The Control of Replication of Shiga Toxin-converting Phages in the Light of Potential New Detection Methods and Therapies of Enterohemorrhagic *Escherichia coli* Infections

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Escherichia coli is a species of bacteria that commonly occurs in the environment and can be found in the human lower intestine. Most *E. coli* bacteria are harmless, but some strains can cause serious food poisoning and urine disorders in humans. Shiga toxin-producing *E. coli* (STEC) is a group of pathogenic strains, which are especially harmful in the case of its subset called enterohemorrhagic *E. coli* (EHEC). The endemic *E. coli* O157:H7 strain belongs to this group. Bacteria are particularly effective in colonization of intestinal epithelium, easily adapt to changes of environmental conditions, and are resistant to low temperature and pH.

Recently, a serious outbreak of epidemy, caused by STEC bacteria was noticed in Northern Germany and 15 other European countries in May 2011. In regards to Word Heath Organization (WHO) reports, the outbreak resulted in 4,075 cases of infection and 50 cases of death. The significance of STEC bacteria as a public health problem was recognized in 1982, following an outbreak in the United States of America. The major reservoirs of these bacteria are gastrointestinal tracts of animals, mostly cattle, goats, and sheep. STEC infection has been associated with eating undercooked, contaminated ground beef (hamburgers, steaks), drinking contaminated water, unpasteurized milk and eating contaminated, raw vegetables.

The main virulence factors of STEC are Shiga toxins, encoded by genes (*stx1* and *stx2*) located on genomes of bacteriophages, which occur in bacteria as prophages. Shiga toxin-converting phages belong to the lambdoid family of phages, of which bacteriophage λ is the best investigated member. All lambdoid phages indicate high similarities in the morphology, lifecycle and genome organization. Depending on intracellular conditions, bacteriophages may develop according to one of two pathways, either lysogenic or lytic. Lysogeny is revealed by integration of the bacteriophage nucleic acid into the host genome. The integrated genetic material, called a prophage, is replicated together with bacterial genome and is transmitted to daughter cells at each subsequent cell division. As a results of the prophage induction caused by different factors, the excision of phage genome occurs. Consequently, the viral DNA exists as a separate molecule within the bacterial cell, and replicates separately from the host bacterial DNA as an extrachromosomal element. The

phage genetic material is replicated many times and the genes for head, tail and lysis proteins are expressed. It leads to assembly of multiple new phage particles within the cell and subsequent cell lysis, releasing assembled virions into the environment. Effective production of Shiga toxins occurs only upon prophage induction and its further lytic development.

Bacteriophage λ DNA replication is a complex and multi-stages process. It involves the bacteriophage O and P proteins as well as bacterial proteins, including heat-shock proteins and all components of the bacterial replisome. The initiator protein λO first binds to four tandemly arranged, 19-nucleotides long repeats (iterons), localized within the replication *origin* region called *oriλ*. Subsequently, phage protein λP delivers bacterial DnaB helicase to the forming replication complex. Restoration of the DnaB protein activity and remodeling of this nucleoprotein complex is performed in the next step by bacterial molecular chaperones DnaK, DnaJ and GrpE. In effect, the DNA duplex is locally unwound and replication fork is created. The unwinding of DNA by the DnaB helicase allows for incorporation of other bacterial proteins, like: gyrase, SSB protein, primase and DNA polymerase III holoenzyme, into the replication complex. The activity of the p_R promoter is required to initiate replication from *oriλ*, as transcription from this promoter leads to transcriptional activation of the *origin*, a process required to start synthesis of the new DNA strand, even in the presence of all proteins. The transcriptional activation of *oriλ* during initiation of DNA replication is positively regulated by the host DnaA, SeqA and DksA proteins, whereas the main agent of the stringent response in starved bacteria - guanosine tetraphosphate (ppGpp) inhibits this process.

After lysis of bacterial cell, Shiga toxins are released in the intestine where they attack cells of epithelial tissue. The toxins require highly specific glycolipids (Gb3) on the cells' surface in order to attach and enter the cell. Within target cell, toxins bind to the 60S subunit of the ribosome and inhibit protein synthesis, leading to the death of the cell. It leads consequently to a breakdown of the intestinal epithelium and to hemorrhage. In effect, bloody diarrhea is the first symptom of the infection. The progressive production of toxins causes also other symptoms like abdominal pain and vomiting. Symptoms of food poisoning maintain around 10 days, however 15-20% of patients infected with STEC progress to severe complications: hemolytic ureamic syndrome, thrombocytopenia or hemorrhagic colitis.

Bacteriophage λ, a paradigm for the family of lambdoid phages, to which Shiga toxin-bearing phages belong, seems to serve as a suitable model in studies on DNA replication process. Although the morphology of virions, organization of genome and nucleotide sequences are similar among lambdoid phages, differences in regulations of basic processes

can occur. For that reason, previous studies on bacteriophage λ may have implications for understanding the replication mechanism occurring in case of Shiga toxin-converting bacteriophages, however, they cannot be directly transferred to these phages without investigation.

Apart from other important mechanisms of regulation of Shiga toxin-converting phage development (e.g. prophage induction), the mechanism of their replication appear to be essential. The replication efficiency of *stx* gene-bearing DNA molecules significantly influences the expression of toxins' genes by affecting their copy numbers in cells, and indirectly affects also the pathogenicity of bacteria and development of infection.

