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Small heat shock proteins IbpA and IbpB cooperate in sequestration of misfolded substrates to promote their refolding

Współpraca pomiędzy małymi białkami szoku cieplnego IbpA i IbpB w wiązaniu i późniejszej dezagregacji zdenaturowanych białek

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1. Streszczenie

Małe białka szoku cieplnego (sHSPs) stanowią pierwszą linię obrony komórkowej proteostazy. Ich podstawową rolą w komórce jest wiązanie rozfałdowujących się, podatnych na agregację polipeptydów - uprzedzając i minimalizując ich samorzutną agregację. sHSPs funkcjonują niezależnie od hydrolizy ATP, a wytworzone kompleksy sHSPs z rozfałdowanymi białkami służą jako rezerwuar polipeptydów dla późniejszej aktywnej dezagregacji prowadzonej przez ATP-zależne białka opiekuńcze Hsp70 i dezagregazy Hsp100.

Większość γ-proteobakterii posiada tylko jedno białko sHSP, które jest w stanie szybko i trwale wiązać rozfałdowane polipeptydy. Jednak duże powinowactwo tych sHSPs do substratów jest niekorzystne podczas dezagregacji, gdy białka Hsp70 muszą związać się do substratu w miejsce uprzednio wypartych sHSPs. Przedstawiona praca ma na celu analizę funkcjonowania sHSPs pochodzących z bakterii posiadających 2 współpracujące białka tej rodziny w miejsce tylko jednego. Analiza filogenetyczna wykazała, że układ 2-białkowy powstał na drodze duplikacji pojedynczego genu sHSP obecnego ongiś u wspólnego przodka *Enterobacterales* i późniejszej specjalizacji w kierunku IbpA oraz IbpB obecnych u znakomitej większości współczesnych gatunków bakterii należących do tego rzędu - np. u bakterii *Escherichia coli*.

Eksperymenty *in vitro* oraz *in vivo* przedstawione w treści tej pracy pokazują zupełnie różne właściwości biochemiczne białek IbpA oraz IbpB. Białka IbpA posiadają właściwości kanoniczne dla większości sHSPs zaangażowanych w ochronę przed nieodwracalną agregacją, takie jak zdolność do szybkiego i trwałego wiązania polipeptydów ulegających rozfałdowaniu z powodu szoku temperaturowego (*ang. heat shock*). Równocześnie te białka wydatnie utrudniają wiązanie Hsp70 do wytworzonych kompleksów sHSPs-substrat poprzez słabą zdolność do dysocjacji. Białka IbpB natomiast mają niewielkie zdolności do wiązania polipeptydów, jednak poprzez oddziaływanie z IbpA są efektywnie włączane do kompleksów sHSPs-substrat. Współobecność IbpB obok IbpA w kompleksach sHSPs-substrat znacząco ułatwia późniejszą dysocjację sHSPs podczas wiązania Hsp70, co w efekcie przekłada się na dużo efektywniejszą dezagregację uwięzionych polipeptydów. Obserwowane współdziałanie tych 2 sHSPs wydaje się więc omijać kompromis pomiędzy efektywnym wiązaniem a dysocjacją od rozfałdowanych polipeptydów, którym obarczone są pojedyncze sHSPs.

Obecność efektywnego układu dwóch sHSPs z pewnością ma korzystny wpływ na przystosowanie (*ang.* fitness) komórek bakteryjnych do przetrwania w warunkach stresu gdy zapotrzebowanie na Hsp70 w komórce znacząco rośnie. Specjalizacja IbpA oraz IbpB nosi znamiona neosubfunkcjonalizacji, podczas której IbpB nabyło nowych właściwości, które w połączeniu z klasyczną aktywnością IbpA zapewniają bakteriom rzędu *Enterobacterales* narzędzie do skuteczniejszej ochrony proteostazy.

2. Abstract

Small heat shock proteins (sHSPs) are a conserved class of ATP-independent chaperones that bind to unfolding, aggregation-prone polypeptides in stress condition. sHSPs encage them in so called sHSPs-substrate assemblies, shielding them from further aggregation and facilitating subsequent solubilization and refolding by Hsp70 and Hsp100 ATP-dependent chaperones.

Most of γ-proteobacteria express a single sHSP, that not only has to tightly bind unfolding polypeptides upon heat shock, but also should effectively dissociate upon Hsp70 action to allow disaggregation initiation. This work is dedicated to investigate the two-sHSP system that has evolved to overcome this bind-or-release trade-off. Initial phylogenetic analysis shows that a single sHSP gene duplication event in the ancestor of *Enterobacterales* (a subgroup of γ-proteobacteria) has given rise to the two sHSPs - IbpA & IbpB, which are now found in the majority of contemporary descendant species, e.g. in *Escherichia coli*.

Both *in vitro* and *in vivo* experiments designed to unravel the molecular basis of IbpA & IbpB cooperation show fundamental differences in their activities, identifying IbpA as a strong canonical polypeptide binder, similar to other single sHSPs. On the other hand, its non-canonical IbpB partner cannot stably bind aggregating substrates. Instead, IbpB presence alongside IbpA enhances dissociation of both sHSPs from polypeptides upon substrate disaggregation from sHSPs-substrate assemblies.

The analysed two sHSP cooperation provides substantial reduction in demand on Hsp70 necessary to perform efficient substrate disaggregation and refolding. It is achieved without compromising the ability of the sHSP system to scavenge aggregating polypeptides upon heat shock, which is hardly achieved by analysed single sHSPs.

The emergence of this effective sHSP system most certainly has an impact on the cells' ability to handle stress conditions, as it allows for employing less Hsp70 in disaggregation when the overall cellular demand for Hsp70 is particularly high. This might have provided the fitness that was necessary to fix both gene copies in the population along bacteria speciation. IbpA and IbpB function drift across the evolution might be considered a neosubfunctionalization as IbpB appeared with new functional properties that provide the new quality but only when in cooperation with IbpA.

3. Abbreviations

AAA+	─ ATPases associated with diverse cellular activities
A. hydrophila	─ *Aeromonas hydrophila*
A. tumefaciens	─ *Agrobacterium tumefaciens*
A. vinelandii	─ *Azotobacter vinelandii*
ATP	─ Adenosine triphosphate
ATPase	─ Protein with the ability to hydrolyze ATP
BLI	─ Bio-Layer Interferometry
BSA	─ Bovine Serum Albumin
B. suis	─ *Brucella suis*
CS	─ Citrate Synthase
C. neteri	─ *Cedecea neteri*
D. radiodurans	─ *Deinococcus radiodurans*
DLS	─ Dynamic light scattering
DTT	─ Dithiothreitol
E. amylovora	─ *Erwinia amylovora*
E. coli	─ *Escherichia coli*
EDTA	─ Ethylenediaminetetraacetic acid
HSP	─ Heat Shock Protein
IbpAB	─ Inclusion body protein A and B
IbpAB-Luc	─ Complexes of IbpA, IbpB, and Luciferase
IPTG	─ Isopropyl β-D-1-thiogalactopyranoside

LB	─ Luria broth
Luc	─ Luciferase
MDH	─ Malate Dehydrogenase
ML	─ Maximum-Likelihood
M. leprae	─ *Mycobacterium leprae*
M. tuberculosis	─ *Mycobacterium tuberculosis*
NBD	─ Nucleotide-binding domain
NEF	─ Nucleotide exchange factor
PDB	─ Protein Data Bank
PSII	─ Photosystem II
RCF	─ Relative centrifugal force (g)
RPM	─ Rotations per minute
SBD	─ Substrate-binding domain
SDS	─ Sodium dodecyl sulphate
SDS-PAGE	─ SDS polyacrylamide gel electrophoresis
sHSP	─ Small heat shock protein
TB	─ Tuberculosis
TCA	─ Trichloric acid
TRIS	─ Tris(hydroxymethyl)aminomethane
V. harveyi	─ *Vibrio harveyi*

4. Introduction

Each and every living cell utilizes proteins as one of their very basic building blocks employed in all activities that define life. Performing such a monumental task, protein networks have grown in complexity across the evolution. This have risen a need for dedicated maintenance taking care of such precise yet vulnerable machinery.

Proteostasis (protein homeostasis) is the term encompassing regulation of proteins levels within the cell in order to maintain the health of the cellular proteome. It is achieved on multiple levels, starting from transcriptional control, through precisely tuned translation and finishing with degradation of excess or unnecessary proteins. This seemingly straightforward, however extremely complex protein life and death pipeline is affected by yet another kind of processes gathered under the name of protein unfolding/misfolding and aggregation.

4.1. The nature of protein aggregation

Each proteins' function is defined by their sequence and proper, so called 'native' fold. Most polypeptides are folded right after synthesis in spontaneous or assisted manner and maintain their fold throughout their whole life cycle until degradation. The lower the energy of the folded state the more stable it is and the harsher conditions it can persist through. However, excessive fold stability is a disadvantage when it comes to degradation, impairing cellular protein turnover. Therefore, across the evolution, proteins tend to be just stable enough to perform their tasks. This phenomenon however, makes protein structures vulnerable when cell comes across harsh conditions like elevated osmotic pressure, high heavy metal ions concentration or raised temperature. Such unfavourable environment may cause the damage of the protein or its native fold - resulting in more or less massive unfolding events throughout the cell.

Except transmembrane domains, most of soluble proteins' folds bury hydrophobic amino acid residues in so-called hydrophobic cores (Munson et al. 1996). Unfolding of such structures leads to their thermodynamically unfavourable exposure to the polar environment. As long as the conditions impede spontaneous or assisted refolding into native state (which is the global energetic minimum for most proteins (Govindarajan and Goldstein 1998)), hydrophobic regions of multiple unfolded polypeptides have a chance

to clash with each other in solution. These clashed polypeptides stay clustered together, providing a thermodynamically favourable energy drop resulting from lower surface to volume ratio of such a hydrophobic arrangement in polar environment (local energy minimum) (Onuchic, Luthey-Schulten, and Wolynes 1997). This arrangement of stochastically clustered polypeptides is called an amorphous aggregate [Fig. 1].

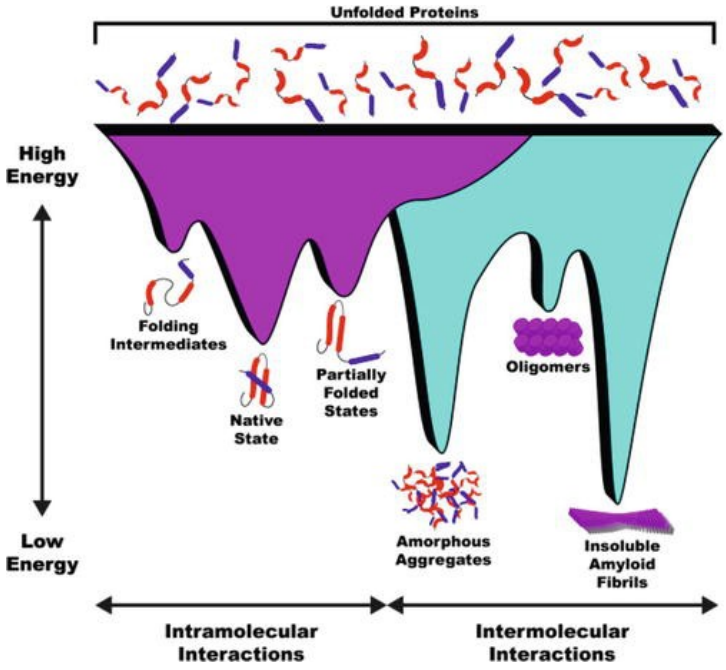

Fig. 1. Energy landscape of protein folding and aggregation. The purple surface shows the range of possible conformations leading to the thermodynamically balanced state (native state). Cyan-colored area of the landscape indicates the conformations moving toward to amorphous aggregates of insoluble amyloid fibrils. Adapted from (Agbas 2019).

Protein aggregation can also proceed in a strictly ordered manner. In favourable conditions (not necessarily harsh), an extensive nucleation of polypeptides may result in formation of highly ordered amyloid fibrils. These are long polymers (although not covalently linked) where monomers of the same protein contact each other through β-sheet interactions running perpendicular to the long axis of the fibril. Such an arrangement provides large contact zones between monomers, resulting in very high overall stability of this structure (Chiti and Dobson 2006; Gillam and Macphee 2013) [Fig. 1].

In general, proteins trapped in aggregates are no longer functional, which may impair cellular functions required for growth and survival. Additionally, misfolded and aggregated protein species appear cytotoxic by interfering and binding to functional proteins and folding intermediates. Yet, the whole spectrum of different possible

mechanisms of aggregate cytotoxicity is very broad and still remains debated (Stefani and Dobson 2003; Zhou and Xu 2014; Grudzielanek et al. 2007).

4.2. Chaperones in counteracting protein aggregation

The vulnerability of precise cellular protein machinery have not been left unaddressed along evolution. In all living organisms we can find systems dedicated to maintaining proteins in good shape, a subgroup of which are those capable of dealing with more prominent proteostasis imbalances like massive protein aggregation. These roles are being fulfilled by several key players that are embedded in different chaperone families: Hsp70 and its cochaperones; Hsp100 disaggregases and small heat shock proteins.

4.3. Hsp70 and its cochaperones

70-kDa heat shock proteins play a role in a vast variety of folding processes, including *de novo* folding after synthesis (Nelson et al. 1992), refolding of misfolded and/or aggregated polypeptides (Hartl 1996), membrane translocation (both import/export between organelles and secretion) (Young, Hoogenraad, and Hartl 2003; Craig 2018) and rearrangements of regulatory complexes (Liberek, Georgopoulos, and Zylicz 1988; Hwang, Crooke, and Kornberg 1990). Performing multiple housekeeping tasks, Hsp70s are highly conserved across evolution (Moran et al. 1983; Amir-Shapira et al. 1990).

Hsp70s are universally arranged in two domains - NBD and SBD (nucleotide and substrate binding domains) linked with a flexible linker involved in allosteric communication between domains (Jiang et al. 2007; Vogel, Mayer, and Bukau 2006). In SBD we can distinguish two subdomains. βSBD subdomain is a β-sandwich of two antiparallel β-sheets with a hydrophobic peptide-binding cavity that is equipped with αSBD subdomain - a helical lid that is able to close on βSBD substrate binding pocket (Bertelsena et al. 2009; Flaherty et al. 1994; Zhu et al. 1996) [Fig. 2].

