

Hydrogen peroxide-mediated induction of the Shiga toxin-converting lambdoid prophage ST2-8624 in *Escherichia coli* O157:H7

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Shiga toxin-producing *Escherichia coli* (STEC); prophage induction; oxidative stress; hydrogen peroxide.

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) may cause bloody diarrhea and hemorrhagic colitis, with sometimes severe complications. Because genes coding for Shiga toxins are located on lambdoid prophages, effective toxin production occurs only after prophage induction. However, although agents that effectively induce prophage λ (a paradigm of the family of lambdoid phages) under laboratory conditions, such as UV irradiation or DNA replication inhibitors, are well known, it is unlikely that such factors are present in human intestine infected with STEC. In this report, we demonstrate that induction of a Shiga toxin-converting prophage in its host (*E. coli* O157:H7) occurs not only in the presence of DNA-interfering antibiotics (mitomycin C and norfloxacin) but also under conditions of oxidative stress [following treatment with hydrogen peroxide (H_2O_2)]. Under these conditions, we observed not only effective prophage induction but also expression of the reporter gene (replacing the original *stx2* gene). In the light of previously published reports, indicating that oxidative stress conditions might occur during colonization of human intestine by enteric bacteria, and that neutrophil-produced H_2O_2 can increase production of the Shiga toxin in a clinical isolate of STEC, these results suggest that oxidative stress may be one of the agents responsible for stimulating the pathogenicity determinants of STEC, leading to induction of Shiga toxin-converting prophages in these bacteria.

Introduction

Although most *Escherichia coli* strains belong to the natural physiological flora of human intestine, there are also pathogenic strains of this bacterium. Shiga toxin-producing *E. coli* (STEC) strains are one such group. STEC infections, which appear to be especially dangerous in children, can cause bloody diarrhea and hemorrhagic colitis, and may also result in severe complications (Nataro & Kaper, 1998; Besser *et al.*, 1999).

STEC encode some general colonization factors and other virulence factors, but pathogenicity of these bacteria is significantly enhanced by synthesis of Shiga toxins, which are encoded by *stx1* and *stx2* genes (Nataro & Kaper, 1998; Besser *et al.*, 1999; Schmidt, 2001). These genes are located on lambdoid prophages (called Shiga toxin-converting prophages), and without prophage induction, their expression is

mostly repressed. Therefore, in most cases, effective production of Shiga toxins requires prophage induction and its further lytic development, including replication of the phage genome as an extrachromosomal element (Schmidt, 2001; Wagner *et al.*, 2001b, 2002; Herold *et al.*, 2004; Waldor & Friedman, 2005). Shiga toxin 1 (contrary to 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. Thus, understanding specific conditions causing induction of Shiga toxin-converting prophages in bacteria occurring in the human intestine is especially important.

Shiga toxin-converting phages are lambdoid phages, a family of viruses with bacteriophage λ as the best-investigated member. The mechanism of λ prophage induction has been investigated in detail; however, this is true only for

standard laboratory conditions and a few induction agents, such as UV irradiation and mitomycin C (Ptashne, 2004; Węgrzyn & Węgrzyn, 2005). Moreover, some antibiotics used routinely in clinical practice, such as norfloxacin, can also induce lambdoid prophages (Matsushiro *et al.*, 1999). Generally, any agent that can provoke the bacterial SOS response is a potential prophage induction agent, as the first step in induction is a RecA-dependent autocleavage of the λ cI repressor and subsequent activation of lytic promoters (for reviews, see Ptashne, 2004; Węgrzyn & Węgrzyn, 2005). On the other hand, Shkilnyj & Koudelka (2007) demonstrated that an increased salt concentration (which does not induce the SOS response) caused induction of a λ^{imm434} prophage, and this induction was RecA-independent.

