Evolution of small Heat Shock Protein function in *Enterobacterales*

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One of the basic mechanisms of protein aggregation control in a cell, crucial for maintaining proteostasis, is activity of molecular chaperones which facilitate refolding of misfolded proteins and disaggregation of protein aggregates.

There are three families of molecular chaperones that play a role in protein disaggregation in bacteria – Hsp70, Hsp100 and small heat shock proteins (sHsp). Hsp70 and Hsp100 proteins cooperate in actively disaggregating aggregated proteins using energy from ATP hydrolysis, while sHsps act at the stage of protein aggregation. While present during aggregation, they are able to bind misfolded proteins and trap them in nearly – native conformation in so called sHsp – substrate assemblies, which prevents uncontrolled protein aggregation and enhances effectiveness of protein disaggregation by Hsp70 and Hsp100.

In majority of *Gammaproteobacteria* there exists a single sHsp – IbpA. In *Enterobacterales* clade (excluding *Erwinacea* and *Pectobacteriacea*), however, there exist two sHsps – IbpA and IbpB. Previous phylogenetic analysis suggests that they are paralogues originating from single gene duplication in the last common ancestor of *Enterobacterales*.

IbpA has very high affinity to substrates which enables it to form assemblies efficiently, but at the same time makes it difficult to be outcompeted from assemblies by Hsp70, which can lead to inhibition of disaggregation at low Hsp70 concentrations. IbpB, on the other hand, has low substrate affinity and is unable to form assemblies on its own, but is able to facilitate IbpA dissociation from assemblies and reduce the demand for Hsp70 in protein disaggregation. The exact mechanism of cooperation between IbpA and IbpB is not yet well understood. In our research we analysed evolution of *E.*coli IbpA and IbpB since duplication event to elucidate molecular basis of differences in their function and cooperation between them.

Using maximum likelihood – based algorithm we reconstructed aminoacid sequences of ancestral proteins corresponding to subsequent nodes in *Enterobacterales* sHsp between (and including) last common ancestor of *Enterobacterales* sHsps and modern day *E.coli* IbpA and IbpB: AncAB, AncA1, AncA2, AncA3, AncA4, AncA5, AncB1, AncB2, AncB4 and AncB5. By analysing the ratio of nonsynonymous to synonymous substitutions we detected probable positive selection acting on the phylogenetic tree branch between proteins AncB2 and AncB4. In order to analyse activities of reconstructed proteins *in vitro*, we overproduced them in *Escherichia coli* and purified using ion-exchange chromatography. All reconstructed proteins were able to reversibly deoligomerise in high temperature *in vitro*. All reconstructed ancestral post-duplication IbpA proteins, as well as the last common ancestor of *Enterobacterales* sHsp were able to efficiently form assemblies with model

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substrate *in vitro*, while reconstructed ancestral IbpBs were not, which suggests that ability to efficiently form substrate-sHsp assemblies was lost in IbpB lineage shortly after duplication event.