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

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**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 (H₂O₂)]. 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 H₂O₂ 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 cl repressor and subsequent activation of lytic promoters (for reviews, see Ptashne, 2004; Węgrzyn & Węgrzyn, 2005). On the other hand, Shklinjy & Koudelka (2007) demonstrated that an increased salt concentration (which does not induce the SOS response) caused induction of a λimm434 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 (H2O2), produced by human neutrophils, may increase the production of Shiga toxin 2 (Wagner et al., 2004; Prerez et al., 2004; Prere & Fayet, 2005; Łos 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 (Astx2::cat gfp) prophage (Łos 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 μg mL−1 mitomycin C, 0.2 μg mL−1 norfloxacin, 200 mM NaCl or 3 mM H2O2.

Monitoring of prophage induction and phage development

Bacteria were cultured in LB or MMGlu medium at 37 °C to an A 600 nm of 0.1, then the culture was divided into two parts. An induction agent (mitomycin C, norfloxacin, NaCl or H2O2) was added to the appropriate concentration (1 μg mL−1, 0.2 μg 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 °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 °C) top nutrient agar (0.7%), and 1 mL of the indicator E. coli strain culture was added. Following supplementation of the mixture with MgSO4 and CaCl2 (to a final concentration of 10 mM each), it was poured on a plate with LB agar (1.5%) supplemented with 2.5 μg mL−1 chloramphenicol, according to a previously published procedure (Łos et al., 2008a). Plates were incubated at 37 °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 (Astx2::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 (Astx2::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
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 (A570 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; Wegrzyń & Wegrzyń, 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 μg mL⁻¹, respectively) caused the appearance of phages in cultures of *Escherichia coli* O157:H7 lysogenic with ST2-8624 (∆strx2::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. 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 μg mL⁻¹ (a), norfloxacin to 0.2 μg mL⁻¹ (b), NaCl to 200 mM (c) or H₂O₂ 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.
Thus, it appears that an increased salt concentration, which provokes induction of \( \lambda_{\text{imm}434} \) prophage (Shkilnyj & Koudelka, 2007), is not an inducer of the ST2-8624 prophage. However, we found that addition of \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \lambda \) (Osterhout et al., 2007 and references therein). Perhaps surprisingly, no decrease in the culture density was evident after prophage induction with 3 mM \( \text{H}_2\text{O}_2 \) (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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 antibiotics and \( \text{H}_2\text{O}_2 \) (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 \( \text{stx} \) genes is crucial. Therefore, we have monitored expression of the reporter gene (\( \text{gfp} \)) in bacteria bearing the modified Shiga toxin-converting prophage ST2-8624 (\( \Delta\text{stx}2::\text{cat gfp} \)), after induction with various agents. About a 10^5-fold increase in fluorescence was detected following prophage induction with mitomycin C, norfloxacin or \( \text{H}_2\text{O}_2 \) (Fig. 3). Interestingly, contrary to mitomycin C and norfloxacin, induction by \( \text{H}_2\text{O}_2 \) caused a relatively rapid appearance of
the signal, which then became gradually weaker. These results were confirmed when another, more specific, method was used for monitoring expression of the \textit{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$_2$O$_2$ 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 \textit{gfp} gene in the cultures of \textit{E. coli} O157:H7 lysogenic with phage ST2-8624 (\textit{Δstx2::cat gfp}) after prophage induction with H$_2$O$_2$ 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$_2$O$_2$ allow prophage induction and expression of the \textit{stx2} (or \textit{gfp}) gene, but partially prevent the formation of mature progeny virions and lysis of the host.

\textbf{Fig. 4.} Relative GFP amounts in cultures of \textit{Escherichia coli} O157:H7, lysogenic with ST2-8624 (\textit{Δstx2::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\,$\mu$g mL$^{-1}$ (black columns), norfloxacin to 0.2\,$\mu$g mL$^{-1}$ (white columns) or H$_2$O$_2$ to 3 mM (gray columns).
cell. On the other hand, both the phage burst size and the kinetics of the phage lytic development after \( \text{H}_2\text{O}_2 \)-mediated induction are similar to those observed in cultures treated with mitomycin C or norfloxacain (Fig. 1). Therefore, it appears that in \( \text{H}_2\text{O}_2 \)-treated cells, the phage ST2-8624 (\( \Delta \text{stx} \_2::\text{cat gfp} \)) completes its development and forms infective progeny virions. One possible explanation of this ‘paradox’ could be a suggestion that phage induction by \( \text{H}_2\text{O}_2 \) 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-convertting prophage in an STEC host can be mediated by oxidative stress (induced in this work by \( \text{H}_2\text{O}_2 \)). 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-convertting 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 \( \text{H}_2\text{O}_2 \), 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) is an actual inducer of the prophage excision, subsequent phage lytic development and efficient expression of \( \text{stx} \) 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; Loś 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 \( \text{stx} \) 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; Loś et al., 2008b; Murphy et al., 2008), could be a basis to develop novel diagnostic and/or therapeutic procedures.

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