UV irradiation and mitomycin C are classical agents that can efficiently induce lambdoid prophages. However, occurrence of these agents in the STEC-infected human intestine is very unlikely. Furthermore, although prophage induction may be stimulated by some antibiotics used for treatment of patients, usually the induction occurs before or in the absence of antibiotic therapy. Thus, a search for other conditions responsible for lambdoid prophage induction, which are also more likely to be present in the intestine, is desirable. Recent studies have demonstrated that oxidative stress conditions may occur during colonization of the human intestine by enteric bacteria (Kumar *et al.*, 2007). Moreover, earlier studies on a clinical isolate of *E. coli* O157:H7 suggested that hydrogen peroxide (H_2O_2), produced by human neutrophils, may increase the production of Shiga toxin 2 (Wagner *et al.*, 2001a). Therefore, we aimed to test whether oxidative stress, simulated under laboratory conditions through treatment of bacterial cultures with H_2O_2 , could cause induction of a Shiga toxin-converting prophage.

The STEC phenotype was initially correlated to the O157 serotype of *E. coli* (Riley *et al.*, 1983). Although subsequent studies demonstrated that some *E. coli* O157 strains are deficient in the production of Shiga toxins and that there are several *E. coli* serotypes other than O157 that can be responsible for the STEC phenotype (Birembaux *et al.*, 1999; Karch & Bielaszewska, 2001; Blanco *et al.*, 2004; Prere & Fayet, 2005; Łoś *et al.*, 2008c), it appears that still many STEC strains have the O157 antigen, and belong to the O157:H7 serotype (Gyles, 2007). Therefore, as a model in our studies, we have chosen a previously characterized *E. coli* O157:H7 strain no. 86-24, bearing a Shiga toxin-converting prophage (Griffin *et al.*, 1988).

Materials and methods

Bacteria and growth conditions

Escherichia coli O157:H7 strain no. 86-24, bearing the Shiga toxin-converting prophage ST2-8624, has already been

described (Griffin *et al.*, 1988). In our experiments, for both safety reasons and to monitor the expression of the *stx* genes, we used the host containing a prophage in which the *stx2* locus (*stxA* and *stxB* genes) has been replaced with the *gfp* gene, the ST2-8624 ($\Delta stx2::cat\ gfp$) prophage (Łoś *et al.*, 2008a). For phage titration, *E. coli* strain C600 (Appleyard, 1954) was used.

Bacteria were cultured in either Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) or a minimal medium MMGlu (Jasiecki & Węgrzyn, 2003). The media were supplemented, when indicated, with the following tested agents (added to indicated final concentrations): $1\ \mu\text{g mL}^{-1}$ mitomycin C, $0.2\ \mu\text{g mL}^{-1}$ norfloxacin, 200 mM NaCl or 3 mM H_2O_2 .

Monitoring of prophage induction and phage development

Bacteria were cultured in LB or MMGlu medium at $37\ ^\circ\text{C}$ to an $A_{600\text{ nm}}$ of 0.1, then the culture was divided into two parts. An induction agent (mitomycin C, norfloxacin, NaCl or H_2O_2) was added to the appropriate concentration ($1\ \mu\text{g mL}^{-1}$, $0.2\ \mu\text{g mL}^{-1}$, 200 mM or 3 mM, respectively) to one of these cultures (the second culture was a control without an induction agent). The cultivation was continued at $37\ ^\circ\text{C}$, and samples were harvested every 30 min. To each serial dilution of the culture sample (0.5 mL), 30 μL of chloroform was added, and the mixture was vortexed and centrifuged for 5 min in a microfuge. The water phase was mixed with 2 mL of a prewarmed (to $45\ ^\circ\text{C}$) top nutrient agar (0.7%), and 1 mL of the indicator *E. coli* strain culture was added. Following supplementation of the mixture with $MgSO_4$ and $CaCl_2$ (to a final concentration of 10 mM each), it was poured on a plate with LB agar (1.5%) supplemented with $2.5\ \mu\text{g mL}^{-1}$ chloramphenicol, according to a previously published procedure (Łoś *et al.*, 2008a). Plates were incubated at $37\ ^\circ\text{C}$ overnight and the phage titer was calculated on the basis of the number of plaques. Each experiment was repeated three times.

Estimation of the expression efficiency of the reporter gene

In the ST2-8624 ($\Delta stx2::cat\ gfp$) phage, the *stx2* locus is replaced with *cat* and *gfp* genes. Thus, efficiency of expression of the latter gene corresponds to that of *stx2* in the wild-type phage. For estimation of expression of the *gfp* gene in bacteria bearing the ST2-8624 ($\Delta stx2::cat\ gfp$) prophage, two methods were used. In both methods, bacteria were cultured as for monitoring of phage development.

