Proteins are not particularly stable molecules. In stress conditions a substantial part of cellular proteins partially unfolds and forms aggregates. This is very dangerous to cells. Chaperone proteins from three families (small heat shock proteins (sHsp), Hsp70 and Hsp100) are capable of protein refolding from aggregates. This process is essential to survive stress conditions for bacteria, fungi and plants. Small Hsps act first in such conditions. These chaperones are responsible for driving protein aggregation toward refolding-prone assemblies consisting of protein substrates and sHsps. The assemblies are processed by Hsp70 and Hsp100 chaperones, which leads to extraction and refolding of single polypeptides from aggregates.

In Escherichia coli, the model organism in molecular biology, there are two small heat shock proteins, IbpA and IbpB. These were created in evolution as a result of gene duplication event at the base of *Enterobacterales*. Each of them possesses strikingly different biochemical and functional properties but they cooperate in interaction with the substrates. The molecular bases of the functional differences between IbpA and IbpB are unknown. It is also not known what are the benefits of possessing two protein sHsps system in comparison to single sHsp.

In the grant we will define which elements in amino acid sequences of IbpA and IbpB are responsible for particular properties of these proteins. Our strategy is to predict and reconstitute several ancestral IbpA and IbpB proteins corresponding to same stages of bacterial evolution between last common ancestor (AncAB) of IbpA and IbpB and the present day *E.coli,* IbpA and IbpB. After reconstruction these proteins will be synthetized and purified for *in vitro, in vivo* and *in silico* functional analysis.

As a proof of concept we reconstituted AncAB protein, the last common ancestor of IbpA and IbpB, the most distantly related sHsp to present day IbpA and IbpB from *E. coli*, which is planned to be investigated. The AncAB ancestral protein was purified and its functional properties were analysed. All performed experiments show that the reconstituted single AncAB is functionally equivalent to present day IbpA and IbpB *E. coli* two protein system. Reconstituted AncAB differs in 34 aa positions from IbpA and in 74 aa positions from IbpB. All these results show that we are capable of reconstructing functional ancestral sHsp even though the number of introduced amino acid substitutions was quite high.

We believe that this approach will let us understand the principles of sHsps functioning both as a single small heat protein and two small heat shock protein system. We will understand which amino acid define that sHsp becomes IbpA-type or IbpB-type sHsp. Understanding of functioning of sHsps in formation of refolding-prone assemblies is crucial for understanding the principles of disaggregation and refolding of proteins from aggregates, the process which is vital to survive stress conditions.