The aim of this work was to understand the regulatory mechanism of replication of Shiga toxin-converting bacteriophages in comparison to the mechanism observed in phage λ, in response to different factors influencing the efficiency of this process and in the light of potentially practical applications.

The presented work was executed on the basis of both phages and phage-derived plasmids as research models. At the beginning of this work, I asked whether replicons derived from phages bearing the Shiga toxin genes can serve as suitable tools for studying the replication process. Plasmids derived from such phages (pRstx2cmr, pR8624cmr, p933Wcmr, p32cmr, p27cmr and p22cmr) were constructed similarly to wild-type λ plasmid (pCB104). Such plasmids bear the replication regions of the phage genomes, encompassing all genes and regulatory sequences required for the initiation of DNA replication from the unique site called *origin* or *ori*. Efficient transformation of bacteria by such plasmids and possibility to passage obtained colonies many times without a loss of plasmid DNA, confirmed that lambdoid plasmids may be convenient models to study the DNA replication process [1].

Subsequently, it was found that similarly to λ plasmid, all other analyzed lambdoid plasmids require functions of molecular chaperones DnaK, DnaJ and GrpE during their replication in bacteria. Nevertheless, contrary to the phenotype observed in λ plasmid, four tested replicons were able to replicate in the *E. coli dnaA46* mutant [1]. It appears that in comparison with DnaA-mediateted stimulation of transcription from the p_R promoter, required during DNA replication of λ, the replication process of these four plasmids is DnaA-independent. It seems that the replication mechanism of replicons of Shiga toxin-converting bacteriophages is similar to that of bacteriophage λ, however there are important differences which influence regulation of this process. Specific nucleotide changes causing amino acid substitutions relative to wild-type λO and λP proteins, appear to be responsible for these differences [1]. It is proposed that nucleotide differences located in the C-terminal part of the

O protein and in the N-terminal part of the P protein (which are known to interact each other) influence the O-P interaction and consequently also formation and rearrangement of the replication complex. In effect, the DnaA-stimulated transcriptional activation of the *origin* is probably not required.

Another discrepancy observed between λ phage and other analyzed lambdoid baceriophages refers to the DksA protein, which (besides ppGpp) is a second main agent of the bacterial response to starvation conditions. In comparison with the p_R promoter of λ phage, the weaker DksA-dependent stimulation of transcription from homologous promoters of Shiga toxin-converting bacteriophages was observed [2]. The previously published (by others) data indicate that the mechanism of DksA-mediated stimulation of transcription from p_R promoter of λ phage involves the DksA protein's ability to facilitate binding of RNA polymerase (RNAP) to that promoter. Perhaps the similar mechanism occurs in Shiga toxin-converting bacteriophages, however, less efficient (relative to λ) DksA-mediated stimulation contributes to the decrease of RNAP association to the promoter sequence, and in effect to less efficient transcription process. In accordance with this proposed mechanism, I found that promoters of lambdoid phages ST2-8624 and 933WΔtox homologous to λ p_R appeared to be weaker than that of λ phage [2]. Most probably, under normal conditions the efficiency of transcription from these promoters is sufficient, however, in amino acid-starved bacteria the efficiency of transcription process responsible for transcriptional activation of the *origin* is not enough and results in the inhibition of DNA replication initiation of Shiga toxin-converting bacteriophages. The proposed mechanism may explain results obtained in this work, concerning the inhibition of Shiga toxin phage-derived plasmids replication, during both stringent and relaxed responses (*relA*$^+$ and *relA*$^-$ strains, respectively), independently of ppGpp occurrence [2]. These results are compatible with findings that in amino acid starvation conditions the lytic development of phage 933WΔtox was inhibited in both the wild-type bacteria (*relA*$^+$) and even in the absence of ppGpp (*relA*$^-$ hosts) [3].

Additional studies performed in the frame of this work indicated that sodium citrate (the compound of oral rehydration solutions, like Orsalit) inhibits Shiga toxin-converting phage development. Interestingly, the addition of glucose (another compound of these solutions) reverses the effect of citrate [3].

Treatment of infections caused by STEC bacteria is difficult because many medicaments (both antibiotics and chemotherapeutics) are prophage inducers which increase toxin gene expression and enhance severity of the disease symptoms. Thus, it was recommended that such medicaments should be avoided in treating patients with infections by

these bacteria. The symptomatic treatment of diarrhea involves two alternative and opposite recommendations. One approach favors either reducing oral intake or even fasting during first stages of illness, whereas another approach involves continued feeding. Results presented in this work support the fasting strategy in the case of STEC infections and may have both basic and potentially practical values. It appears that the DksA protein rather than ppGpp plays a fundamental role in the inhibition of the replication process of Shiga toxin-converting phages under starvation conditions. Further studies are required to completely understand this mechanism, however the impact of starvation conditions seems to be significant and may have an influence on development of these bacteriophages and consequently also on the progress of STEC infection. Perhaps, the modification of oral rehydration solution compounds and/or inclusion of the starvation stage during treatment would be favourable for the management of STEC infections and might decrease the severity of subsequent complications. Results presented in this work provided principles of understanding of the large scientific problem, and although further analyses are required, they potentially may facilitate development of novel procedures for treatment of STEC-infected patients.

References

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