„Links between DNA replication and other cellular processes in *Escherichia coli* and their role in bacterial cell cycle coordination"

Joanna Morcinek-Orłowska, M.Sc.

The bacterial cell cycle is a set of consecutive events that leads to the formation of two daughter cells. It comprises cell growth, DNA replication, and cell division. DNA replication is central to the cell cycle, as it ensures the fidelity and integrity of the genetic material passed to progeny cells. This process relies on the coordinated activity of highly specialized protein machinery known as the replisome.

During replication initiation, the initiator protein DnaA unwinds the DNA duplex within the *oriC* region, allowing the helicase, primase, and the multisubunit DNA polymerase III to assemble. The activity of DnaA is tightly regulated throughout the cell cycle by regulatory proteins – a positive regulator, DiaA, which promotes initiation, and negative regulators, Hda and SeqA, which prevent premature reinitiation. During elongation, the catalytic subunit of DNA polymerase III extends the DNA strand in the 5'-3' direction, using deoxyribonucleotides supplied by ribonucleotide reductase. Polymerase processivity is ensured by the sliding clamp (β subunit), which is loaded onto DNA by the clamp-loader complex. Replication proceeds bidirectionally on the chromosome until it reaches the *ter* region, where the process is completed.

Key events in the bacterial cell cycle – DNA replication and cell division – need to be not only strictly controlled but also tightly coordinated with cell growth, whose rate can change rapidly under the diverse environmental conditions in microorganisms' habitats. The mechanisms underlying this coordination still remain not fully understood. Increasing evidence, however, suggests that growth-dependent regulation of replication is coupled to various metabolic processes.

The goal of my doctoral thesis is to characterize the links between DNA replication and other cellular processes in the model Gram-negative bacterium *Escherichia coli*. The central research hypothesis posits that replication proteins can directly interact with metabolic proteins and/or small-molecule metabolites. These interactions – occurring in the cell at specific stages of the cell cycle and under defined growth conditions – may modulate the

activity of replication proteins and constitute a mechanism coordinating DNA replication with bacterial cell growth.

The study focused on eight key *E. coli* proteins involved in the progression and regulation of DNA replication: DnaA (the initiator protein), DiaA, Hda, SeqA (regulatory proteins), DnaB (helicase), DnaG (primase), HolD (the ψ subunit of DNA polymerase III, part of the clamp-loader complex), and NrdB (the β subunit of ribonucleotide reductase). I mapped the interaction networks formed by these proteins under conditions supporting fast and slow bacterial growth. Isolation of protein complexes formed directly in the bacterial cell was enabled by chromosomal fusion of the respective coding sequences with SPA-tag sequence. This tag allows affinity purification of the bait protein together with its interacting partners, followed by liquid chromatography-tandem mass spectrometry to identify complex components. The experimental design included appropriate control samples, based on which we developed tailored qualitative and quantitative data processing strategies to filter out non-specific interactions.

Interaction profiles formed by replication proteins exhibit strong dependence on bacterial growth conditions used. Moreover, each bait protein forms a distinct interaction network, and the set of proteins interacting with more than one bait is relatively small. Proteins associating with the replication machinery represent several functional categories, including ribosome biogenesis, RNA processing and degradation, and nucleotide metabolism. Particularly notable is the identification of proteins involved in the synthesis of cell envelope components – membrane phospholipids, peptidoglycan, and lipopolysaccharide (LPS). Different representatives of this functional group appear consistently across nearly all analyzed interactomes.

From among the identified interaction partners, I selected a few proteins representing distinct functional classes for further analysis. I determined the effect of their gene deletion on DNA content in fast- and slow-growing cells using flow cytometry after replication run-out. I showed that depletion of RfaD, an enzyme involved in one of the steps of LPS core biosynthesis, leads to asynchronous replication initiation and disturbances in replication-timing control within the cell cycle, particularly evident under slow-growth conditions. Deletion of *rlmE*, encoding an rRNA methyltransferase delays replication initiation

and disrupts its synchrony, but this effect manifests under conditions of rapid cell growth. My results indicate that the potential coordination mechanisms involving proteins interacting with the replication machinery are strongly dependent on growth conditions.

As part of a scientific collaboration, I also participated in studies aimed at elucidating the function of accessory lipoproteins of the Bam complex, which is responsible for the folding of outer membrane proteins. Given the observed genetic interactions of the *bamB* gene with subunits of DNA polymerase III and replication regulatory proteins, I investigated the effect of its deletion on the regulation of DNA replication during the cell cycle using flow cytometry after replication run-out. I demonstrated that the *bamB* gene deletion can lead to earlier and partially asynchronous replication in the cell cycle. These results further support the functional interplay between cell envelope homeostasis and replication regulation in *E. coli*.

The structure of DiaA, a positive regulator of replication initiation, contains phosphosugar-binding domain, characteristic of sugar isomerases, which prompted us to ask not only about its role, but also about possible interactions between replication proteins and metabolites. We showed that DiaA binds sedoheptulose-7-phosphate (S7P) *in vitro*, and that this interaction impedes the stimulatory effect of DiaA on DnaA oligomerization at *oriC*. I also demonstrated that lowering intracellular S7P levels deregulates replication initiation timing in the cell cycle, resulting in earlier initiation. S7P is an intermediate of the pentose phosphate pathway, and a key metabolite for the bacterial lipopolysaccharide biosynthesis. The identified interaction points to interconnection between nucleotide biosynthesis, cell envelope biogenesis and replication regulation, which may coordinate the bacterial cell cycle in response to metabolite concentration and metabolic enzyme activities.

The results of this dissertation provide a foundation for future studies aimed at determining the functional significance of the identified interactions. It would ultimately contribute to a deeper understanding of the mechanisms coupling DNA replication with other cellular processes. Coordination of the bacterial cell cycle is crucial not only for microbial survival in dynamically changing environments, but also for the virulence of pathogenic bacteria. Consequently, discovered mechanisms may constitute novel molecular targets

for antimicrobial therapies and, conversely, may support bacterial growth where desirable, such as in bioreactors.