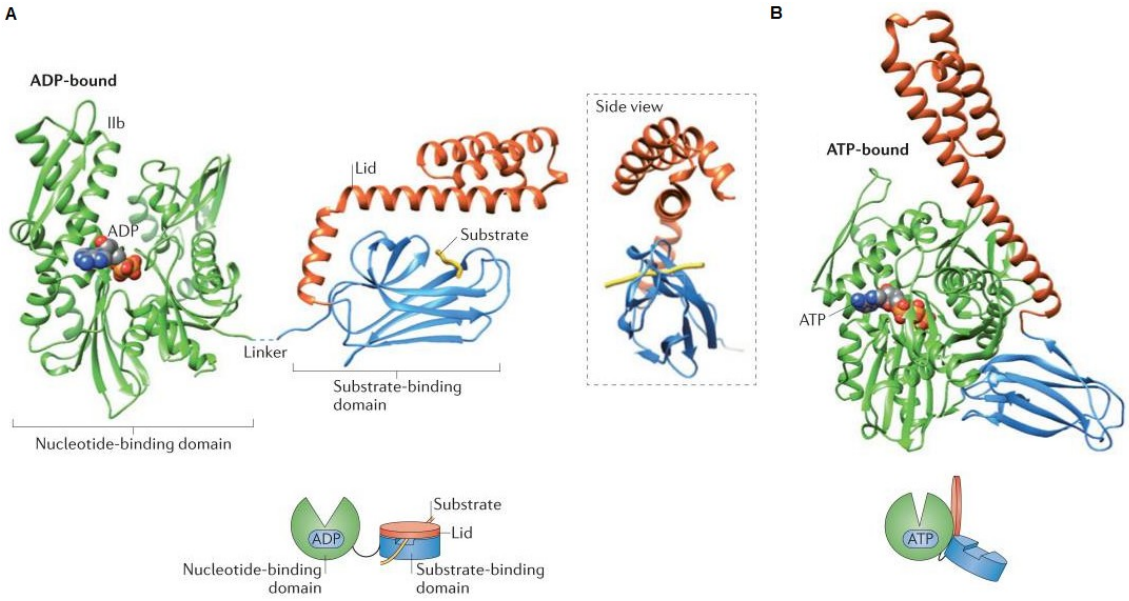

Fig. 2 Structure and conformations of Hsp70. (A) ADP-bound or nucleotide-free state, the nucleotide-binding domain NBD (green; Protein Data Bank (PDB) code: 3HSC), substrate-binding domain SBD (blue; PDB code: 1DKZ), with the lid domain (red) locking a peptide substrate (yellow) into the binding pocket of Hsp70. A side view of the SBD is shown on the right. A cartoon depicting the two-domain complex is shown below. The bound nucleotide is shown in space filling format. (B) The ATP-bound, docked Hsp70 state, (PDB code: 4B9Q). NBD is docked to SBD with lid subdomain bound to the surface of NBD, resulting in a widely opened substrate-binding site. Adapted from (Saibil 2013).

Hsp70 substrates are principally short, hydrophobic stretches of residues in full-length proteins, whose binding and release is specifically coupled with ATP hydrolysis in Hsp70 ATPase cycle (Russell, Jordan, and McMacken 1998). In ATP-bound state Hsp70 NBD and SBD are docked together with the αSBD lid being in an open conformation [Fig. 2b]. This state provides low substrate affinity and high K_{ON}/K_{OFF} rates. Binding of the substrate stimulates ATP hydrolysis, which results in drastic conformational change. NBD and SBD undock and αSBD lid closes on βSBD peptide cavity [Fig. 2A], securing the bound substrate. In this state Hsp70 shows high substrate affinity with very low K_{ON}/K_{OFF} rates (Mapa et al. 2010; Laufen et al. 1999; Qi et al. 2013). A further ADP/ATP exchange regenerates an open conformation, where substrate is released and Hsp70 is ready for another cycle to begin (Silberg and Vickery 2000; Packschies et al. 1997). Hsp70 substrate bind-and-release cycle serves as highly universal cell motor that functions together with a class of proteins that are dedicated for targeting and stimulating Hsp70 activity. These are its cochaperones - Hsp40s/J-proteins and nucleotide exchange factors (NEFs).

Hsp40s constitute a class of cochaperones universally equipped with a J-domain, named after its homology to an approximately 73 amino acid region in DnaJ protein from *Escherichia coli* (F. Hennessy et al. 2000). J-domains contain an extremely conserved HPD motif that is directly responsible for contact with Hsp70s and provide a stimulus for ATP hydrolysis (Laufen et al. 1999; Jiang et al. 2007). Together with the conserved, omnipresent J-domain, different Hsp40s possess a variety of different domains that together are responsible for targeting Hsp70 to different cellular processes/substrates. These include protein folding, disaggregation, protein translocation and iron-sulphur clusters biogenesis (Fritha Hennessy et al. 2005; Kampinga and Craig 2010). Hsp40s are generally responsible for targeting Hsp70 activity by binding dedicated substrates and bringing them to Hsp70 in ATP state to form a generally short-living triple complex. Substrate binding and J-domain interaction cooperatively trigger Hsp70 to hydrolyse ATP (Jiang et al. 2007), securing the substrate and dissociating the J-domain protein due to Hsp70 conformational change (Marcinowski et al. 2011; Ahmad et al. 2011).

In order to proceed with its cycle, Hsp70 has to exchange ADP for yet another ATP molecule, changing the conformation back and releasing the substrate. In Hsp70s this exchange can occur spontaneously (Silberg and Vickery 2000), however in many of them at very low rates (Packschies et al. 1997). As it can be concluded from the name, nucleotide exchange factors (NEFs) are cochaperones dedicated for facilitation of this process. In fact, their name is misleading in term of their mechanism of action as NEFs do not exchange nucleotides but rather only stimulate ADP release (Blatch, Brodsky, and Bracher 2007). A subsequent ATP binding is then driven just by cellular high ATP/ADP concentrations ratio. Along evolution, multiple NEFs seem to have independently evolved for the same function as it is common for proteins exerting this function to be completely unrelated to each other (Bracher and Verghese 2015).

Hsp70 and its cochaperones are canonically considered a standalone machinery that acts most efficiently as a triad in all the processes that Hsp70 is involved in. As Hsp70 is doubtlessly the main performer in these tasks (Kampinga and Craig 2010), J-proteins and NEFs synergistically act to stimulate its basal ATPase activity even up to 5000-fold (Liberek et al. 1991). This provides necessary control and targeting for such potent machinery. The best studied Hsp70 & cochaperones triad is formed by proteins originating from *E. coli* - DnaK (Hsp70), DnaJ (Hsp40) and GrpE (NEF), being the model for research, which allowed most of findings described above.

4.4. Hsp100 disaggregases

Depending on the environmental niche, different organisms have to cope with more or less challenging thermal amplitudes. Especially vulnerable species include unicellular organisms - lacking any thermal inertia and plants - being generally non mobile, therefore unable to escape from unfavourable conditions. Severe heat shock is a well known factor causing extensive protein aggregation, which sometimes happens to be beyond Hsp70 management abilities. To fight such a scenario, bacteria, yeast and plants utilize yet another chaperone family - Hsp100 disaggregases.

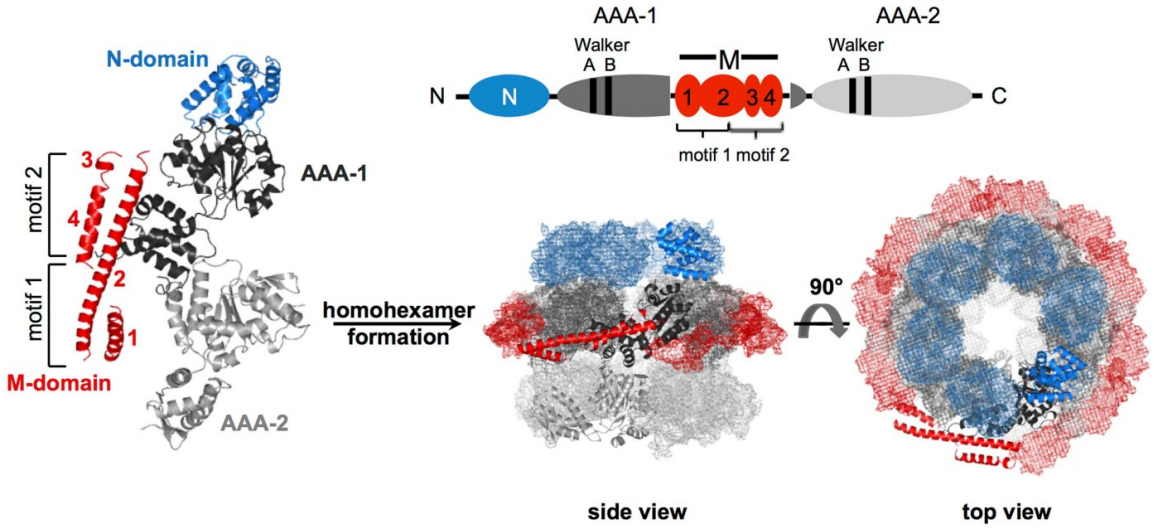

Fig. 3. Structure of ClpB/Hsp104 disaggregases. Domain organization, structure, and hexameric model of ClpB/Hsp104. The ClpB/Hsp104 protomer consists of an N-terminal (N) domain, two AAA domains (AAA-1, AAA-2) encompassing conserved Walker A and B motifs for ATP binding and hydrolysis, and an inserted ClpB/Hsp104-specific coiled-coil middle (M) domain. The M domain consists of four α-helices that are numbered accordingly. Motif1 comprises helices 1 and 2; motif2 consists of helices 2, 3, and 4. The monomer assembles into a hexamer consisting of three layers (rings) formed by N-domains, AAA-1/M-domains, and AAA-2 domains. Adapted from (Mogk, Kummer, and Bukau 2015).

Hsp100 disaggregases are hexameric AAA+ ATPases, which form asymmetric ring-like structures with a prominent axial channel (Erzberger and Berger 2006) [Fig. 3]. Equipped with two nucleotide binding domains NBD1 and NBD2, Hsp100 is able to transform ATP hydrolysis energy into coordinated motions, allowing translocation of substrate polypeptides through its central channel (Yokom et al. 2016; Yu et al. 2018). As a result

of this process, the aggregated polypeptide is tethered from the aggregate and gains yet another chance for proper folding after translocation (Doyle and Wickner 2009).

Being potentially dangerous for intrinsically unstructured cellular proteins (that may resemble their natural substrates), Hsp100 disaggregases have to be tightly controlled. Unfavourable ATP/ADP affinities make Hsp100s highly repressed by cellular ADP concentration, therefore in order to effectively process polypeptides, disaggregases tightly cooperate with Hsp70 system (Kłosowska, Chamera, and Liberek 2016). Similarly to J-proteins targeting Hsp70s to their substrates, Hsp70s already bound to the aggregate surface recruit Hsp100 disaggregases (Winkler et al. 2012). This provides substrate preference for Hsp100 (Chamera et al. 2019). Additionally, in this process Hsp70 also stimulates Hsp100 ATPase through direct interaction with axially protruding Hsp100 middle domain [Fig. 3] (Sielaff and Tsai 2010; J. Lee et al. 2013; Seyffer et al. 2012). The set of dependencies and interactions described above constitutes fully functional disaggregating machinery in yeast and bacteria.

4.5. Small heat shock proteins*

Apart from actively disaggregating chaperones that mostly act to restore already aggregated proteins back to their functional state, there is another class of chaperones involved in the process. Small heat shock proteins (sHSPs) constitute the very first line of cellular defence from irreversible protein aggregation, canonically being able to influence ongoing protein aggregation for the benefit of the cell. sHSPs are found widespread in all kingdoms of life and tend to be very plastic along evolution, acquiring multiple functions apart of typical protein quality control. Bacterial sHSPs being the main topic of this dissertation, I decided to introduce their functions in greater details especially because the central interest of my research oscillates around such a manifestation of a new function.

4.5.1. From the discovery to common features

The first-ever discovered member of small heat shock protein family was α-crystallin and has been known for more than a century to be the major structural protein of the vertebrate eye lens (Mörner 1894). Many years later, a well-established family of

* This chapter was published as 'Small but mighty: a functional look at bacterial sHSPs' (Obuchowski✉ and Liberek 2020).

α-crystallins was found to be related to *Drosophila melanogaster* small heat shock proteins (abbreviated as sHSPs), which justified joining them into common classification group of sHSPs (Ingolia and Craig 1982). This, together with heat shock response-focused research boom of these years, has led to the broad investigations on small heat shock proteins.

Although sequence conservation of sHSPs is rather limited, especially in contrast to other heat shock proteins, they started to be identified by homology to α-crystallins and the fruit fly sHSPs (Key, Lin, and Chen 1981; Russnak et al. 1983; Booth et al. 1988; Nerland et al. 1988; Verbon et al. 1992; B. Y. Lee, Hefta, and Brennan 1992). As the number of identified sHSPs has been growing, their polypeptides were found to be typically subdivided into an α-crystallin domain, the most homologus region in their sequence, and much less conserved flanking N- and C-termini (Van Montfort et al. 2001). At the same time, multiple sHSPs were shown to form large dynamic complexes (A. P. Arrigo and Welch 1987; Behlke et al. 1991) and later, their dissociation was found to be regulated by temperature changes (Fu and Chang 2004). Accompanying structural studies have shown that sHSPs are especially rich in β-structures (L. K. Li and Spector 1974; Merck et al. 1993), finally leading to the first resolved bacterial sHSP structure (K. K. Kim, Kim, and Kim 1998; R. Kim et al. 1998) [Fig. 4]. It was later found that β-sandwich fold [Fig. 4B] is a common, highly conserved feature of all sHSPs.

Along structural studies, a lot of effort was put into determination of functional traits of sHSPs. Initial studies on α-crystallins focused on the medical contexts, especially prevention of cataract formation (Roy and Spector 1976) and later on roles in tumour cells growth (Gaestel et al. 1989) and cell differentiation (Stahl et al. 1992). Concerning presence of sHSPs in organisms from every kingdom of life, it appeared challenging to elucidate their common functions (Schlesinger 1986) suggested by their striking fold conservation. sHSPs were often found to be highly overexpressed at heat stress - conferring thermotolerance in some organisms (Loomis and Wheeler 1982; Berger and Woodward 1983) - but showing no feasible phenotype when disrupted in others (Susek and Lindquist 1989; Praekelt and Meacock 1990). It took detailed biochemical studies to demonstrate that sHSPs act as molecular chaperones both in animals (Jakob et al. 1993; Wang and Spector 1995), plants (G. J. Lee, Pokala, and Vierling 1995) and bacteria (Chang et al. 1996; Thomas and Baneyx 1998).

