shown are mean ± SD from three experiments.

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-I, 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-

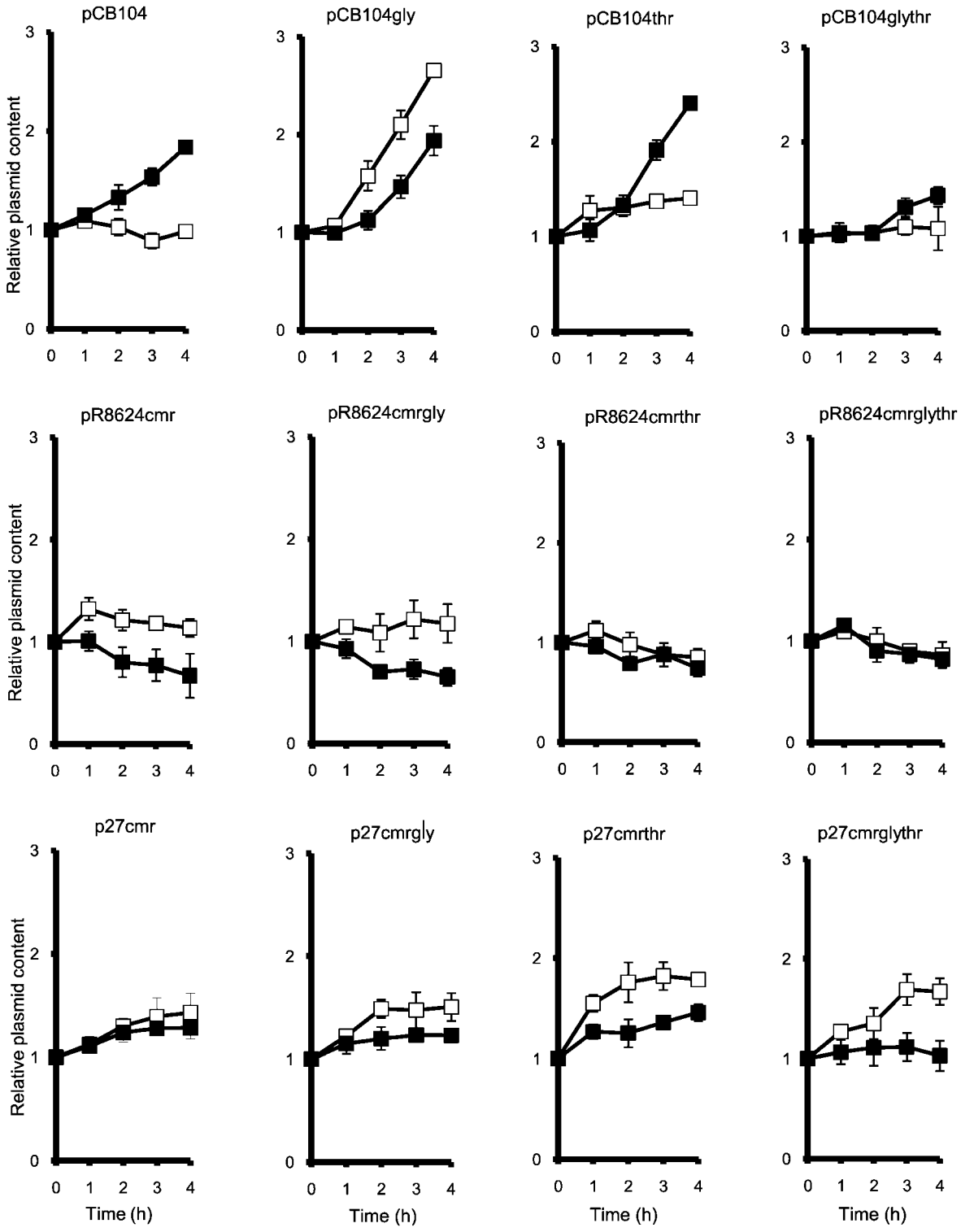

Fig. 3. Replication of various lambdoid plasmids estimated by measurement of relative plasmid content at various times, in isoleucine-starved *E. coli relA*$^+$ (open symbols) and *relA*$^-$ (closed symbols) bacteria. The 'gly' and 'thr' abbreviations in the names of plasmids indicate mutations causing a Ser282Gly substitution in the 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^{-1}. Results shown are mean ± SD from three experiments.