In the first method, following addition of the induction agent, at each time point, 0.1 mL of the culture was harvested and transferred to the well of the enzyme-linked

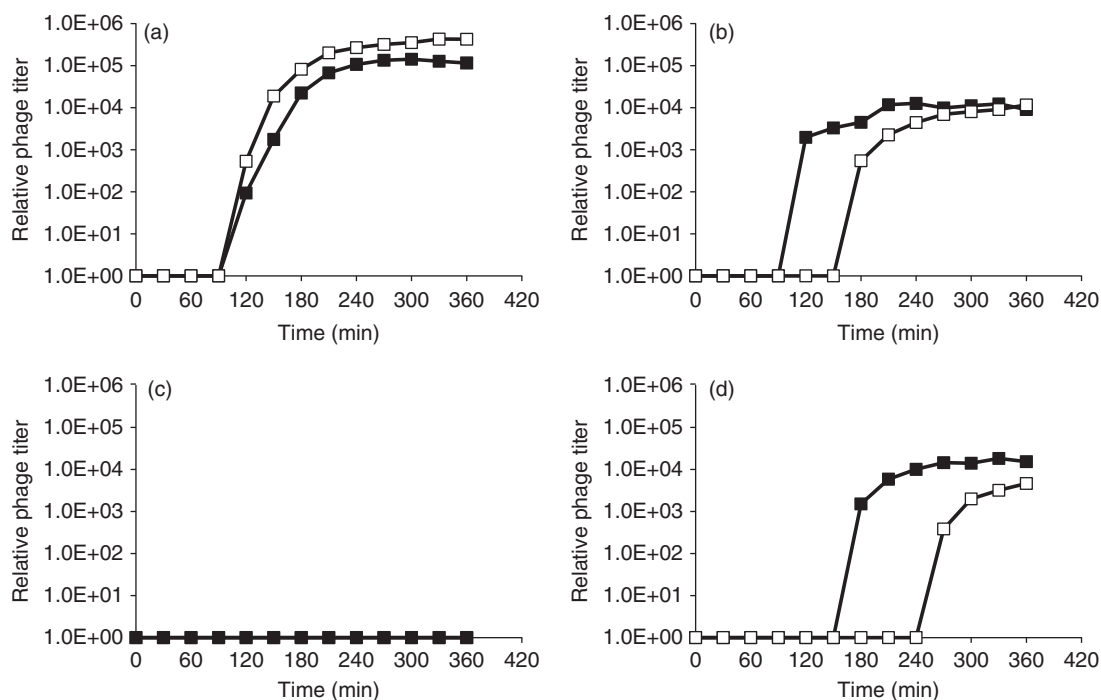


Fig. 1. Efficiency of prophage induction and further development of the ST2-8624 phage in the *Escherichia coli* O157:H7 host growing in LB (■) or MMGlu (□) media. The following inducers were added to bacterial cultures, to indicated concentrations, at time = 0: mitomycin C to $1 \mu\text{g mL}^{-1}$ (a), norfloxacin to $0.2 \mu\text{g mL}^{-1}$ (b), NaCl to 200 mM (c) or H_2O_2 to 3 mM (d). The relative phage titer was calculated by subtraction of the phage titer determined in an uninduced culture from the titer determined in a particular induced culture. Experiments were repeated three times with a high reproducibility (SD < 20%), and representative results are shown.

immunosorbent assay plate (each sample was in triplicate). Green fluorescent protein (GFP) fluorescence (induction at 485 nm, emission at 535 nm), and OD of the bacterial culture ($A_{570 \text{ nm}}$) were measured for 1 s in the Victor spectrophotometer (Perkin Elmer). Each experiment was performed three times.