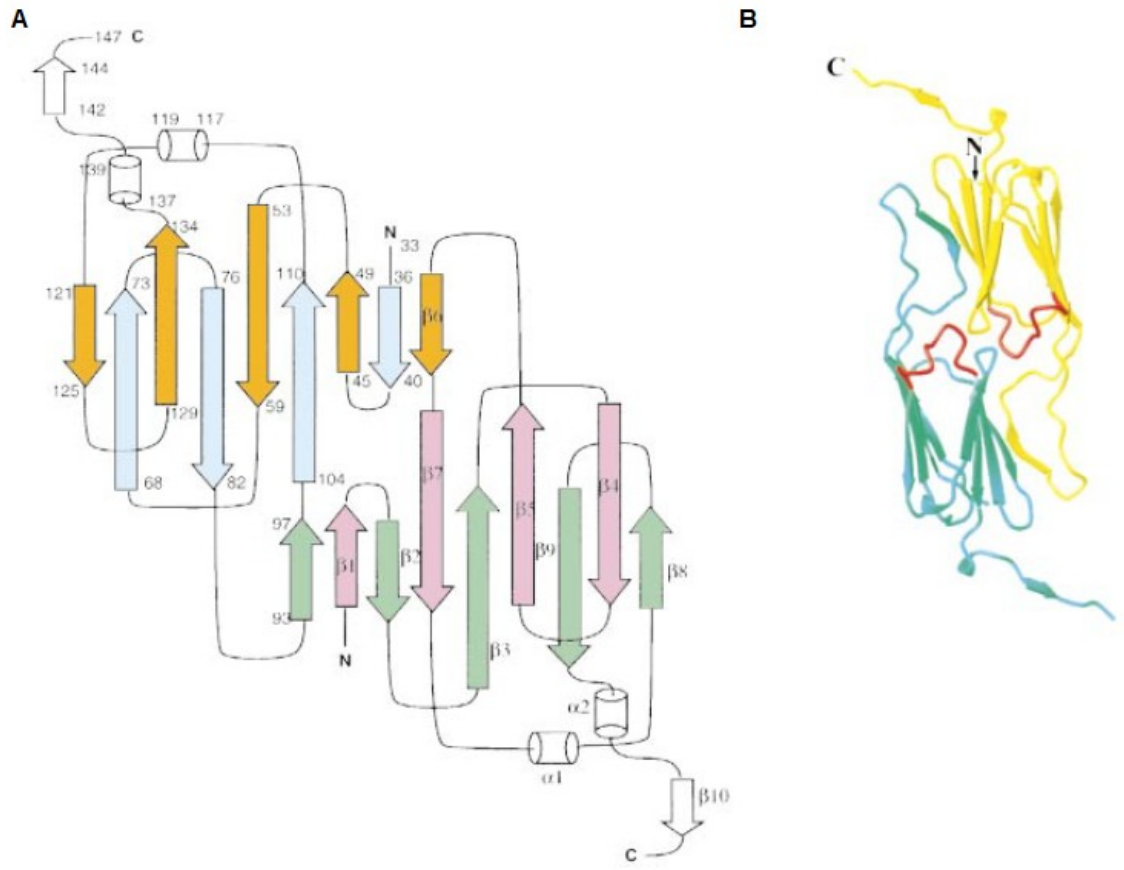

Fig.4. First-ever solved sHSP structure. (A) Topology of the secondary structure of a MjHSP16.5 dimer. The first and last residue numbers for each secondary-structural element are indicated in the top (left) monomer; the secondary-structural elements are labelled in the monomer at the bottom (right). The first β-sheet of the top monomer is in blue and the second β-sheet is inyellow, but b6 (also yellow) is from the adjacent subunit. The first and second β-sheets of the bottom monomer are also shown in different colours (green and pink, respectively) (B) Ribbon diagram of an HSP16.5 dimer viewed along the non-crystallographic two-fold symmetry axis. The N and C termini are indicated. Adapted from (K. K. Kim, Kim, and Kim 1998)

From the evolutional point of view, small HSPs divergence across all kingdoms of life seems to have been driven with great plasticity towards acquiring different functions (De Jong, Leunissen, and Voorter 1993; Carra et al. 2017). There is a clear tendency to increase the number of sHSP-coding genes with increasing organism complexity, which comes along with increased diversity of performed functions. Bacteria, considered to be the least complex organisms, usually express only one or two sHSPs (Haslbeck et al. 2005) that can still perform several functions in bacterial cell. Up till now, most important reported bacterial sHSPs functions are: (i) molecular chaperone function, which can be subdivided into two distinct modes of action: transient interactions with unfolding polypeptides at mild proteotoxic stress and high-affinity interactions observed at massive

aggregation events, (ii) protection of cell membrane components and membrane integrity and (iii) - a handful of more specific functions dedicated to survival in adverse environment.

4.5.2. sHSPs chaperone activity: stable interactions

The best studied chaperone activity of sHSPs is preventing formation of large amorphous aggregates, which seems to be evolutionarily the oldest and most important sHSPs function for bacteria. To perform it, sHSPs intercept unfolding intermediates and co-assemble in so called sHSPs-substrate assemblies which serve as the safe-storage for polypeptides before refolding. This section will focus on formation, architecture and refolding of substrates from sHSPs-substrate assemblies.

In 1996, after multiple evidence of eukaryotic sHSPs being molecular chaperones appeared (Jakob et al. 1993; Boyle and Takemoto 1994; Singh et al. 1995; Wang and Spector 1995; Raman, Ramakrishna, and Mohan Rao 1995), Hsp16.3 from *Mycobacterium tuberculosis* was shown to suppress citrate synthase (CS) thermal aggregation, though it could not protect CS activity nor refold it afterwards (Chang et al. 1996). The same year *Escherichia coli* IbpA & IbpB, previously described as inclusion body associated proteins (Allen et al. 1992), were found to colocalize with aggregated protein fraction in heat shock conditions (Laskowska, Wawrzynów, and Taylor 1996). Later, they were also shown to confer thermotolerance and stabilize aggregated proteins for further refolding (Thomas and Baneyx 1998; Veinger et al. 1998). These observations, taken together with sequence homology to eukaryotic sHSPs, gave a solid basis for considering bacterial sHSPs molecular chaperones.

The chaperone activity of sHSPs is exerted by stabilization of structurally damaged proteins for subsequent refolding by Hsp70-Hsp100 bichaperone system. It is achieved by sHSPs binding to partially unfolded polypeptides in stress conditions and driving their aggregation towards characteristic complexes called sHSPs-substrate assemblies. sHSPs showing this activity are often called aggregases, which might be misleading as sHSP-substrate assemblies and the assembly process itself differ from amorphous aggregates and aggregation. Up to date, direct molecular mechanism of the assembly formation process remains elusive except of several details. It is known that substrate binding, executed by the N-terminus and α-crystalline domain (Fu et al. 2005; Tomoyasu, Tabata,

and Nagamune 2010; Fu, Shi, Yin, et al. 2013; Fu and Chang 2006), is preceded by dissociation of sHSP oligomers into smaller species - preferably dimers (Fu and Chang 2004) - that are postulated to be the active species in this process. It is typically observed also among non-bacterial sHSPs (Haslbeck and Vierling 2015).

Efficient polypeptide sequestering in assemblies requires the presence of stoichiometric amounts of sHSPs - at least *in vitro* (Friedrich et al. 2004; Fu and Chang 2004; Jiao et al. 2005). *In vivo* sHSP genes are commonly found to undergo massive expression upregulation in stress conditions. It is more pronounced than upregulation of any other chaperone as judged by cellular protein content and transcription profiling (Richmond et al. 1999; Münchbach, Nocker, and Narberhaus 1999; S. Lee et al. 1998) - presumably providing enough sHSPs for efficient *in vivo* substrate sequestering. On the other hand, little is known of the substrates trapped by bacterial sHSPs. It can only be induced from studies on yeast sHSPs that these are stored in a near-native conformation (Ungelenk et al. 2016), which is probably one of the factors facilitating further disaggregation and refolding. Speaking of assemblies architecture, substrate molecules are postulated to be held in the core of the assembly with only limited number of sHSPs and shielded from the environment by sHSP outer shell (Żwirowski et al. 2017), however there is no direct structural data on this subject.

Up till now, multiple studies describing sHSPs action in protein aggregation were carried out in the *E. coli* system, where two sHSPs - IbpA & IbpB - cooperate with each other. Interestingly, IbpA, when present during substrate aggregation, is enough to generate assemblies with the substrate but also inhibits its further disaggregation. This inhibition is lost in the presence of IbpB alongside IbpA. However, IbpB alone has a much less pronounced effect on disaggregation, being also hardly able to generate assemblies with the unfolding substrate (Ratajczak, Zietkiewicz, and Liberek 2009; Matuszewska et al. 2005; Thomas and Baneyx 1998).

An alternative example of two-sHSP bacterial system comes from *Deinococcus radiodurans*, where sHSPs act separately. One of them, Hsp20.2, is effective in assembly generation when in the presence of the aggregating substrate and the other, Hsp17.7, is capable of sustaining substrate activity (or postponing activity loss) in otherwise denaturing conditions (Bepperling et al. 2012). The abovementioned activity protection is

achieved by more transient interactions with the substrate, which will be discussed as a standalone phenomenon in the next section.

Overall, both *E. coli* and *D. radiodurans* systems seem rather atypical as most bacteria express only one sHSP (Haslbeck et al. 2005). The best studied single sHSP is Hsp16.3 from *M. tuberculosis*, which was used for functional studies showing typical assembly-forming chaperone activity (Chang et al. 1996), surface hydrophobicity changes (Yang et al. 1999) and oligomer dissociation (Fu and Chang 2004) upon heat treatment. Intensive studies on Hsp16.3 have also revealed its non-chaperone functions that will be described in another section.

4.5.3. sHSPs chaperone activity: transient interactions

Another important, yet less studied example of sHSPs chaperone activity is their ability to protect enzymes from inactivation or postpone their activity loss upon mild denaturing conditions. It is exerted via transient, cyclic interactions (in contrast to stable assembly generation-driving interactions) with hydrophobic regions of slightly damaged protein substrates, somehow directing them back to native fold. This section will focus on several bacterial sHSPs that were shown to act in this mode of chaperone activity.

From the mechanistic point of view, it is highly elusive how bacterial sHSPs protect enzymes activity, however, it can be induced from several studies on vertebrate sHSPs. These have been shown to interact weakly and transiently with misfolded intermediates - forming dynamic high molecular weight assemblies (Kulig and Ecroyd 2012). Target substrate hydrophobicity and stability largely determines if sHSPs would tightly interact with the substrate, stabilizing it for further refolding, or transiently bind and release. As the misfolding intermediate is subsequently bound and released, it is secured from aggregation and gets the chance to fold into the native structure (Kulig and Ecroyd 2012; Hatters et al. 2001).

In bacteria, enzyme activity protection assay was initially applied for *M. tuberculosis* Hsp16.3. Although it showed chaperone activity towards citrate synthase as monitored by static light scattering, the authors could not observe any protection of citrate synthase activity (Chang et al. 1996). On the other hand, *E. coli* IbpA & IbpB turned out to be more successful in this type of experiment. Together, they were shown to protect luciferase from thermal inactivation (although weakly) (Matuszewska et al. 2005) and

both separately and together - to protect several other enzymes from oxidative and freeze-thaw inactivation (Kitagawa et al. 2002). Interestingly, authors claim IbpB to be more effective than IbpA in enzyme activity protection (Kitagawa et al. 2002), which is consistent with the later reported *in vivo* IbpB ability to protect metabolic enzymes activities during heat stress (Fu, Shi, Yan, et al. 2013). In contrast, IbpA was shown to be much more potent than IbpB in forming stable assemblies with aggregating polypeptides. This suggests a diversity in their activities although they cooperate acting as mixed complexes (Ratajczak, Zietkiewicz, and Liberek 2009; Matuszewska et al. 2005; Hochberg et al. 2018). Finally, it seems that cooperation is not a key feature for this type of sHSP activity. Here an example is *D. radiodurans,* which Hsp17.7 was shown to effectively protect CS from thermal inactivation in contrast to its paralog Hsp20.2 - that can neither protect CS activity nor cooperate with Hsp17.7 (Bepperling et al. 2012).

Leaving the cooperation issue behind, it seems that the ability to form assemblies with aggregating substrate and protect enzyme activity is somehow in opposition. This is supported by research on single sHSPs: *M. tuberculosis* Hsp16.3 that is only able to form assemblies (Chang et al. 1996) and - in contrast - its close relative sHSP18 from *Mycobacterium leprae*, which effectively protects restriction enzymes from thermal inactivation however was not assayed for generating stable complexes/assemblies with aggregating substrate (Lini et al. 2008).

Although sHSPs-dependent enzyme protection and sHSPs-substrate complex formation were already shown in the very first publication attributing sHSPs with chaperone activity (Jakob et al. 1993), these modes of action are rarely assayed when a new bacterial sHSP appears. The most exploited assay in this field (and technically the easiest) is *in vitro* aggregation monitored *via* static light scattering. It does not directly indicate whether prevention of sample scattering increase is achieved by protecting the substrate's native fold or scavenging unfolded polypeptides within soluble assemblies. Therefore it does not allow to discriminate sHSPs chaperone activity modes. The same concern may apply to widely exploited experiments on *in vivo* aggregation, where the amount of cellular insoluble (aggregated) protein is compared between strains. Here again one could deliberate if the volume of aggregates is lower due to sHSPs-dependent substrate activity (fold) maintenance or providing a more potent substrate for effective disaggregation (sHSPs-substrate assemblies instead of amorphous aggregates).

On the other hand, from the 'end user' point of view it is the final outcome that matters e.g. reduced aggregation/aggregates volume. Therefore we can see two different strategies to achieve that. It can only be speculated that aggregation modulation path may be more effective in counteracting severe proteotoxic stress when unfolding events occur frequently and rapidly among greater pool of polypeptides. On the contrary, the activity protection path might be favourable at less severe conditions, when only a smaller pool of less stable substrates is exposed to the risk of unfolding.

Finally, evidence can also be found for a third option. Klein and colleagues (Klein et al. 2001) have shown that IbpA, a single sHSP from marine bacterium *Vibrio harveyi*, complexes *in vivo* with aggregated protein fraction similarly to most typical sHSPs. However, one of their experiments suggests that the aggregated protein fraction (containing IbpA) is highly stable during cells recovery. This in turn could suggest a dilution of IbpA-detoxified, stable aggregate species (IbpA-substrate assemblies?) along cell divisions as a mechanism of aggregate handling in *V. harveyi*, however it would require further research.

4.5.4. Membrane-focused sHSPs

Along chaperone-focused research, accompanying localization studies have shown several bacterial sHSPs to be located in cell membranes (B. Y. Lee, Hefta, and Brennan 1992; Laskowska, Wawrzynów, and Taylor 1996; Otani et al. 2005). This feature was further exploited in several different bacteria species, giving rise to the concept of sHSPs as lipochaperones (Maitre et al. 2014). This section will attempt to briefly summarize what is known on sHSPs-membrane relation.

Starting from the work of Horvath and colleagues, who identified *hsp17* as a 'fluidity gene' in *Synechocystis* PCC 6803 (Horvath et al. 1998), it was shown that several sHSPs are capable of reducing membrane fluidity in permissive or heat stress conditions and in the presence of an organic solvent (Torok et al. 2001; Capozzi et al. 2011). To perform this task sHSPs associate with membranes not as higher-order oligomers but in the form of dissociated species (Zhang et al. 2005; Maitre et al. 2012) which are also active substrate-binding forms concerning the canonical chaperone activity. Later, Maitre and colleagues have proposed a joined model of chaperone and lipochaperone *Oenococcus oeni* Lo18 activities that summed up extensive studies in this field (Maitre et al. 2014).

However no further molecular details of Lo18 lipochaperone sHSPs activity are available.

Moving from general to more specific lipochaperone activity, the ability of cyanobacteria sHSPs to maintain thylakoid membrane integrity and their canonical chaperone activity towards phycocyanins (Nakamoto and Honma 2006) were integrated and analysed in the context of resistance to UVB-induced damage (Balogi et al. 2008) and oxidative stress (Sakthivel, Watanabe, and Nakamoto 2009) in *Synechocystis*. Presented data strongly highlights the importance of HspA, cyanobacterial sHSP, for preserving photosynthetic thylakoid functions - both through maintaining general thylakoid membrane integrity (lipochaperone) and specific protection of phycobilisomes and PSII complexes from inactivation (dedicated chaperone).

4.5.5. sHSPs for special tasks

Small HSPs are featured with the least conserved sequence among all chaperone families, which goes along with great evolutionary plasticity towards acquiring new functions. This is especially evident in multiple sHSP-expressing organisms like plants or animals, where many of the sHSPs perform functions other than the general chaperone function. In bacteria it is less evident (or less investigated), although there are several case-studies that demonstrate specific, dedicated chaperone-target interactions or other non-chaperone sHSP function. This section is an attempt to briefly summarize the activities of bacterial sHSPs that are apart from the already discussed canonical chaperone activities.