8624. However, this does not seem to have any significant effect on phage DNA replication (Nejman *et al.*, 2009). Homologous proteins encoded by phages Stx2Φ-I, 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 *relA*$^+$ (wild-type, CF1648) and *relA*$^-$ (Δ*relA*251::*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 *oriλ* 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 *relA*$^+$ and *relA*$^-$ 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.

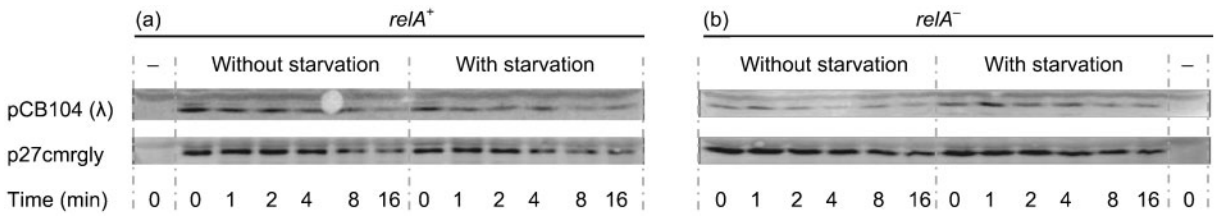

Fig. 4. Evidence for suitability of anti-λO antibodies to detect the O protein encoded by Shiga toxin-converting bacteriophage φPT27Δtox, and an example of the determination of the stability of the O protein. Bacterial strains (*relA*$^+$ or *relA*$^-$) bearing either no plasmid (–) or one of the plasmids derived from lambdoid bacteriophages (pCB104 or p27cmgly) were used for determination of O protein stability, employing anti-λO antibodies, as described in Methods.