In the second method, following addition of the induction agent, at each time point, a sample of 5×10^9 cells was harvested. The sample was centrifuged for 5 min at 2000 g, and the pellet was frozen at -80°C . After thawing, DNase and RNase solution was added according to the standard procedure (Sambrook *et al.*, 1989), and the mixture was incubated at room temperature for 15 min. Proteins were separated during 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis under denaturing conditions, according to Sambrook *et al.* (1989). The Western-blotting procedure was performed with mouse anti-GFP antibodies (Sigma) and biotin-conjugated anti-mouse IgG antibodies (Sigma). The blots were developed using extravidin-conjugated alkaline phosphatase and the Alkaline Phosphatase Blue Membrane Substrate Solution (Sigma). The results were quantified densitometrically, using QUANTITY ONE software (Bio-Rad).

Results and discussion

As reported previously for bacteriophage λ and other lambdoid phages, mitomycin C (an agent that interacts with DNA, interfering with genome replication and inducing the SOS response) provokes lambdoid prophage induction (for reviews, see Ptashne, 2004; Węgrzyn & Węgrzyn, 2005). Therefore, this compound was used as a positive control for the prophage inducer. In fact, antibiotics that induce the bacterial SOS response were demonstrated to enhance prophage induction (Matsushiro *et al.*, 1999) and expression of the *stx* genes (Kimmitt *et al.*, 2000). Hence, in our experiments, norfloxacin was used as an example of such antibiotics.

As expected, both mitomycin C and norfloxacin (at final concentrations of 1 and $0.2 \mu\text{g mL}^{-1}$, respectively) caused the appearance of phages in cultures of *E. coli* O157:H7 lysogenic with ST2-8624 ($\Delta\text{stx}2::\text{cat gfp}$) (Fig. 1a and b), which can be interpreted as evidence for prophage induction and lytic development of the phage. This occurred in both rich (LB) and minimal (MMGlu) media.

Treatment of the tested bacterial culture with 200 mM NaCl did not cause any effects on prophage induction

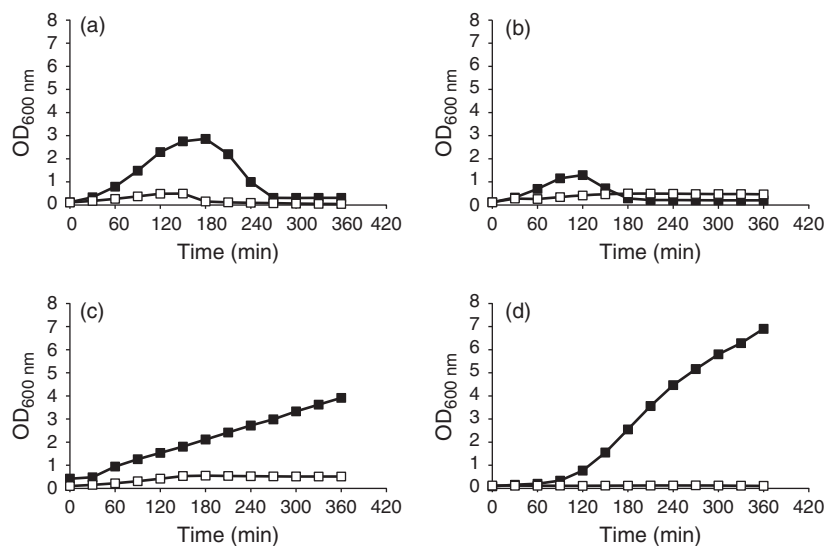


Fig. 2. Monitoring of the culture density (by measurement of $A_{600\text{ nm}}$, $OD_{600\text{ nm}}$) of the *Escherichia coli* O157:H7 host, lysogenic with the ST2-8624 phage, growing in LB (■) or MMGlu (□) media. The following inducers were added to bacterial cultures, to indicated concentrations, at time = 0: mitomycin C to 1 μg mL⁻¹ (a), norfloxacin to 0.2 μg mL⁻¹ (b), NaCl to 200 mM (c) or H₂O₂ to 3 mM (d). Experiments were repeated three times with a high reproducibility (SD < 20%), and representative results are shown.

(Fig. 1c). Thus, it appears that an increased salt concentration, which provokes induction of λ^{imm434} prophage (Shkilyj & Koudelka, 2007), is not an inducer of the ST2-8624 prophage. However, we found that addition of H₂O₂ at a final concentration of 3 mM resulted in the appearance of infective ST2-8624 virions in amounts comparable to those observed after induction with norfloxacin (Fig. 1b and d). Therefore, it appears that H₂O₂ is an inducer of the ST2-8624 prophage.