An example of a specific sHSP function was found in *Agrobacterium tumefaciens*. HspL, one out of four sHSPs expressed in this bacterium, was found important for *A. tumefaciens* virulence towards plant cells e.g. transferring its DNA into plant cells in order to take-over plant metabolism. HspL, but no other *A. tumefaciens* sHSP, effectively protects VirB8 protein (Tsai et al. 2012) that is an essential assembly factor for type IV secretion system responsible for DNA injection into plant cells (Baron and Cellulaire 2006). VirB8 protein is conserved across evolution (Baron and Cellulaire 2006), therefore a follow-up study, concerning VirB8-dependent mammal pathogen, appeared. *Brucella suis*, a facultative intracellular bacterial pathogen of mammals, also uses a type IV secretion system for virulence particle delivery to the host cells. Unlike in *A. tumefaciens*, it was shown that *B. suis* IbpA is not required for virulence (Berta et al. 2014). The second *B. suis* sHSP (also annotated as IbpA) was not analysed based on significantly

lower homology to *A. tumefaciens* HspL (Berta et al. 2014). Considering this data, it seems that VirB8 relation to HspL found in *A. tumefaciens* is rather limited to narrow species group.

As sHSPs are responsible for protecting proteostasis on the molecular level, they may also play a more general role in some of the strategies for survival in a hostile environment. One of these strategies is the formation of biofilm,which is a three-dimensional, complex structure formed of bacteria settled in extracellular matrix. Biofilms are more resistant to various stress conditions (antibiotics, heavy metal ions, oxidation) than free-living bacterial cells, enhancing their ability to survive (Flemming et al. 2016). In *E. coli*, it was shown that IbpA and IbpB indirectly influence biofilm formation, delaying its establishment in their absence. In *ΔibpAB* strains, cells are affected by endogenous oxidative stress, which results in overproduction of indole, that in turn inhibits formation of the biofilm (Kuczyńska-Wiśnik, Matuszewska, and Laskowska 2010).

Another strategy for survival in adverse environment, where sHSPs may interfere, is cysts formation. In opposition to biofilm, microbial cyst is a resting/dormant stage, dedicated to passive survival in harsh conditions. *Azotobacter vinelandii* is a free-living soil bacterium whose sHSP - Hsp20 was shown to be essential for cyst desiccation resistance. Consistently with the function, *hsp20* gene in *A. vinelandii* is under the control of RpoS sigma factor (Cocotl-Yañez et al. 2014) that governs the expression of many genes crucial for bacteria survival in adverse environments. This is however atypical for sHSPs as most of them in related bacterial species are under control of RpoH paralogs - master regulators of heat shock response in these species (Tilly et al. 1986).

The most complete story in terms of sHSPs-affected survival in adverse environment comes from *M. Tuberculosis*. Its Hsp16.3 sHSP is associated with dormancy and stationary phase, where it was shown to be expressed the most. Hsp16.3 expression results in lower cell susceptibility to autolysis at the cost of a slower growth rate (Ying Yuan, Crane, and Barry 1996). Hsp16.3 was also shown to be instrumental for cell wall thickening that provides additional protection during dormancy (Cunningham and Spreadbury 1998). Similarly to the growth on media, research conducted in a pathogen-host system has also shown that Hsp16.3 plays a role in slowing the growth of *M. tuberculosis* during infection (Hu et al. 2006) – being important for TB-characteristic

infection latency. Hsp16.3 is highly induced upon entry into macrophages and is crucial for both pathogen survival and virulence in the host organism (Y. Yuan et al. 2002; Hu et al. 2006).

4.5.6. The interplay of sHSPs and disaggregating chaperones

The ability of sHSPs to stabilize unfolded polypeptides provoked obvious concerns about the later fate of trapped polypeptides. In 1998, Veigner and colleagues have shown that *E. coli* IbpB, when present during malate dehydrogenase thermal aggregation, improves further disaggregation by dedicated chaperones (Veinger et al. 1998). This finding, which was also established for eukaryotic sHSPs (Garrett J. Lee et al. 1997), has led to integration of sHSPs as a part of the chaperone network. It became clear that bacterial (and other) sHSPs modulate protein aggregation in order to hold unfolded polypeptides in a refolding-competent state (Matuszewska et al. 2005; Ratajczak, Zietkiewicz, and Liberek 2009).

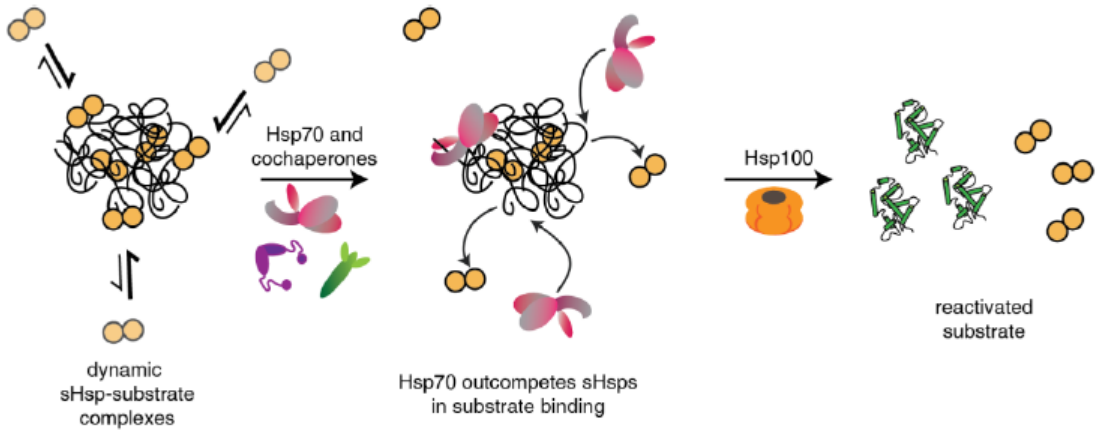

Fig. 5. Schematic model of the Hsp100-Hsp70 bichaperone system action on sHSP–substrate assemblies. Surface-exposed sHSPs dynamically bind and dissociate from the sHSP–substrate assemblies, with the equilibrium shifted towards the bound state. Hsp70 outcompetes sHSPs in binding to the assembly surface, rendering sHSPs in the unbound state. Once the sHSPs are displaced from the surface, the misfolded polypeptides are extracted from the assemblies and refolded by a concerted action of the Hsp100-Hsp70 bichaperone system. Adapted from (Żwirowski et al. 2017).

The details by which sHSPs and disaggregating chaperones interplay was unknown until recently, when Żwirowski et al. (2017) have proposed the mechanism of extraction and

refolding of misfolded polypeptides from sHSPs-substrate assemblies. It was shown that specifically Hsp70 chaperone binds to the assemblies to outcompete sHSPs, which allows to start single polypeptide extraction by the recruited Hsp100 disaggregase [Fig. 5]. The authors suggested a lack of direct interaction of Hsp70 with sHSPs being removed from assemblies - just affinity competition for unfolded polypeptides trapped in assemblies. Additionally, several experiments were performed with yeast proteins suggesting commonness of described mechanism (Żwirowski et al. 2017).

In most Bacteria species, there are usually one or two sHSP-coding genes (Haslbeck and Vierling 2015). As mentioned in the previous sections, in *E. coli* IbpA and IbpB sHSPs act together in counteracting extensive protein aggregation. IbpA is responsible for efficient assembling with aggregating substrate (canonical function) and IbpB seems to have a strong impact on further disaggregation (Ratajczak, Zietkiewicz, and Liberek 2009; Matuszewska et al. 2005; Thomas and Baneyx 1998). Together, they realize the very same task that is fulfilled by a single sHSP in many other species. In the light of the mechanism described by Żwirowski et al. (2017), the yet unresollved IbpB functional cooperation with its canonical partner IbpA in the context of Hsp70/Hsp100-driven disaggregation now appears tackleable.

5. Aim of the project

The goal of the research performed within my doctoral project was to dissect the cooperation of *E. coli* IbpA and IbpB proteins in conferring disaggregation potential onto aggregating substrates. This was attempted by employing the biochemical comparison of *Enterobacterales* 2-sHSPs systems and single-sHSP systems found in their close relatives.

6. Materials

6.1. Strains

***E. coli* DH5α** supE44 _lacU169 (φ80 lacZ_M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1

***E.coli* BL21(DE3)** ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])

***E.coli* BL21(DE3)ΔibpAB** Δibp::kan ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) Source: Tania Baker's lab

E.coli* Mc4100 P_{IPTG}*dnaKJ [araD139D(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR] (PA1/lacO-1 dnaK, dnaJ, lacIq) (Mogk et al. 2003)

6.2. Plasmids

pET-3a vector (69418, Novagen) used for overproduction of $IbpA_{Cn}$, $IbpB_{Cn}$, $IbpA_{Ah}$, AmpR, T7 lac promoter, IPTG-induced

pET-15b vector (69661-3, Novagen) used for overproduction of $IbpA_{Ec}$, $IbpA_{Vh}$ and $IbpA_{Ea}$ AmpR, T7 lac promoter, IPTG-induced

pOPINE vector used for overproduction of luciferase-His, AmpR, T7 lac promoter, IPTG-induced (Berrow et al. 2007)

pBB540 vector used for overproduction of GrpE, CmR, IPTG-regulated promoter PA1/lacO-1 (de Marco et al. 2007)

pCas9cr4 vector expressing Cas9 nuclease for no-SCAR technique (Reisch and Prather 2015)

pKDsgRNA vector for siRNA expression (Reisch and Prather 2015)

pBR322-*ibpAB* vector harbouring whole WT *ibpAB* operon for *in vivo* expression (department collection)

pBR322-*ibpAB-ibpA* R11Stop vector with *ibpA* knock-out (department collection)

pBR322-*ibpAB-ibpB* M12Stop vector with *ibpB* knock-out (department collection)

pBR322-*ibpAB-ibpA*$_{Ea}$ vector with *ibpB* knock-out and *ibpA*$_{Ec}$ seamlessly replaced with *ibpA*$_{Ea}$ (this work)

pBR322-*ibpAB-ibpA*$_{Vh}$ vector with *ibpB* knock-out and *ibpA*$_{Ec}$ seamlessly replaced with *ibpA*$_{Vh}$ (this work)

6.3. Proteins

6.3.1. Chaperones

IbpA$_{Ec}$, IbpA$_{Cn}$, IbpA$_{Vh}$, IbpA$_{Ea}$, IbpA$_{Ah}$, IbpB$_{Cn}$, GrpE - this work

IbpB$_{Ec}$, DnaK, DnaJ and ClpB - departmental collection (Strózecka et al. 2012; Ratajczak, Zietkiewicz, and Liberek 2009; Matuszewska et al. 2005; Żwirowski et al. 2017)

6.3.2. Chaperone substrate proteins

Luciferase (**Luc**) *P. pyralis*, recombinant (Promega)

Luciferase-His (**Luc-His**) - this work

L-Malate Dehydrogenase (L-MDH) from pig heart (mitochondrial, Sigma)

6.4. Antibodies

αIbpA$_{Ec}$ (crossreactive), **αIbpB$_{Ec}$** (crossreactive), **αIbpA$_{Vh}$** (Genscript)

(H+L) HRP conjugated anti-rabbit IgG as secondary antibodies (BIO-RAD)

6.5. Broths

LA 0.5% yeast extract, 1% tryptone, 1% NaCl, 1.5% agar

LB 0.5% yeast extract, 1% tryptone, 1% NaCl, 1.5% agar

6.6. Antibiotics

Ampicillin (100 μg ml^{-1})

Chloramphenicol (20 µg ml^{-1})

Spectynomycin (50 µg ml^{-1})

Anhydratetracyclin (100 ng ml^{-1})

6.7. Oligonucleotides

Name	Sequence 5' -> 3'	Description
f_siRNA_A	CCAATAGCAGAACGGTAAAGgttttagagctagaaatagcaag	no-SCAR siRNA preparation primer, protospacer in capital letters
r_siRNA_A	CTTTACCGTTCTGCTATTGGgtgctcagtatctctatcactga	no-SCAR siRNA preparation primer, protospacer in capital letters
f_siRNA_B	TTGACGCATCAGTGGGGATAgttttagagctagaaatagcaag	no-SCAR siRNA preparation primer, protospacer in capital letters
r_siRNA_B	TATCCCCACTGATGCGTCAAgtgctcagtatctctatcactga	no-SCAR siRNA preparation primer, protospacer in capital letters
OLIGO_A	TTAAACAAACGGTCAAATCCAATAGCAGATTAGTAAAGCGGGGAT AAATCAAAGTTACGCATAATCAATAGCTCC	no-SCAR mutagenic nucleotide for *ibpA*
OLIGO_B	TTTGTCAAAACCGATCCATTGACGTTACAGTGGGGATAAATCGAA GTTACGCATAGTCATTTCTCCTTCTAAGAA	no-SCAR mutagenic nucleotide for *ibpB*
Aop_for	AATCAATAGCTCCTGAAATCAGC	Fast Cloning, for amplifying pBR322_*ibpAB* without *ibpA*
Aop_rev	TTCCCTAAGGCCGCCTG	Fast Cloning, for amplifying pBR322_*ibpAB* without *ibpA*
Erw_for	GATTTCAGGAGCTATTGATTATGCGTAATTTCGACCTTGCC	Fast Cloning. for amplifying $ibpA_{Ea}$ with adaptors
Erw_rev	CGCCAGGCGGCCTTAGGGAATTATTTCACAATTTCAATGCGTCGT GGC	Fast Cloning. for amplifying $ibpA_{Ea}$ with adaptors
Vib_for	GATTTCAGGAGCTATTGATTATGCGTAATGTAGATTTCTCACCA	Fast Cloning. for amplifying $ibpA_{Ea}$ with adaptors
Vib_rev	CGCCAGGCGGCCTTAGGGAATTAGCTGTTTTCAATCAGGTTG	Fast Cloning. for amplifying $ibpA_{Ea}$ with adaptors

7. Methods

7.1. Phylogenetic reconstruction

50 γ-proteobacteria proteomes from OMA database (Altenhoff et al. 2015) were selected with maximum divergence. Using OMA we were able to identify 20,982 Orthology Groups (= OGs; sets of genes in which all representatives are Orthologous to all other members). From these we selected OGs with a minimum of 25 species represented (>50% of species with a member of the OG). To improve the overall quality of the concatenated alignment the 2 least complete or redundant taxa were now deleted (had over 40% of missing data). 1,489 OGs were kept at this stage, aligned using Clustal Omega v1.2.2 and concatenated into 200,800 amino acids alignment (Sievers et al. 2011). The positions with more than 10% of missing data were removed, which resulted in 163,081 amino acids alignment. The γ-proteobacteria species phylogeny was reconstructed using the maximum likelihood (ML) approach using RAxML 8.2.10 (Stamatakis 2014) with general time reversible model of amino acid substitution and GAMMA model of rate heterogeneity (CAT + GTR) with 1,000 ML searches and with 1000 rapid bootstrap replicates. sHSP genes were localized in the bacterial genomes using reciprocal-best-BLAST algorithm using both IbpA and IbpB as a query.

7.2. sHSPs phylogeny

The protein sequences of the IbpA and IbpB orthologs were obtained from OMA Hierarchical Orthologous Groups (Altenhoff et al. 2013). The sequences were aligned using Clustal Omega v1.2.2 with default parameters (Sievers et al. 2011). The alignment was corrected and trimmed manually. To infer protein phylogeny, 10,000 ML searches were performed using RAxML v8.2.10 (Stamatakis 2014) with 100 rapid bootstrap replicates with constrained for species containing both IbpA and IbpB to prevent Long Branch Attraction artifact. LG model of amino acid substitution and GAMMA model of rate heterogeneity with four discrete rate categories and the estimate of proportion of invariable sites (LG + I + G) (Le and Gascuel 2008) was determined as the best-fit model by ProtTest v3.2 following Akaike criterion (Darriba et al. 2011) was selected for the analysis.