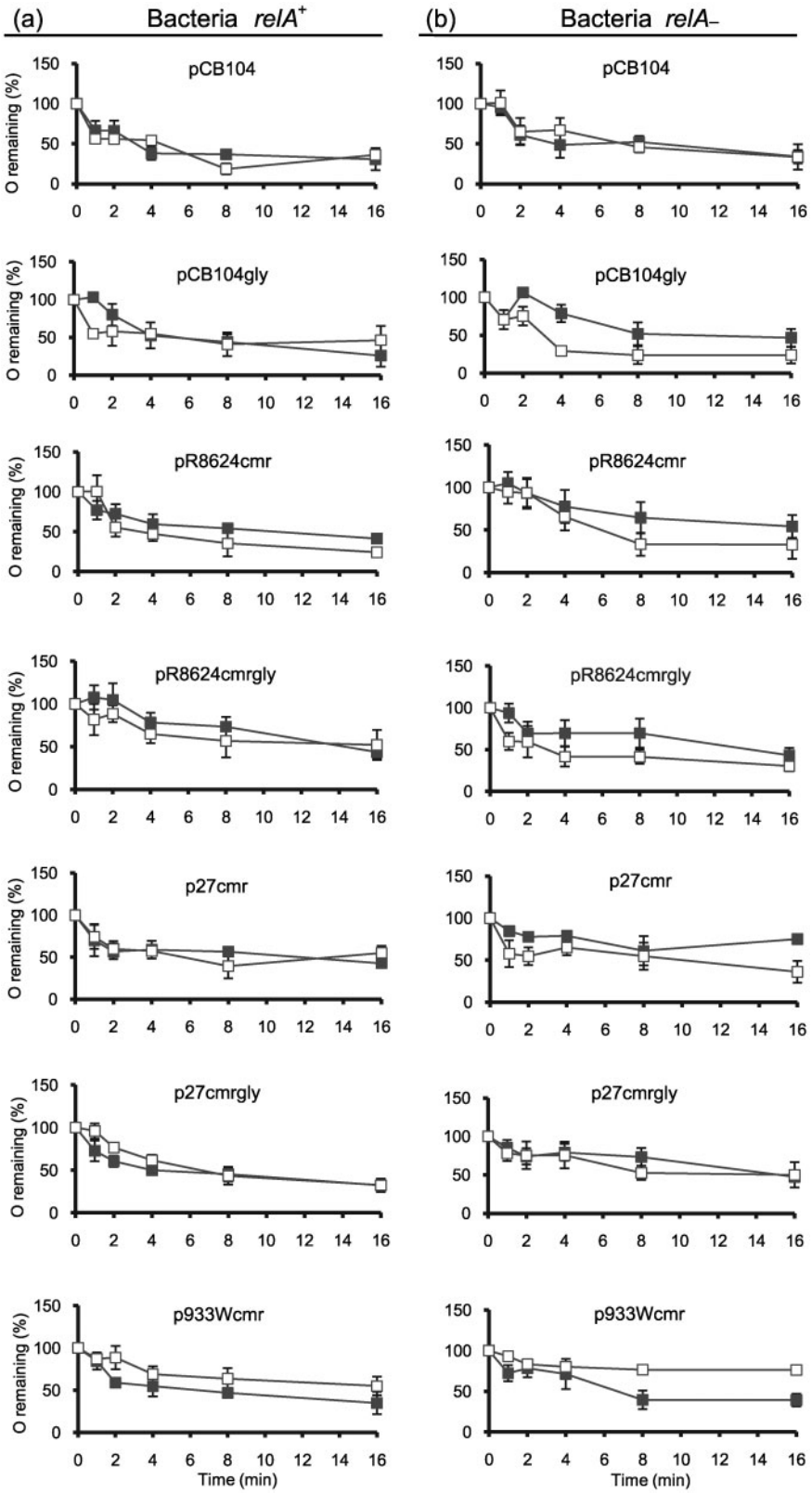

Fig. 5. Stability of the O protein in *relA*$^+$ (left panels) and *relA*$^-$ (right panels) *E. coli* cells bearing the plasmids indicated in each panel. The bacteria were either starved for isoleucine (open symbols) or not starved (closed symbols). Results shown are mean ± SD from three experiments.

Regulation of the activity of P_R promoters of lambdoid phages by ppGpp and DksA

Inhibition of λ-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 (Łyżeń *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 (Łyżeń *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, φPT22Δtox, φPT27Δtox and φPT32Δtox are identical, though different from those of phages λ and ST2-8624 (Nejman *et al.*, 2009), we used constructs bearing P_R sequences of λ, 933W and ST2-8624 in our experiments.

In control experiments with multiple rounds of *in vitro* transcription from the λ P_R promoter, we confirmed previous results from single-round transcription studies (Łyżeń *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 λ P_R.

The results of the *in vitro* studies were corroborated by *in vivo* experiments, in which the activities of P_R promoters

