Molecular basis of small heat shock protein evolution in *Erwiniaceae*

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Small heat shock proteins (sHsp) bind aggregating protein substrates and sequester them in nearly – native conformation in sHsp - substrate assemblies, preventing uncontrolled protein aggregation. Nearly – native conformation of the sequestered substrates combined with relatively small size of sHsp – substrate assemblies facilitates later disaggregation and refolding of those substrates by Hsp70 system and Hsp100 disaggregases. Initiation of the disaggregation process requires Hsp70 system replacing sHsp bound to the substrate, which leads to the tradeoff between sHsp ability to bind and sequester substrates (sequestrase activity) and their ability to stimulate Hsp70 – Hsp100 - mediated disaggregation and refolding.

Majority of *Enterobacterales* (including family *Enterobacteriaceae*) contains a system of two cooperating sHsps – IbpA and IbpB, which most likely evolved by a duplication of ancestral, primarily single IbpA. IbpA protein from that two – protein system strongly interacts with the substrate and exhibits strong sequestrase activity. IbpB, on the other hand, on its own interacts weakly with most substrates, but can facilitate sHsp displacement from sHsp – substrate assemblies during disaggregation, enabling the sHsps to efficiently stimulate this process even at lower Hsp70 concentrations. In *Erwiniaceae* family the IbpB protein was lost and the secondarily single IbpA evolved the ability to facilitate Hsp70 – Hsp100 – mediated disaggregation and refolding with effectiveness similar to two – protein sHsp system, even in the absence of the IbpB partner.

In this study I investigated how the secondarily single IbpA from *Erwiniaceae* developed its new functionality. I reconstructed the evolutionary history of IbpA in *Enterobacterales*, showing that the characteristic activity of *Erwiniaceae* secondarily single IbpA developed in the last common ancestor of that family, in parallel to the loss of paralogous protein IbpB, most likely due to positive selection. By comparing the sequences and biochemical properties of reconstructed ancestral proteins, I identified two substitutions (Q66H and G109D) that enabled *Erwiniaceae* IbpA to much more effectively stimulate Hsp70 – Hsp100 - mediated disaggregation and refolding of sequestrated proteins. I have shown that those substitutions weakened interactions between ACD domain and CTE region of IbpA, as well as between IbpA and substrate, which in turn facilitated IbpA displacement from the substrate by Hsp70, increasing the effectiveness of the disaggregation stimulation. Moreover, amino acid residues on positions homologous to the two identified play a key role in functional differences between extant sHsp from families *Erwiniaceae* and *Enterobacteriaceae*.