7.3. Preparation of *E. coli* competent cells

100 ml of Luria Broth (LB) medium was inoculated with 1 ml of night *E. coli* culture and grown in a shaking water bath at 37°C until OD ≈ 0.5. The culture was then chilled on ice for 20 min and harvested by centrifugation (3000 RCF, 10 min, 4°C). Later, cells were gently resuspended in 50 ml of ice-cold 100 mM $CaCl_2$ solution and left on ice for 20 min. After centrifugation (3000 RCF, 10 min, 4°C) cells were again resuspended in 5ml of 100 mM $CaCl_2$ and incubated on ice for 1 hour. Finally, glycerol was added to the concentration of 10% (v/v) and cells were aliquoted to 100 µl prior freezing at -80°C.

7.4. Plasmid DNA isolation

Plasmid DNA isolation from *E. coli* night cultures was performed with use of standard plasmid DNA isolation kit (A&A Biotech) according to the enclosed protocol.

7.5. Transformation of *E. coli* competent cells

Aliquots of competent cells were thawed on ice and subsequently supplemented with plasmid DNA (20-100 ng per 100 µl of cells) and incubated on ice for 30 min. Then, cells were heat-shocked at 42°C for 45 sec and again incubated on ice for another 2 min. Subsequently, 900 µl of warm LB medium was added to the cells and these were shaked at 37°C for at least 45 min. The cells where then plated on freshly prepared LA medium plates supplemented with antibiotics according to transformed strain and plasmid resistance.

7.6. Protein purification

7.6.1. Luciferase-His

BL21 (DE3) *E coli* cells were transformed with pOPINE plasmid harbouring *P. pyralis* luciferase gene with C-terminal His-tag. Cell culture was grown in 5-liter flasks at 37°C (orbital shaker) until OD ≈ 0.6, when IPTG was added to a final concentration of 0.5 mM. The cells were shifted to 30°C for 3.5h and later grown overnight with shaking at room temperature. Cells were then harvested by centrifugation (5000 RCF, 8 min, 4°C) and resuspended in buffer A (10% Glycerol; 150 mM NaCl; 50 mM Tris-HCl pH 7.5; 25 mM Imidazole; 5 mM -Mercaptoethanol) prior to lysis with French cell press under 20 000

psi. The lysate was then centrifuged (50 000 RCF; 30 min; 4°C) and the supernatant was applied to 5 ml HisTrap™ prepacked collumn (GE Healthcare). The resin was then extensively washed with buffer A and eluted with a linear gradient of buffer B (10% Glycerol; 150 mM NaCl; 50 mM Tris-HCl pH 7.5; 500 mM Imidazole; 5 mM 2-Mercaptoethanol). Samples containing pure Luc-His were then dialysed against buffer C (20% Glycerol; 150 mM KCl; 50 mM Tris-HCl pH 7.5; 20 mM $MgCl_2$; 2 mM DTT) and stored at -80°C.

7.6.2. *E. coli* IbpA

E. coli BL21 (DE3) *ΔibpAB* competent cells were transformed with pET15b-ibpAEc plasmid harbouring WT *E. coli ibpA* gene. The culture was grown in 5-liter flasks in an orbital shaker at 37°C. After the cultures have reached OD = 0.6, they were supplemented with IPTG to a final concentration of 0.5 mM. The cells were grown for another 2 h in 37°C, then were harvested (5000 RCF, 8 min, 4°C). Bacterial pellet was then gently resuspended in buffer D (50 mM Tris-HCl pH 7.4; 100 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol; 0,5 mM EDTA) and lysed with French press under 20,000 psi. The lysate was then centrifuged (50 000 RCF, 60 min, 4°C) and the pellet was resuspended in buffer E (50 mM Tris-HCl pH 7.4; 50 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol; 2 M Urea) and shaked for 1 h at 4°C, followed by centrifugation as above. Next, the pellet was resuspended in buffer F (50 mM Tris-HCl pH 7.4; 50 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol; 6 M Urea), shaked for 1 h at 4°C and centrifuged as above. The supernatant was then diluted 4-fold with buffer F and loaded onto 20 ml Q-Sepharose resin (GE Healthcare). The resin was extensively washed with buffer F and eluted with linear buffer G (50 mM Tris-HCl pH 7.4; 500 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol; 6 M Urea) gradient. Fractions containing pure $IbpA_{Ec}$ were pooled and dialysed against buffer H (50 mM Tris-HCl pH 8,2; 100 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol; 6 M Urea) for 2h, then dialysed stepwise (2h for each step) against buffer H and I mixed (50 mM Tris-HCl pH 8,2; 100 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol) in 2:1, 1:2 ratio and next against buffer I only. Finally, the sample was dialysed against buffer J (50 mM Tris-HCl pH 7,4; 150 mM KCl; 10% Glycerol; 5 mM 2-Mercaptoethanol) and stored at -80°C.

7.6.3. *E. coli* IbpB, DnaK, DnaJ and ClpB

DnaK, DnaJ, ClpB and $IbpB_{Ec}$ (both WT and His-tagged) were from the laboratory collection, purified for earlier conducted studies (Strózecka et al. 2012; Ratajczak, Zietkiewicz, and Liberek 2009; Matuszewska et al. 2005; Żwirowski et al. 2017).

7.6.4. *V. harveyi* and *E. amylovora* IbpA

E. coli BL21 (DE3) *ΔibpAB* competent cells were transformed with pET15b-ibpAVh plasmid harbouring WT *V. harveyi ibpA* gene. The culture was grown in 5-liter flasks in an orbital shaker at 37°C. After the cultures have reached OD = 0.6, they were supplemented with IPTG to a final concentration of 0.5 mM. The cells were grown for another 2 hat 37°C, then were harvested (5000 RCF, 8 min, 4°C). Bacterial pellet was then gently resuspended in buffer K (50 mM Tris-HCl pH 7.4; 50 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol; 0,5 mM EDTA) and lysed with French press under 20,000 psi. The soluble fraction was applied onto Q-Sepharose resin and flow-through fractions containing IbpAVh were dialysed against buffer F prior to a next round of Q Sepharose chromatography in denaturing conditions (elution with buffer G linear gradient). Fractions containing $IbpA_{Vh}$ were then dialysed against buffer F and resubjected to Q Sepharose chromatography, eluted with a linear gradient of buffer L (50 mM Citrate buffer pH 5.0; 50 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol; 6 M Urea). Fractions containing pure $IbpA_{Vh}$ were then dialysed against buffer M (50 mM Tris-HCl pH 8.5, 150 mM KCl, 10% Glycerol, 5 mM 2-Mercaptoethanol) and stored at -80°C . *E. amylovora* IbpA ($IbpA_{Ea}$) was purified according to the foregoing protocol, however the final dialysis against buffer M was preceded by additional dialysis against buffer H.

7.6.5. *A. hydrophila* and *C. neteri* IbpA

E. coli BL21 (DE3) *ΔibpAB* competent cells were transformed with pET3a-ibpAAh plasmid harbouring synthetic (optimized for *E. coli* expression) *A. hydrophila ibpA* gene (Genscript). The culture was grown in 5-liter flasks in an orbital shaker at 37°C. After the culture has reached OD = 0.6, it was supplemented with IPTG to a final concentration of 0.5 mM. The cells were grown for another 2 h at 37°C, then were harvested (5000 RCF, 8 min, 4°C). Bacterial pellet was then gently resuspended in buffer D and lysed with French press under 20,000 psi. The lysate was then centrifuged (50 000 RCF, 60 min, 4°C) and

the pellet was resuspended in buffer F and shaked for 1 h at 4°C followed by centrifugation as above. The supernatant was then diluted 2-fold with buffer F and loaded onto Q-Sepharose resin (GE Healthcare). The resin was extensively washed with buffer F and eluted with linear buffer G gradient. Fractions containing pure protein were then dialysed against buffer M and stored at -80°C. *C. neteri* IbpA ($IbpA_{Cn}$) was purified according to the foregoing protocol.

7.6.6. *C. neteri* IbpB

E. coli BL21 (DE3) *ΔibpAB* competent cells were transformed with pET3a-ibpBCn plasmid harbouring synthetic (optimized for *E. coli* expression) *C. neteri ibpB* gene. The culture was grown in 5-liter flasks in an orbital shaker at 37°C. After the culture has reached OD = 0.6, it was supplemented with IPTG to a final concentration of 0.5 mM. The cells were grown for another 2 h in 37°C, then were harvested (5000 RCF, 8 min, 4°C). Bacterial pellet was then gently resuspended in buffer D and lysed by French press under 20,000 psi. The lysate was then centrifuged (50 000 RCF, 60 min, 4°C) and proteins from supernatant were salted-out by adding NH_4SO_4 to 50% saturation. After 1h incubation at 10°C, the protein pellet was centrifuged (50 000 RCF, 45 min, 4°C) and later dissolved in buffer F. The sample was then applied onto Q-Sepharose resin (GE Healthcare), subsequently washed with buffer F and eluted with a linear gradient of buffer G. Samples containing $IbpB_{Cn}$ were then dialysed against buffer N (50 mM Tris-HCl pH 8.5, 50 mM KCl, 10% Glycerol, 5 mM 2-Mercaptoethanol), negatively purified with Q-Sepharose resin and finally dialysed against buffer M and stored at -80°C.

7.6.7. GrpE

BL21 (DE3) *E coli* cells were transformed with pBB540 plasmid harbouring WT *E. coli grpE* gene under the control of Plac promoter (de Marco et al. 2007). Cell culture was grown in 5-liter flasks at 37°C (orbital shaker) until OD ≈ 0.6, when IPTG was added to a final concentration of 0.5 mM. After another 2h cells were harvested (5000 RCF, 8 min, 4°C), resuspended in buffer K and lysed with French press under 20,000 psi. The lysate was then centrifuged (50 000 RCF, 60 min, 4°C) and both used WT and His-tagged he supernatant was applied onto Q-Sepharose resin (GE Healthcare), washed with buffer K and eluted with linear gradient of buffer O (50 mM Tris-HCl pH 7.4; 500 mM NaCl; 10% Glycerol; 5 mM 2-Mercaptoethanol). Fractions containing GrpE were then dialysed

against buffer P (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 25 mM Imidazole; 10% Glycerol; 5 mM 2-Mercaptoethanol). Simultaneously, 5 ml HisTrap™ prepacked column (GE Healthcare) was saturated with ~50 mg of His-DnaK (lab collection), washed with 2 column volumes of 5 mM ATP in buffer P and equilibrated with buffer P. The sample containing GrpE was then applied onto the prepared column, extensively washed with buffer P and eluted stepwise with 10 mM ATP in buffer P. Fractions containing pure GrpE were then dialysed against buffer J and stored at -80°C. His-DnaK was eluted stepwise with 500mM Imidazole in buffer P and stored for further use at -80°C.

7.7. Drop test experiment

7.7.1. Plasmid preparation

pBR322-*ibpAB*-*ibpB*11Stop plasmid (department collection) was a base for seamless *ibpA* replacements for native promoter expression in *E. coli*. Genes were replaced with FastCloning method (C. Li et al. 2011) using primers listed in 'Materials' and Pfu Turbo II polymerase (Sigma), always with annealing temperature of 53°C and standard PCR conditions suggested by the polymerase producer.

7.7.2. Drop test

E. coli MC4100 P_{IPTG} *dnaKJ ΔibpAB* strains (Mogk et al. 2003) carrying pBR322 plasmids with cloned *E. coli ibpAB* operon or with stop codon introduced in either *ibpA* or *ibpB* gene (F4Amber in both cases) or with *ibpA* gene seamlessly replaced with *E. amylovora* or *V. harveyi ibpA* (additionally with stop codon in *ibpB* as above) were grown at 30°C in LB supplemented with 1mM IPTG until OD≈1. Serial dilutions were plated on LA medium supplemented with 100 µM, 75 µM, 50 µM or without IPTG and grown for 24 h in 37°C.

7.8. *In vitro* disaggregation experiments

Luciferase (1.5 µM, Promega) was denatured at 44°C for 10 min in buffer Q (50 mM Tris-HCl pH 7.4; 150 mM KCl; 20 mM $MgCl_2$; 2 mM DTT) in the presence or absence of 10 µM sHSPs as indicated (3 µM $IbpA_{Ec}$ and 7 µM $IbpB_{Ec}$ in case of $IbpAB_{Ec}$) and subsequently shifted to room temperature. Protein refolding was started by 40-fold dilution of denatured luciferase in the Hsp70-Hsp100 chaperone cocktail. Unless noted

otherwise, the chaperone concentrations used were as follows: Limiting Hsp70 - DnaK 0.7 μM, DnaJ 0.28 μM, GrpE 0.21 μM, ClpB 2 μM; saturating Hsp70 - DnaK 3.5 μM, DnaJ 1.4 μM, GrpE 1.05 μM, ClpB 2 μM. All assays were performed in the presence of an ATP-regenerating system (18 mM creatine phosphate, 0.1 mg/ml creatine kinase, 5 mM ATP). The disaggregation reaction was carried out at 25°C. Luciferase activities were measured at time points using a Sirius Luminometer (Berthold) and presented as a mean ±SD from at least 3 independent experiments.

7.9. DLS measurements

Particle size was determined using Malvern Instruments ZetaSizer Nano S dynamic light scattering instrument. Measurements were taken in buffer Q. Luciferase (Promega) was present at a fixed 1.5 μM concentration and sHSP concentrations ranged from 0 to 10 μM. The conditions were as follows: measurement volume - 40 μl, scattering angle - 173°, wavelength - 633 nm, temperature - 25°C. For every data point minimum 10 measurements of ten 10s runs were averaged and particle size distribution was calculated by fitting to 70 size bins between 0.4 and 10,000 nm. Results are shown as average diameter of the main peak with SD (at least 80% of total measured particle mass was contained in the main peak) plotted against sHSP concentration. $IbpA_{Ec}$ and $IbpB_{Ec}$ ($IbpAB_{Ec}$) were always used in a fixed 1:2 ratio when together, the concentration depicted is for both proteins in total.

7.10. Analysis of assemblies formation by sedimentation

Luciferase (1 μM, Promega) was denatured at 44°C for 10 min in buffer Q in the presence of 5 μM sHSPs (1.66 μM $IbpA_{Ec}$ and 3,34 μM $IbpB_{Ec}$ in case of $IbpAB_{Ec}$). To verify the ability of sHSPs to form assemblies, 150 μl of each sample was applied on a 3.6 ml 10–60% glycerol gradient in buffer U (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM Mg acetate, 2 mM DTT). The samples were then centrifuged at 10°C in a Beckman SW 60 rotor at 160,000g for 1 h, fractions were collected from the top. Protein distribution in each fraction was verified by SDS–PAGE followed by Oriole (Bio-Rad) fluorescent staining.

7.11. Static light scattering analysis of aggregation

Luciferase (1.5 μM, Promega), malate dehydrogenase (2 μM, Sigma) or citrate synthase (2 μM, Sigma) was mixed with $IbpA_{Ec}$ (3 μM), $IbpB_{Ec}$ (7 μM), both $IbpA_{Ec}$ and $IbpB_{Ec}$ (3 μM and 7 μM respectively) or without sHSPs at room temperature (0°C in case of luciferase) and injected to preheated (temp. as indicated in figure) spectrofluorometric cuvettes prior to scattering measurement. Scattering wavelengths were 605 nm for luciferase and citrate synthase and 565 nm for malate dehydrogenase.