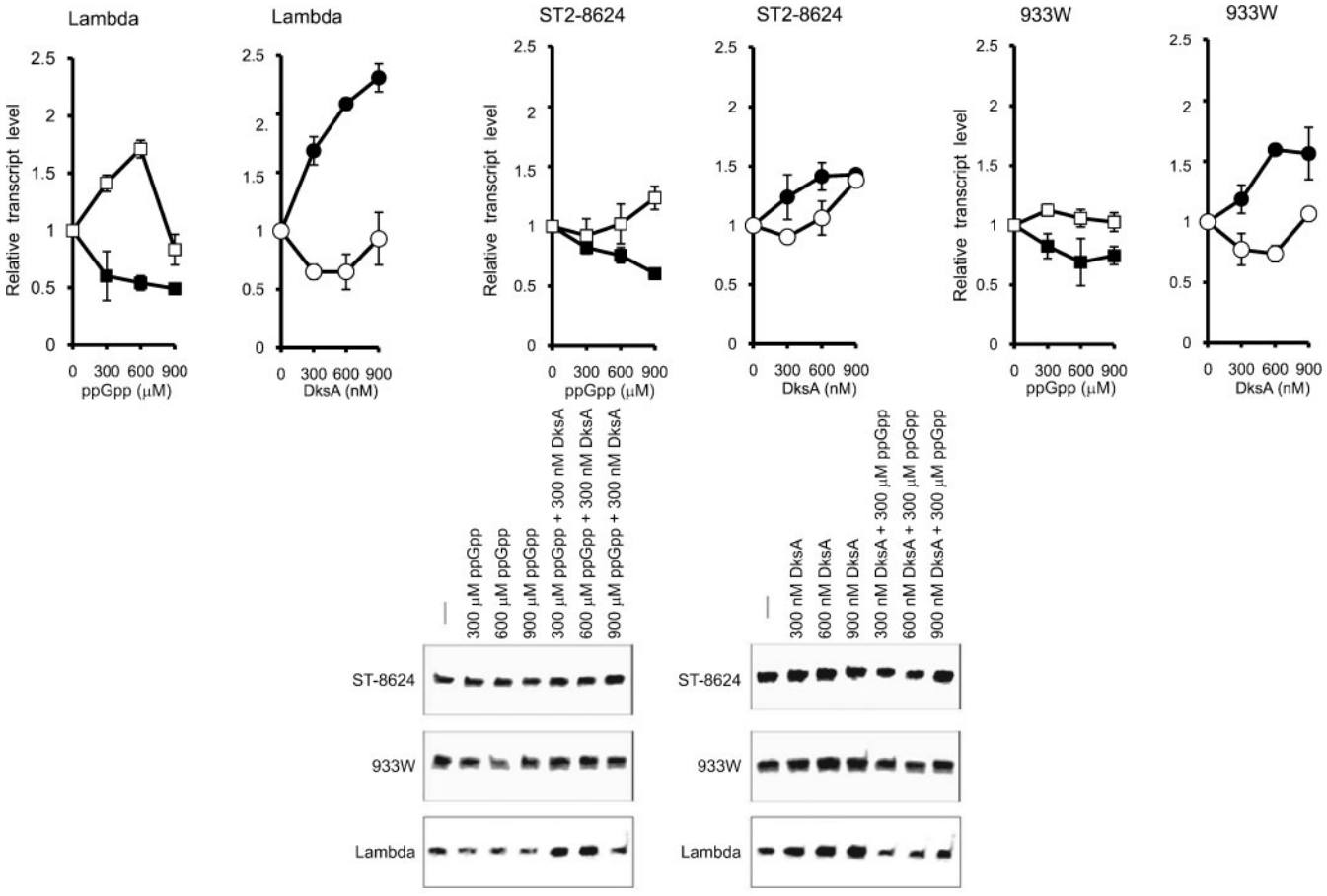

Fig. 6. *In vitro* transcription from the P_R promoter of phage λ (panels 'Lambda'), or a corresponding promoter from the ST2-8624 and 933W phages in the presence of ppGpp and/or DksA. Results of experiments with various concentrations of ppGpp (■), DksA (300 nM) together with various concentrations of ppGpp (□), various concentrations of DksA (●), and ppGpp (300 μM) 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.

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 $\pm$ 350 Miller units) was about twofold higher than that of P_R from 933W (1903 $\pm$ 407 Miller units), and about threefold higher than that of P_R from ST2-8624 (1179 $\pm$ 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