When the $A_{600\text{ nm}}$ of the bacterial culture was monitored after prophage induction with mitomycin C and norfloxacin, a decrease in the culture density was observed (Fig. 2a and b), which was most probably caused by lysis of bacterial cells and liberation of progeny virions, analogously to the phenomenon occurring after induction and lytic development of bacteriophage λ (Osterhout *et al.*, 2007 and references therein). Perhaps surprisingly, no decrease in the culture density was evident after prophage induction with 3 mM H₂O₂ (Fig. 2d).

The experiments depicted in Figs 1 and 2 were performed with concentrations of particular agents found (in our preliminary experiments; data not shown) to be optimal for efficient prophage induction. Antibiotic and H₂O₂ concentrations lower than those shown in Figs 1 and 2 caused less efficient prophage induction (results not shown). Moreover, higher antibiotic concentrations did not result in an increase in the phage burst size and did not cause a more pronounced decrease in the bacterial culture density, and an H₂O₂ concentration of 0.5 mM caused a significant inhibition of the growth of bacterial cultures and inefficient (over 100-fold less effective) prophage induction; this was true for experiments conducted in both rich (LB) and minimal (MMGlu) media (results not shown). Therefore, we conclude that the differences between results obtained with

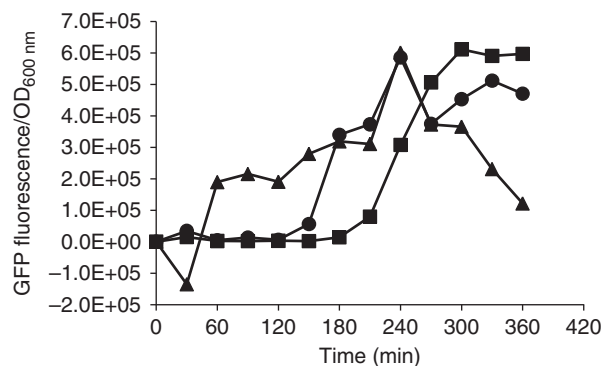


Fig. 3. GFP fluorescence in cultures of *Escherichia coli* O157:H7, lysogenic with ST2-8624 ($\Delta stx2::cat gfp$), after prophage induction in LB medium. The following inducers were added to the bacterial culture to indicated concentrations at time = 0: mitomycin C to 1 μg mL⁻¹ (■), norfloxacin to 0.2 μg mL⁻¹ (●), or H₂O₂ to 3 mM (▲). The results are presented as relative fluorescence (in arbitrary units) per OD of bacterial culture.

antibiotics and H₂O₂ (Fig. 2) were not caused by putative, various thresholds of the concentrations of the inducing agents in different growth media, but rather reflected differences in the nature and mode of action of these agents (see below for a more detailed discussion).

For maximal pathogenicity of STEC, expression of the *stx* genes is crucial. Therefore, we have monitored expression of the reporter gene (*gfp*) in bacteria bearing the modified Shiga toxin-converting prophage ST2-8624 ($\Delta stx2::cat gfp$), after induction with various agents. About a 10⁵-fold increase in fluorescence was detected following prophage induction with mitomycin C, norfloxacin or H₂O₂ (Fig. 3). Interestingly, contrary to mitomycin C and norfloxacin, induction by H₂O₂ caused a relatively rapid appearance of