7.12. BLI experiments

BLI experiments were performed on BLItz device using Dip and Read™ Ni-NTA (NTA) Biosensors (ForteBio) at room temperature with 2000 rpm mixing. Basal anchoring luciferase layer was immobilized on the sensor in denaturing conditions with 0.6 mg/ml His-tagged luciferase in buffer W (6M Urea, 50 mM Tris pH 7.5, 150 mM KCl, 20 mM $MgCl_2$, 2mM DTT) for 15 min. Excess luciferase and denaturant were washed away with buffer Q. The top luciferase aggregate layer was formed by transferring the sensor with the anchoring layer to a test tube with 0.1 mg/ml His-tagged luciferase in buffer Q and subsequent incubation at 44°C for 10 min. Next, the sensor was transferred back to the BLItz device where non-bound luciferase aggregates were washed away in buffer Q for 10 min. 5 μM sHSPs in buffer Q (or 1.66 μM $IbpA_{Ec}$ with 3,34 μM $IbpB_{Ec}$ in case of $IbpAB_{Ec}$) were heat-activated at 44°C for 5 min and immediately transferred to the BLItz instrument. sHSPs were allowed to bind the immobilized luciferase aggregate for 10 min then dissociated either in buffer Q or in the presence of Hsp70 chaperone system (limiting and saturating concentration) for 1 h. Proteins bound to the sensor were stripped with Laemmli buffer before and after Hsp70 system incubation (separate experiment) and subjected to SDS-PAGE followed by Western blot or Oriole® staining.

7.13. Hsp70-dependent release of sHSPs from assemblies

Luciferase (3 μM, Promega) was denatured at 44°C for 10 min in buffer Q in the presence of $IbpA_{Ec}$ (6 μM) with and without His-$IbpB_{Ec}$ (14 μM). Alternatively, 1.5 μM Luciferase was denatured at 44°C for 10 min in buffer D in presence of: 3 μM $IbpA_{Cn}$ with or without 7 μM $IbpB_{Cn}$ or 10 μM $IbpA_{Vh}$ or 10 μM $IbpA_{Ah}$

To isolate the IbpA/IbpAB–luciferase assemblies from unbound sHSPs, 400 µl of preformed assemblies was applied on a 3.4 ml 10–60% glycerol gradient in buffer U. Samples were centrifuged at 10°C in a Beckman SW 60 rotor at 160,000g for 1 h, fractions were collected from the top. Protein distribution in each fraction was verified by SDS–PAGE followed by Oriole (Bio-Rad) fluorescent staining [Figs. 21, 23A]. Fractions containing IbpAB- and IbpA-luciferase assemblies were pooled together and the substrate concentration was determined by optical densitometry following SDS–PAGE at 21 ng/µl for Luc-IbpAB$_{Ec}$, 6 ng/µl for Luc-IbpA$_{Ec}$, 29 ng/µl for Luc-IbpAB$_{Cn}$, 33 ng/µl for Luc-IbpA$_{Cn}$, 42 ng/µl for Luc-IbpA$_{Vh}$ and 42 ng/µl for Luc-IbpA$_{Ah}$ relative to the luciferase concentration. To analyse the effect of chaperones on isolated assemblies, the assemblies were diluted to 1.5 ng/µl of luciferase in limiting and saturating Hsp70 system (DnaK 0.7 µM, DnaJ 0.28 µM, GrpE 0.21 µM or DnaK 3.5 µM, DnaJ 1.4 µM, GrpE 1.05 µM respectively), incubated for 45 min at room temp. and subjected to a second round of sedimentation under the conditions listed above. Fractions containing unbound sHSPs (fractions 1-2; 1 for IbpA$_{Ah}$) and sHSPs-luciferase assemblies (fractions 4-7 for IbpA$_{Ec}$ and IbpAB$_{Ec}$; 3-5 for IbpA$_{Cn}$ and IbpA$_{Vh}$; 4-6 for IbpAB$_{Cn}$ and 2-4 for IbpA$_{Ah}$) were pooled and precipitated with trichloroacetic acid. Pellets were then resuspended in Laemmli buffer and together with the bottom fraction analysed by Western blot using antibodies against IbpA$_{Ec}$ (for IbpA$_{Ec}$; IbpA$_{Cn}$ and IbpA$_{Ah}$) and against IbpA$_{Vh}$. The experiment employing citrate synthase as a sHSPs substrate was performed as above, however, the initial denaturation temperature was 52°C instead of 44°C.

7.14. Isolation of aggregated protein fraction

E. coli MC4100 *ΔibpA* and *ΔibpB* strains were developed by introducing single stop codons on the chromosomal DNA at positions M12 (*ibpB*) and R11 (*ibpA*) respectively using no-SCAR method (Reisch and Prather 2015), for mutagenic nucleotides and siRNA see 'Materials'. *E. coli* MC4100 *ΔibpAB* strain was kindly provided by B. Bukau.

E. coli strains were cultured at 30°C in Luria Broth (LB). Temperature-shift experiments were performed in shaking water baths. The cells were grown overnight, diluted 1:50 in fresh LB and grown until OD = 1. Next, the cultures were pre-shocked at 42°C for 10 min, then heat-shocked at 48°C for 5 min. Finally, the cells were shifted to 30°C for recovery. Aliquots of bacterial cultures (10 ml) were rapidly cooled to 0°C in ice-water bath and centrifuged for 6 min at 4000g to harvest the cells. Pellets were resuspended in

80 µl of buffer X (10 mM KPi pH 6.5; 1 mM EDTA; 20% Sucrose; 1 mg/ml Lysozyme) and incubated on ice for 30 min. The cells were then lysed by addition of 700 µl of buffer Y (10 mM KPi pH 6.5; 1 mM EDTA) and sonicated (Qsonica Q700, tip no. 4418, amplitude 4). Cell debris was removed by 5 min centrifugation at 1000g at 4°C. The supernatant was supplemented with IGEPAL CA-630 non-ionic detergent to a final concentration of 4% (v/v) and vortexed vigorously. The aggregated protein fraction was harvested by centrifugation at 20000 g at 4°C for 20 min. Pellet fractions were washed twice with buffer Z (10 mM KPi pH 6.5; 1 mM EDTA; 5% (v/v) IGEPAL CA-630) in order to remove the membrane fraction and finally washed with buffer Y for detergent removal. The resulting pellet was resuspended in 40 µl of Laemmli buffer prior to SDS-PAGE and WB analysis.

8. Results

E. coli, a very well studied model organism, express 2 sHSPs, whose genes are arranged in single, heat shock-inducible operon. They were named as inclusion body associated proteins A and B (IbpA and IbpB) since first they were found to be associated inclusion bodies (Allen et al. 1992). Only later they were shown to cooperate against irreversible protein aggregation (Laskowska, Wawrzynów, and Taylor 1996). Although they were studied by several research groups, the nature of their cooperation still remains elusive. In order to tackle this issue, I decided to diversify my approach by reinforcing the classical biochemistry with evolutionary analysis of sHSPs from related species.

8.1. Evolutionary history of sHSPs in γ-proteobacteria

While *E. coli* possesses two sHSP-coding genes, multiple other γ-proteobacteria possess only one sHSPs gene. This might suggest that these modern-day chaperone systems have been shaped by gene duplication or gene loss event/s. To trace these hypothetical event/s, we carried out evolutionary analysis of γ -proteobacteria and their sHSPs.

Since the internal phylogeny of γ-proteobacteria was not fully resolved, and the position of one of the key phyla, *Erwiniaceae*, remained debated (Lerat, Daubin, and Moran 2003; Williams et al. 2010; Gao, Mohan, and Gupta 2009; De Maayer and Cowan 2016), we improved on previous phylogenetic analyses by fully sampling 50 γ-proteobacteria genomes and obtained a tree of γ-proteobacteria with strong statistical support (all nodes with at least 99% bootstrap support)[Figs. 6A, S1]. Consistently with the recent analysis (Adeolu et al. 2016), *Erwiniaceae* (the family of *Erwinia amylovora*) and *Enterobacteriaceae* (the family of *E. coli*) have been reconstructed as sister groups within *Enterobacterales* (the taxonomic subgroup of γ-proteobacteria). We found that *Enterobacterales*, except of *Erwiniaceae*, possess two sHSPs (IbpA and IbpB) which is in contrast to other γ-proteobacteria taxa (*Vibrionaceae*, *Aeromonadaceae*, *Psychomonadaceae*, *Shewanellaceae*), wich express only one sHSP [Fig. 6A].

To further investigate sequence the evolution, we reconstructed the phylogeny of sHSPs. We deduced that there had been a single *ibpA* gene in basal γ-proteobacteria species, which underwent duplication in an ancestor of *Enterobacterales.* This resulted in the

emergence of an operon composed of *ibpA* and *ibpB* that is found in modern-day *Enterobacterales* [Fig. 6A]. After the duplication event, *ibpB* sequences evolved faster than *ibpA* [Figs. 6B, S2], indicating that IbpB protein might have become also functionally divergent from IbpA. Additionally, the data indicates that *ibpB* has been lost in the *Erwiniaceae* clade, and only post-duplication single *ibpA* is present there [Figs. 6A, S2].

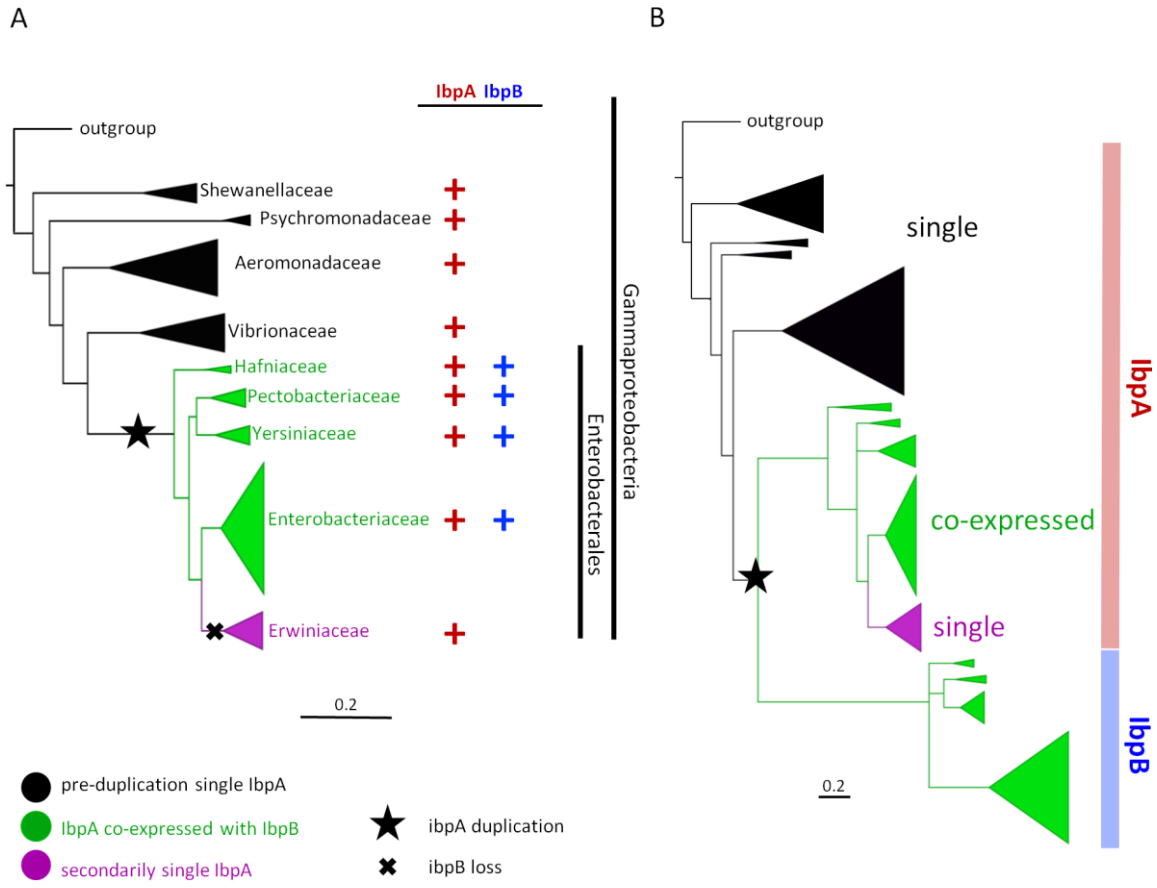

Fig. 6. Evolutionary history of sHSPs in γ-proteobacteria. (A) The distribution of sHSP genes (*ibpA* red; *ibpB* blue) at the species phylogeny of γ-proteobacteria (calculated with a supermatrix of 1500 orthologs from 50 γ-proteobacteria genomes using Maximum Likelihood method with GTR+GAMMA model) indicates the presence of a single copy *ibpA* (black) in γ-proteobacteria before the speciation of Enterobacterales, both *ibpA* and *ibpB* (green) are co-expressed in the subset of *Enterobacterales* (*Hafniaceae, Pectobacteriaceae, Yersiniaceae, Enterobacteriaceae*), and *ibpB* is absent in *Erwiniaceae* (purple). Based on parsimony two evolutionary events in γ-proteobacteria were inferred, the duplication of *ibpA* at the base of *Enterobacterales* (star) and loss of *ibpB* in *Erwiniacea* (cross). (B) The evolutionary history of sHSPs (IbpA red; IbpB blue) in γ-proteobacteria, calculated with Maximum Likelihood method with CAT+I+LG model, indicates the presence single duplication event at the base of *Enterobacterales*, resulting in a presence of both IbpA and IbpB in *Hafniaceae, Pectobacteriaceae, Yersiniaceae, Enterobacteriaceae* (green). In all these lineages *ibpB* evolved faster than *ibpA*, but no *ibpB* is present in *Erwiniacea* (purple). Scale bar indicates number of substitution per site. Calculations performed by M. Stolarska and B. Tomiczek.

I*n vitro* studies of the *E. coli* proteins show that IbpB, contrary to IbpA, does not form characteristic sHSP-substrate assemblies (Ratajczak, Zietkiewicz, and Liberek 2009). Why then, the second copy of sHSP gene has been maintained in the majority of the families within the *Enterobacterales* order? To understand the nature of IbpA and IbpB functional divergence I decided to compare their biochemical activities with single small heat shock protein systems found in *Enterobacterales* relatives. I took advantage of the reconstructed evolutionary history of sHSP genes in γ-proteobacteria to select proteins representative for a sHSP that has always been single ($IbpA_{Vh}$ from *Vibrio harveyi*), post-duplication IbpA and IbpB ($IbpA_{Ec}$ and $IbpB_{Ec}$ from *E. coli*) and post-duplication secondarily single IbpA ($IbpA_{Ea}$ from *Erwinia amylovora,* where IbpB has been lost).

8.2. IbpB, in contrast to IbpAs, does not suppress the phenotype

In order to maintain the second gene copy after duplication, it is indispensable for the additional gene to become vital or at least provide additional fitness for the organism. However, before the after-duplication divergence, it is vulnerable to loss as there is no selective pressure to maintain the two identical copies. Paradoxically, it is this lack of purifying pressure that also allows random mutations (sometimes otherwise deleterious) to be tolerated. By this mechanism the second gene copy becomes unrestrained, allowing it to be driven aside from its previous function optimum, exploring both the mutational and the functional space. This process still may lead to gene loss or pseudogenization (when there is not much pressure for the genome size e.g. in eukaryotes) or increase of system complexity without affecting the function (Finnigan et al. 2012), but can also give rise to some key mutations that will later drive the second gene copy towards fitness-inducing functional changes (Wagner 1998).

In order to investigate the functional perturbations in two-sHSPs system, I took advantage of an *E. coli* strain developed by Mogk and colleagues (Mogk et al. 2003), in which the *ibpAB* operon has been deleted and *dnaK* and *dnaJ* are under the *pLac* promoter - allowing to regulate their expression. I evaluated this expression regulation myself, showing WT-like Hsp70 levels in the cells when grown with 75μM IPTG [Fig. 7].