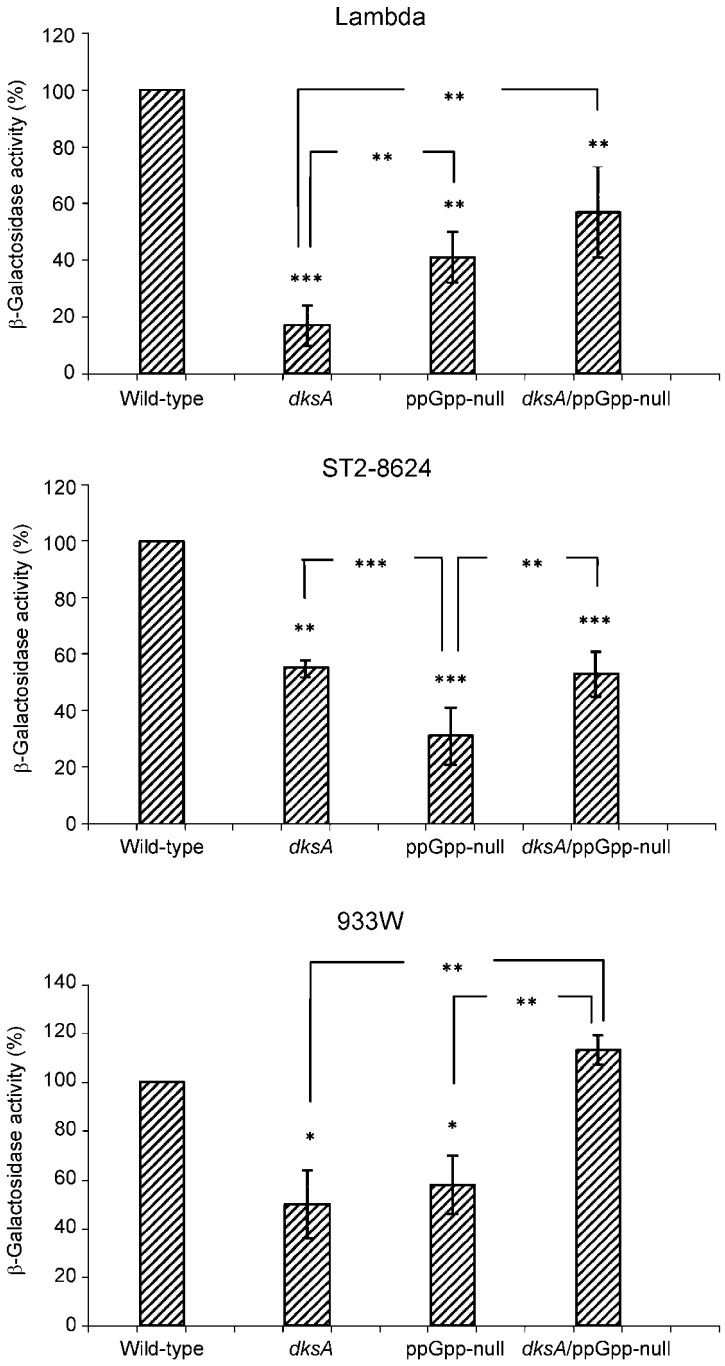

Fig. 7. Estimation of activities of P_R promoters from lambdoid phages (λ, ST2-8624 and 933W) in ppGpp-null (*relA spoT*) and *dksA* mutants by measurement of β-galactosidase activities in cells bearing corresponding fusions of the tested promoters with *lacZ*. The relative values were normalized to the activities measured in samples from wild-type hosts, which were 3772 $\pm$ 350, 1179 $\pm$ 89 and 1903 $\pm$ 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 $\pm$ 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_R promoter contributes to lambdoid plasmid replication. In fact, an impairment of transformation of *dksA* mutants by λ plasmids has already been reported (Łyżeń *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 *oriλ*, 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 *oriλ* region, the corresponding regions of lambdoid phages 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 *origin* sequences are stronger than those occurring at *oriλ*. 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.

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

Plasmid	*dksA* transformation rate (e.o.t.$_{dksA}$/e.o.t.$_{wt}$)*
pCB104	0.17±0.10
pCB104gly	0.99±0.17
pCB104thr	0.67±0.11
pCB104glythr	0.90±0.04
pR8624cmr	0.02±0.01
pR8624cmrgly	0.21±0.01†
pR8624cmrthr	0.13±0.02†
pR8624cmrglythr	0.65±0.10†
p27cmr	0.02±0.02
p27cmrgly	0.01±0.01
p27cmrthr	0.01±0.01
p27cmrglythr	0.25±0.01
pRstx2cmr	<0.01‡
p933Wcmr	<0.01‡
p32cmr	<0.01‡
p22cmr	<0.01‡

*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 1.0×10^2 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.

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; Łoś *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 *origin*s for DNA replication of Shiga toxin-converting bacteriophages is impaired in starved *E. coli* cells, and that both ppGpp-mediated inhibition of the P_R 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 (Łoś *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

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; Łyżeń *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.

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