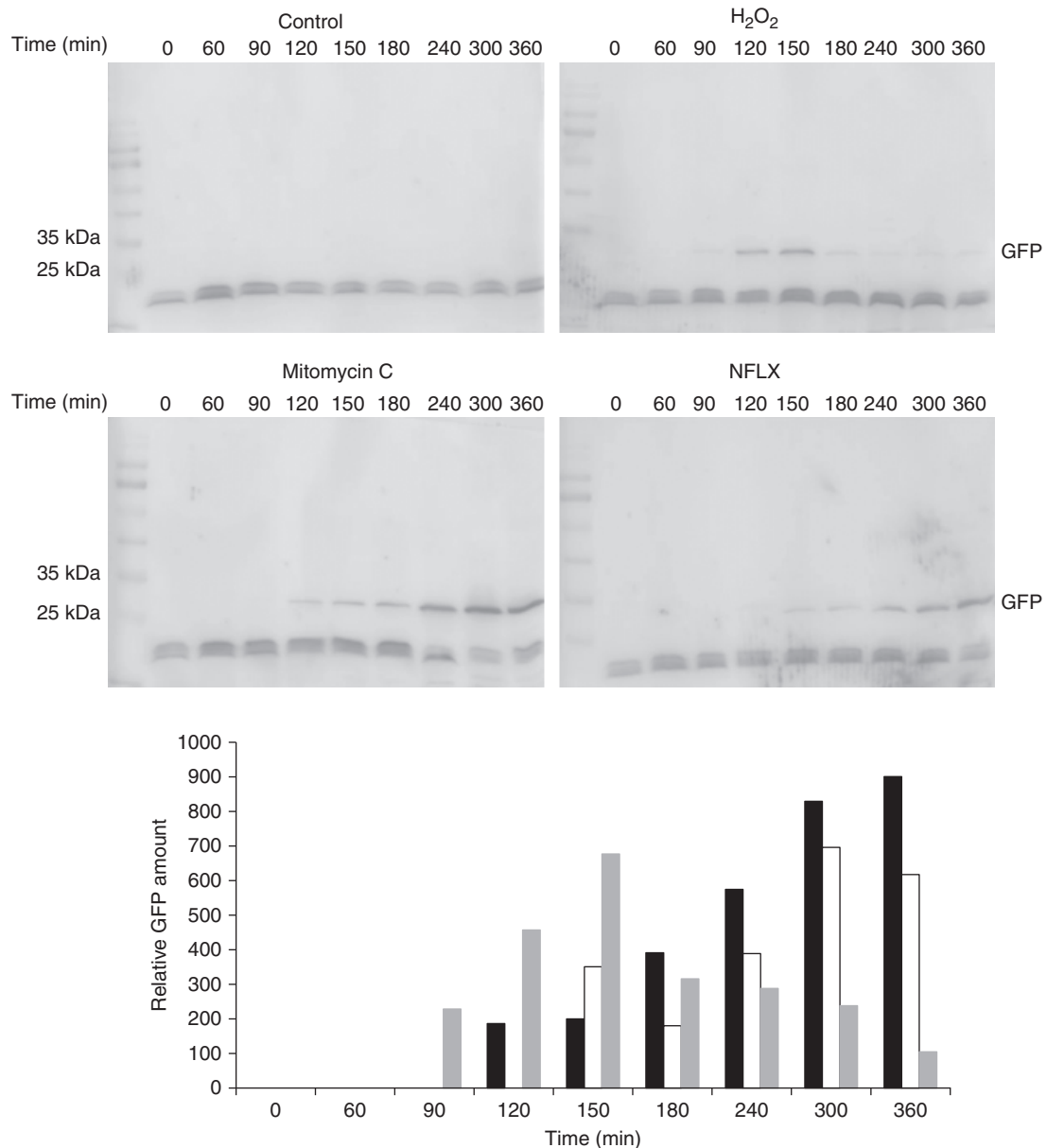


Fig. 4. Relative GFP amounts in cultures of *Escherichia coli* O157:H7, lysogenic with ST2-8624 (Δ stx2::cat *gfp*), after prophage induction in LB medium, as estimated by Western blotting. The upper panel represents blots obtained in experiments with different induction agents and without induction (control); positions of GFP and two size markers are indicated. The lower panel shows results of a densitometric analysis (values are presented in arbitrary units) of the blots. The following inducers were added to bacterial cultures, to indicated concentrations, at time=0: mitomycin C to $1 \mu\text{g mL}^{-1}$ (black columns), norfloxacin to $0.2 \mu\text{g mL}^{-1}$ (white columns) or H_2O_2 to 3 mM (gray columns).

the signal, which then became gradually weaker. These results were confirmed when another, more specific, method was used for monitoring expression of the *gfp* gene. Western-blotting analysis corroborates the results of the fluorescence-based experiments by showing that after prophage induction with mitomycin C or norfloxacin, the GFP protein gradually increased in number (Fig. 4), while induction with H_2O_2 caused a relatively early peak of GFP, followed by a decrease in the amount of this protein (Fig. 4).