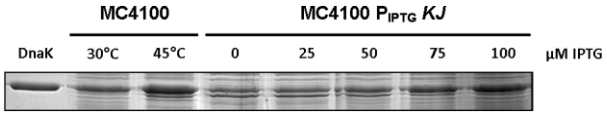

Fig. 7. DnaK expression in *E. coli* MC4100 P_{IPTG} *dnaKJ ΔibpAB* strain. *E. coli* cells were grown in LB supplemented with chloramphenicol at 30°C overnight. Cultures were then diluted in fresh LB with chloramphenicol and indicated concentration of IPTG and grown in 37°C for 3 h prior harvesting. Cells were then subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

Using this strain, the authors have developed a drop-test assay, where they could easily observe double knock-out Δ*ibpAB* phenotype, but only when *dnaK* and *dnaJ* expression was reduced compared to the wild type strain. I expanded their approach by including single sHSP knock-outs (Δ$ibpA_{Ec}$ and Δ$ibpB_{Ec}$) and heterologus sHSP genes that represent sHSPs systems that either lost their IbpB partner ($ibpA_{Ea}$) or have never had one ($ibpA_{Vh}$) in their evolutionary history. In order to achieve that, I cloned the native $ibpAB_{Ec}$ operon onto pBR322 plasmid, introducing stop codons to disrupt the expression of each sHSP and/or replacing homologus $ibpA_{Ec}$ with heterologus $ibpA_{Ea}$ or $ibpA_{Vh}$ genes. Next I used these plasmids to supplement chromosomal Δ*ibpAB* knock-out in *E. coli* cells with regulated *dnaK* and *dnaJ* expression.

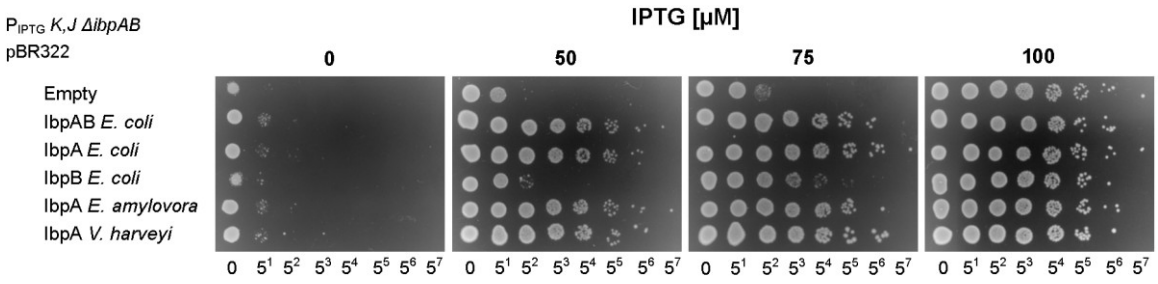

Fig. 8. IbpA-like but not IbpB small heat shock proteins complement Δ*ibpAB* phenotype in *E. coli* with reduced DnaK levels. *E. coli* P_{IPTG} *dnaKJ* strains expressing indicated sHSPs from pBR322 plasmid were grown at 30˚C in presence of 1 mM IPTG until late logarithmic phase. 5-fold serial dilutions of bacterial cultures were plated on LA medium supplemented with indicated concentration of IPTG and grown at 37˚C overnight.

As reported (Mogk et al. 2003), expression of $IbpAB_{Ec}$ is capable of rescuing the cells grown with lowered DnaK and DnaJ levels. Interestingly, after splitting the two-sHSPs system, $IbpA_{Ec}$, but not $IbpB_{Ec}$ is capable of providing the observed phenotype rescue

[Fig. 8] - even though $IbpB_{Ec}$ was highly overexpressed [Fig. 9]. Both tested heterologus IbpAs ($IbpA_{Ea}$ and $IbpA_{Vh}$) were also noticeably expressed [Fig. 9], however, in contrast to $IbpB_{Ec}$, provide complete *E. coli* cells growth restoration [Fig. 8]. Thus, all IbpA proteins must share common functional traits regardless of the existence of their IbpB partner and the latter most probably provides some other features that seem to be irrelevant for this assay.

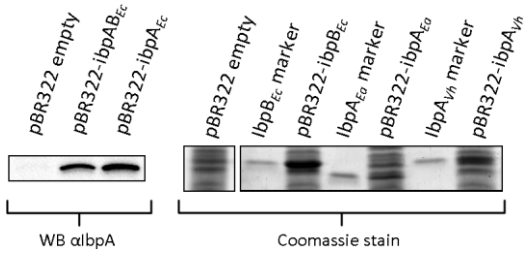

Fig. 9. sHSPs levels in drop test experiment. *E. coli* MC4100 P_{IPTG} *dnaKJ ΔibpAB* strains expressing indicated sHSPs from pBR322 plasmid were grown in LB medium supplemented with ampicillin and 100 μM IPTG at 37°C until late logarithmic phase. Cells were harvested and subjected to SDS-PAGE and Western blot analysis. Purified sHSPs were used as markers for respective sHSP

8.3. IbpB alongside IbpA facilitates potent Hsp70-dependent disaggregation

Interestingly, the phenotype exploited in [Fig. 8] can only be investigated in quite a narrow range of cellular DnaK levels. Cells growth is almost completely abolished at 0 μM IPTG and fully supported at 100 μM IPTG irrespective of the expressed sHSP [Fig. 8]. In light of this obvious dependence, I turned to the process, where DnaK meets sHSPs - protein disaggregation. Presence of sHSPs during protein aggregation alters the morphology of resulting aggregates and influences their subsequent disaggregation (Mogk and Bukau 2017). Although *E. coli* IbpA and IbpB interplay in this process was already exploited in literature (Matuszewska et al. 2005; Ratajczak, Zietkiewicz, and Liberek 2009; Żwirowski et al. 2017), it was never looked at in the context of related single-sHSP systems. Here, I directly compare several single and two sHSP systems in terms of their influence on disaggregation rates and effectiveness. Although *E. coli* Hsp70-Hsp100 systems are heterologus to $IbpA_{Ea}$ and $IbpA_{Vh}$, sHSPs were generally shown not to require homologus Hsp70 for disaggregation as there is no direct interaction involved between them (Żwirowski et al. 2017). Additionally, both $IbpA_{Ea}$ and $IbpA_{Vh}$ already proved themselves fully functional in *E.coli* cells [Fig. 8].

Based on our previous work with IbpAB$_{Ec}$ and firefly luciferase as a model protein substrate (Żwirowski et al. 2017), I chose two concentrations of the Hsp70 system for disaggregation experiments: limiting KJE (0.7 µM DnaK, 0.28 µM DnaJ, 0.21 µM GrpE) and saturating KJE (3.5 µM DnaK, 1.4 µM DnaJ, 1.05 µM GrpE). I heat-aggregated luciferase alone or together with each sHSP/s (IbpA$_{Vh}$, IbpA$_{Ea}$, IbpA$_{Ec}$, IbpB$_{Ec}$ or IbpAB$_{Ec}$) and then assessed the efficiency of luciferase recovery by aforementioned Hsp70 systems together with ClpB disaggregase - that reconstitute a fully functional *E. coli* disaggregating and refolding machinery.

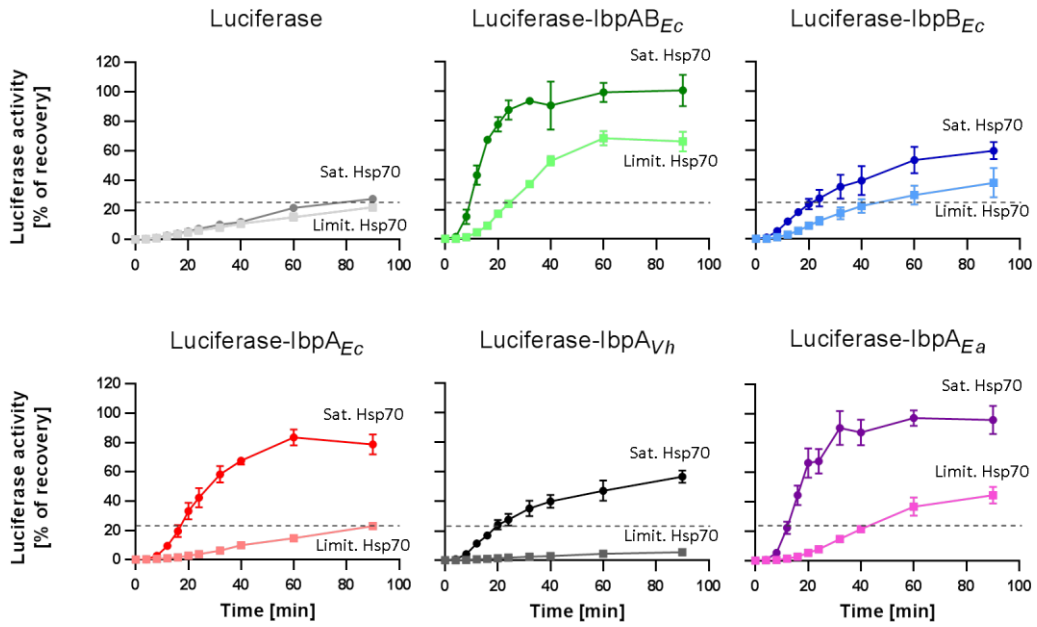

Fig. 10. Refolding potential of sHSPs-substrate assemblies. Luciferase (1.5 µM) was denatured in the presence of 10 µM sHSPs (IbpAB$_{Ec}$ was 3.33 µM IbpA$_{Ec}$ and 6.67 µM IbpB$_{Ec}$) and refolded at limiting (DnaK 0.7 µM; DnaJ 0.28 µM; GrpE 0.21 µM) or 5x higher saturating (DnaK 3.5 µM; DnaJ 1.4 µM; GrpE 1.05 µM) Hsp70 machinery concentrations and ClpB at 2 µM concentration. Data are the mean ± SD of three independent experiments.

Using limiting KJE, I was able to recover up to 20% of luciferase aggregated in the absence of sHSPs. The presence of IbpAB$_{Ec}$ in the same conditions allowed up to 70% recovery. IbpB$_{Ec}$ alone and IbpA$_{Ea}$ (that has lost its IbpB partner across evolution) were also beneficial for the process, providing ~40% luciferase activity recovery. On the other hand, the presence of IbpA$_{Ec}$ and IbpA$_{Vh}$ provided either no improvement or even disaggregation inhibition compared to luciferase aggregated alone [Fig. 10]. Increasing KJE concentration by the factor of 5 (saturating KJE) does not improve recovery of

luciferase aggregated in the absence of sHSPs, which may indicate, that the substrate (aggregate) binding sites are saturated. Contrarily, IbpAB$_{Ec}$-luciferase aggregates are now disaggregated with very high rate and efficiency, reaching 100% recovered luciferase activity in 30min. The very same tendency is now observed for IbpA$_{Ea}$, but aggregates containing just IbpB$_{Ec}$ are disaggregated only slightly better. Surprisingly, now luciferase from both inhibited samples of IbpA$_{Ec}$ and IbpA$_{Vh}$ is being effectively recovered - at the level similar to IbpAB$_{Ec}$-luciferase observed at limiting KJE concentration [Fig. 10]. Summing up, Hsp70 system seems to be the limiting factor in disaggregation from aggregates/assemblies formed in the presence of any IbpA protein, although to a different extent. In the two-protein system, the presence of IbpB alongside its IbpA partner during aggregation substantially lowers the demand for Hsp70 in refolding.

In literature, IbpB$_{Ec}$ was shown to *in vivo* protect several basic metabolism enzymes from inactivation (Kitagawa et al. 2002), therefore I decided to additionally check if this effect might introduce relevant bias in the very effective IbpAB$_{Ec}$-luciferase disaggregation described above. Once again I heat-aggregated luciferase in the presence or absence of IbpAB$_{Ec}$ and traced luciferase activity loss in the very same aggregation conditions. I could not observe any differences in luciferase inactivation patterns [Fig. 11], therefore I can surely attribute the high luciferase activity observed in IbpAB$_{Ec}$-luciferase sample to somehow introduced disaggregation potential but not to luciferase activity protection.

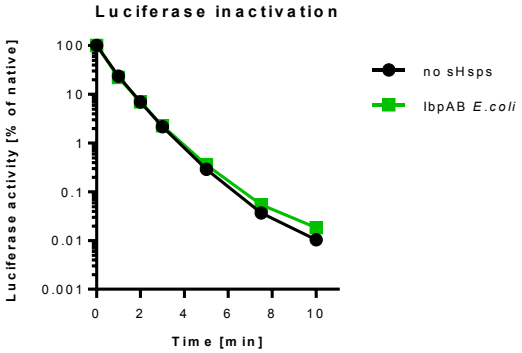

Fig. 11. Luciferase activity protection. Luciferase (1.5 μM) was denatured in the presence or absence of IbpAB$_{Ec}$ (3.33 μM IbpA$_{Ec}$ and 6.67 μM IbpB$_{Ec}$) Along denaturation, Luciferase was sampled and assayed for its residual activity. Data is depicted as percentage of initial activity.

8.4. IbpA proteins, but not IbpB, can form sHSPs-substrate assemblies

Scavenging and securing unfolding proteins in assemblies is a hallmark of sHSPs functioning in protein quality control network. sHSPs-substrate assemblies serve as safe-storage, preventing polypeptides from amorphous aggregation until the conditions become favourable for active protein refolding. Since sHSPs-substrate assemblies are a source of polypeptides to be recovered, their properties are more than expected to impact later substrate disaggregation. One of such suspected impacts may be the resulting substrate-sHSP assembly size, theoretically providing much more binding sites for Hsp70-Hsp100 machinery, as assemblies are considerably smaller than amorphous aggregates (higher surface/volume ratio). Thus one could expect that the smaller assemblies, the more efficient substrate disaggregation.

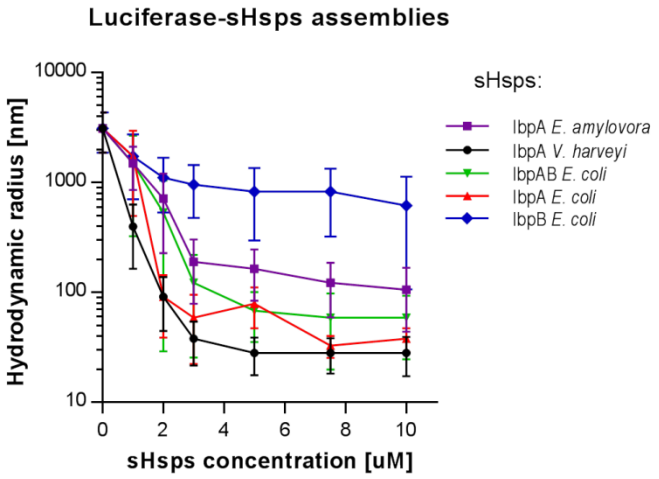

Fig. 12. DLS measurements of sHSPs-luciferase assemblies size. sHSPs-luciferase assemblies were prepared as follows: luciferase at a fixed 1.5 µM concentration in the presence of sHSPs (0 to 10 µM as depicted) was denatured at 44˚C for 10 min. When $IbpA_{Ec}$ and $IbpB_{Ec}$ were tested together ($IbpAB_{Ec}$), 1:2 stoichiometry was used (e.g. 10 µM $IbpAB_{Ec}$ is 3.33 µM $IbpA_{Ec}$ and 6.67 µM $IbpB_{Ec}$). Data presented as average hydrodynamic radius (±SD) of the most occupied peak (min. 80% of total aggregate volume) from DLS size distributions of sHSPs-luciferase assemblies.