The transient expression of the reporter *gfp* gene in the cultures of *E. coli* O157:H7 lysogenic with phage ST2-8624 (Δ stx2::cat *gfp*) after prophage induction with H_2O_2 may be compatible with the lack of an observable decrease in the density of bacterial culture (compare Figs 2d, 3 and 4). Namely, one might assume that conditions occurring in the host cells treated with H_2O_2 allow prophage induction and expression of the *stx2* (or *gfp*) gene, but partially prevent the formation of mature progeny virions and lysis of the host

cell. On the other hand, both the phage burst size and the kinetics of the phage lytic development after H₂O₂-mediated induction are similar to those observed in cultures treated with mitomycin C or norfloxacin (Fig. 1). Therefore, it appears that in H₂O₂-treated cells, the phage ST2-8624 (Δ stx2::cat gfp) completes its development and forms infective progeny virions. One possible explanation of this 'paradox' could be a suggestion that prophage induction by H₂O₂ occurs only in a fraction of lysogenic cells. If such a fraction were high enough to produce a significant number of progeny phages, and small enough to ensure an increase in OD of the whole bacterial culture, we would observe results exactly as depicted in Figs 1d and 2d.

The physiological meaning of the results presented in this report is that induction of Shiga toxin-converting prophage in an STEC host can be mediated by oxidative stress (induced in this work by H₂O₂). Contrary to other known inducers of lambdoid prophages, such as UV irradiation, oxidative stress conditions may occur in the intestine of an infected human. In fact, bacteria present in the human intestine can induce the generation of reactive oxygen species (Kumar *et al.*, 2007). If this is the case during colonization of the human intestine by STEC, the oxidative stress conditions might result in efficient Shiga toxin-converting prophage induction (at least in a fraction of bacterial cells), and subsequent production and liberation of significant amounts of the toxin. Moreover, neutrophils can produce H₂O₂, and it was suggested that this can stimulate the production of Shiga toxin 2 in one clinical isolate of STEC (Wagner *et al.*, 2001a). Very recent studies by Lainhart *et al.* (2009) suggested that production of H₂O₂ by eukaryotic cells may be a signal recognized by bacteria, leading to prophage induction and production of Shiga toxins. Our results corroborate this suggestion and may further suggest that H₂O₂ is an actual inducer of the prophage excision, subsequent phage lytic development and efficient expression of *stx* genes.

Finally, results presented in this report corroborate previous observations that both induction of lambdoid prophages and lytic development of the phages depend on the physiological state of their hosts, and are impaired in slowly growing cells (Gabig *et al.*, 1998; Czyż *et al.*, 2001; Łoś *et al.*, 2007). This also appears to be the case for phage ST2-8624, as it developed significantly slower in the host growing in a minimal medium relative to bacteria cultured in LB (Fig. 1d). This observation might be important in the light of results of recent studies, indicating that infection with STEC may be asymptomatic relatively often, perhaps due to a lack of efficient prophage induction and lytic phage development (Hong *et al.*, 2009). In fact, it was suggested that the efficiency of processes occurring after prophage induction, such as phage DNA replication, might also be important for the regulation of expression of *stx* genes, and thus, for the

expression of STEC pathogenicity (Nejman *et al.*, 2009). Therefore, one might predict that a better understanding of mechanisms of lambdoid prophages' induction and factors causing this phenomenon, as well as regulation of phages' development under conditions similar to those occurring in the human intestine (note that there are relatively few reports published to date on these subjects; see Acheson *et al.*, 1998; Zhang *et al.*, 2000; Wagner *et al.*, 2001a, 2002; Gamage *et al.*, 2003, 2006; Tóth *et al.*, 2003; Livny & Friedman, 2004; Aertsen *et al.*, 2005; Ochoa *et al.*, 2007; Shkilnyj & Koudelka, 2007; Łoś *et al.*, 2008b; Murphy *et al.*, 2008), could be a basis to develop novel diagnostic and/or therapeutic procedures.

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