To investigate the size of sHSP-substrate assemblies I thermally denatured firefly luciferase in the presence of increasing concentration of sHSPs and measured size distributions of the resulting assemblies using dynamic light scattering (DLS). As expected, the presence of each IbpA protein was able to substantially reduce the size

(hydrodynamic radius) of aggregating luciferase, whereas IbpB$_{Ec}$ was not [Fig. 12]. Interestingly, IbpA$_{Vh}$, being the most effective in providing small-size assemblies, also provided the most evident inhibitory effect on disaggregation - at least at the limiting KJE concentration. What is more, IbpA$_{Ea}$, being least efficient assembly former, provided the most superior recovery among single sHSPs. IbpA$_{Ec}$, that is moderately efficient among assembly forming IbpAs, provides either low/no inhibition and moderately effective disaggregation [Fig. 10]. Therefore all three IbpAs when analysed separately show a directly opposite tendency compared to the hypothesis of the smaller the better. Two-sHSP IbpAB$_{Ec}$ system, however, does not seem to follow this dependency, combining superior disaggregation with moderate assembly forming abilities [Figs. 10, 12].

To assess the aforementioned results, I repeated the DLS titration and Hsp70-Hsp100 disaggregation for IbpAB$_{Ec}$ system, using the very same assemblies in order to achieve the most reliable data, as both assembly size and recovery slightly differs between experimental repeats. This repeated experiment shows a clear, expected inverse dependency between assembly size and disaggregation potency [Fig. 13] (the smaller - the better).

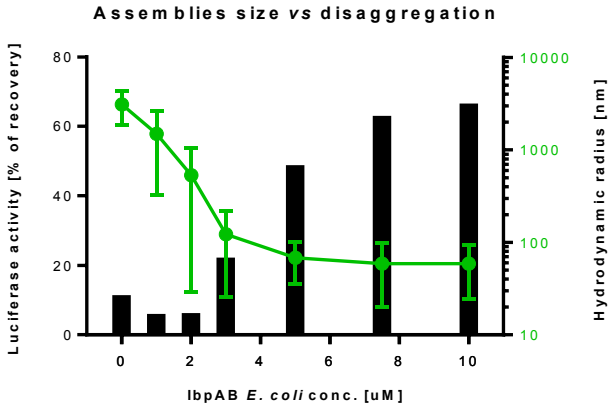

Fig. 13. Luciferase disaggregation effectiveness inversely correlates with sHSPs-Luciferase assemblies size, both being dependent on sHSP concentration used. Luciferase (1.5 μM) was denatured in the absence and presence of increasing *E. coli* IbpAB concentrations (total sHSPs concentration depicted, IbpAB$_{Ec}$ were present at 1:2 stoichiometry) and refolded with Hsp70/Hsp100 machinery (DnaK 1 μM; DnaJ 0.4 μM; GrpE 0.3 μM; 2 μM ClpB) for 40 min. Resulting Luciferase activities (black bars) were plotted against DLS-measured corresponding assemblies sizes (green curve, data from Fig. 12).

As the sHSP oligomers that are not in complex with a substrate may interfere with the DLS measurement, I also analysed sHSP-substrate assemblies using glycerol gradient centrifugation. This technique allows easy separation of unbound sHSPs, which stay in the top fractions from assemblies that sediment to the middle of the gradient and whose colocalization with the substrate can be therefore confirmed by SDS-PAGE. Consistently with DLS measurements, all IbpA- and $IbpAB_{Ec}$-assemblies range from the least-sedimenting $IbpA_{Vh}$ to the deepest-sedimenting $IbpA_{Ea}$. In all IbpA-containing assemblies sHSPs colocalize with luciferase, directly indicating assembly formation. On the other hand, in the case of $IbpB_{Ec}$, although some minor fraction of $IbpB_{Ec}$ can be noticed in the middle of the gradient, luciferase is found exclusively on the bottom of the gradient, indicating amorphous aggregate formation. Thus, glycerol gradient centrifugation results match very well with DLS measurements [Figs. 12, 14].

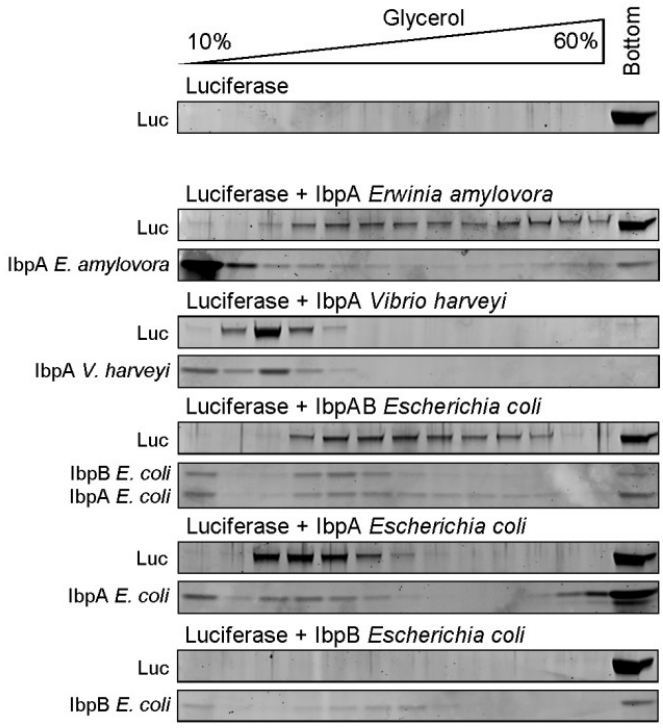

Fig. 14. Sedimentation profiles of sHSPs-luciferase assemblies. 1 μM luciferase was denatured at 44˚C for 10 min in the presence of 5 μM sHSPs ($IbpAB_{Ec}$ were present at 1:2 stoichiometry) and subjected to sedimentation in glycerol gradient. Fractions were collected from the top and analysed with SDS-PAGE followed by Oriole staining.

I also decided to check if the differences between the assembly-forming properties of IbpA$_{Ec}$ and IbpB$_{Ec}$ are not limited to luciferase. To achieve that, I used two other chaperone substrates: citrate synthase (CS) and malate dehydrogenase (MDH). In this experiment I employed static light scattering as it is technically easy to perform and also provides some kinetic data. Although each tested substrate required a different temperature to efficiently aggregate, in all cases IbpAB$_{Ec}$ two-sHSPs system was able to keep the sample scattering low in contrast to Luc, MDH or CS heated alone. Accordingly, IbpB$_{Ec}$ without its IbpA$_{Ec}$ partner was never able to achieve that. IbpA$_{Ec}$ in the absence of IbpB$_{Ec}$, however, showed complete, IbpAB$_{Ec}$-like scattering protection for Luc and CS but not for MDH, which required both sHSPs for any protection to be noticed. When heated in the absence of the substrate, no sHSP developed high scattering signal, thus they do not aggregate in conditions tested [Fig. 15]. Since, Luc and CS were treated at the most distantly ranging temperatures, both showing only IbpA$_{Ec}$-dependent protection, I would consider MDH-IbpAB$_{Ec}$ relation to be of more peculiar nature in contrast to more general trend presented by CS and Luc.

Summing up, these results indicate that IbpB$_{Ec}$, in contrast to all IbpAs, is unable to hold the substrate in the assemblies [Figs. 12, 14, 15]. Therefore, the presence of IbpB$_{Ec}$ in these is rather due to the previously reported interaction with IbpA$_{Ec}$ (Strózecka et al. 2012), not with unfolded substrate. Potentially, it could provide an area of less tightly bound substrate for Hsp70 system docking in IbpAB$_{Ec}$-substrate assemblies - explaining the observed high reactivation potential. On the other hand, assemblies containing IbpA$_{Ea}$ seem to be highly polymorphic or collapsing as they range across most of the fractions [Fig. 14] along the sedimentation analysis. Their potential fragility (as IbpA$_{Ea}$ almost fail to protect them from collapsing) could at least partially explain their relatively effective disaggregation (high substrate availability?).

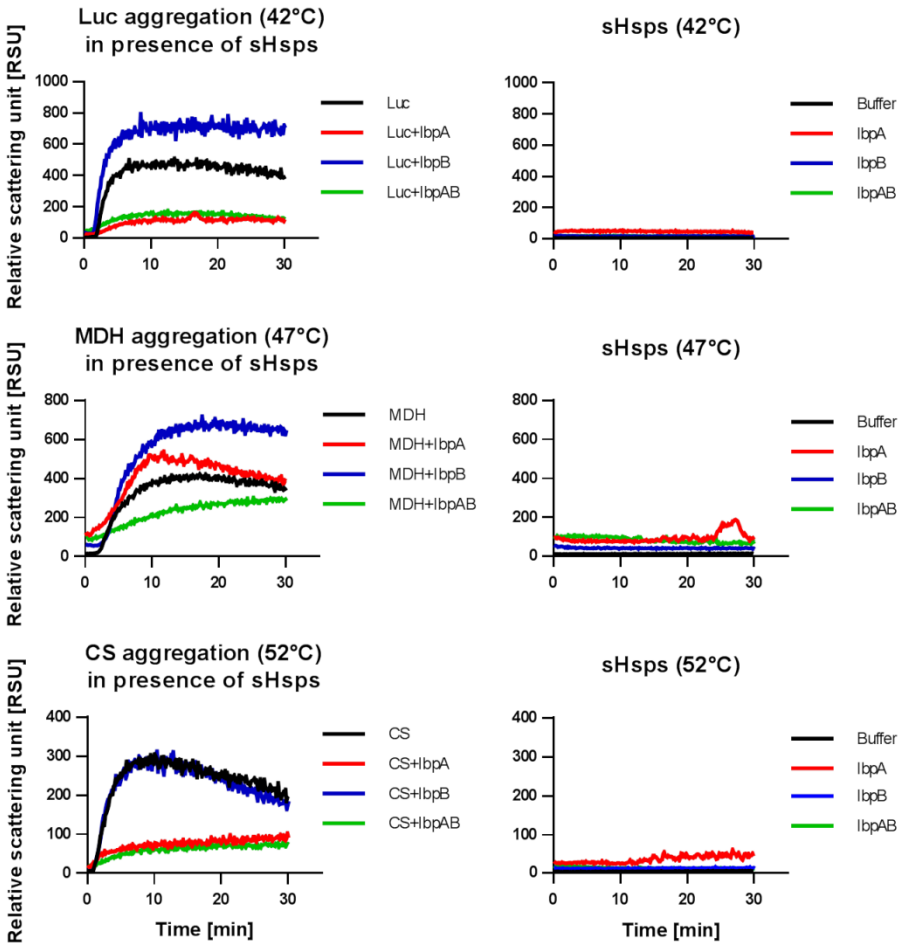

Fig. 15. $IbpA_{Ec}$ and $IbpB_{Ec}$ ability to protect luciferase (Luc), malate dehydrogenase (MDH) and citrate synthase (CS) from aggregation. Luciferase (1.5 μM), malate dehydrogenase (2 μM) or citrate synthase (2 μM) were mixed with $IbpA_{Ec}$ (3 μM, red), $IbpB_{Ec}$ (7 μM, blue), both $IbpA_{Ec}$ and $IbpB_{Ec}$ (3 and 7 μM respectively, green) or with no sHSPs (black) in room temperature (0°C on ice in the case of luciferase) and injected to preheated (temp. as indicated) spectrofluorometric cuvettes prior to scattering measurement. Measurement wavelengths were 605 nm for luciferase and citrate synthase and 565 nm for malate dehydrogenase. Right are sHSPs heated without aggregating substrate (controls).

8.5. IbpAs, but not IbpB, stably bind aggregated substrate

Formation of the assemblies is driven by the ability of sHSPs to interact with an unfolded substrate. Since bacterial sHSPs form large, undefined oligomers and their substrates are heterogeneous, unfolded polypeptides, it is challenging to design any experimental setup capable of assessing this interaction with classic (e.g. SPR) methodology. Having considered many other approaches, I employed a recently developed biolayer interferometry (BLI) technique. BLI technology is based on a dichroic sensor, where white light is partially reflected inside the sensor and partially at the solid/liquid interphase. The difference in reflected light beams interference pattern generated by varying analyte layer thickness on the sensor allows for real-time (kinetic) thickness measurements. Using this approach, I developed luciferase on-the-sensor aggregation methodology, broadening BLI application with very flexible ligand-aggregate measurement setup. I was then able to apply heat-activated sHSPs on the sensor, recording their rapid binding to the substrate aggregate and later dissociation in buffer [Fig. 16A].

In this assay, each IbpA protein binds to the sensor within single seconds, which is rational in light of their assembly-generation activity. After the initial binding, the thickest layer is generated by $IbpA_{Ec}$ (~2.1 nm), closely followed by both $IbpA_{Vh}$ and $IbpA_{Ea}$ (~1.8 nm), however, their binding rates are identical. $IbpB_{Ec}$ binding is strikingly different - slow and generating only a ~0.6 nm layer after the first minute [Fig. 16B]. Finally, $IbpAB_{Ec}$ mixture is found somewhere in between, binding at moderate rates and generating rougly a ~1.3 nm layer. Dissociation of all the IbpAs and $IbpAB_{Ec}$ is comparable, with $IbpA_{Ec}$ being the most stable as most of it remained on the sensor even after 60 min of washing. In contrast, $IbpB_{Ec}$ is the only one to be completely washed away from the sensor (after ~25min) during the experiment [Fig. 16B].

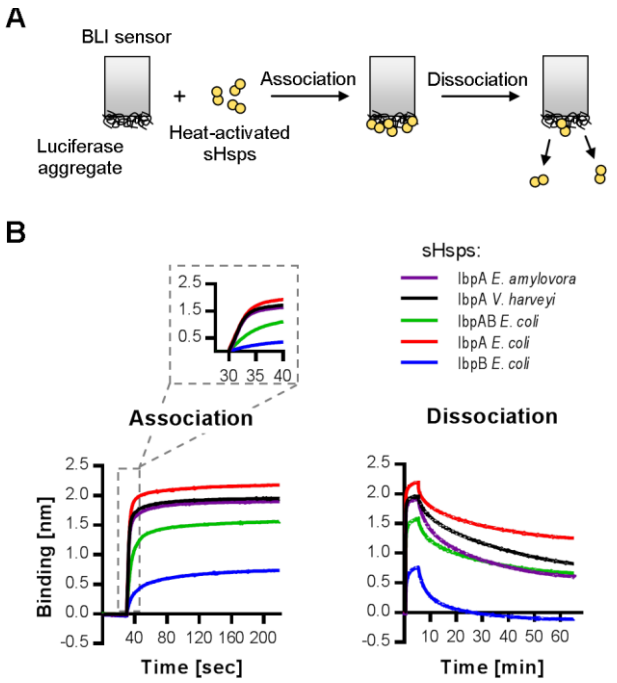

Fig. 16. IbpA small heat shock proteins stably bind aggregated substrates. (A) Experimental scheme. Heat-activated sHSPs (44˚C, 5 min) were applied onto BLI sensor with immobilized aggregated luciferase and subsequently dissociated in the plain buffer. (B) Association and dissociation curves. sHSPs were used in 5 μM concentration, $IbpAB_{Ec}$ stoichiometry was 1:2 as in Fig 14.

This experiment shows that generally the action of all tested IbpAs on the aggregate is very similar, emphasizing the need for fast and tight substrate binding. This is expected, as their function is to prevent excessive conglomeration of unfolded polypeptides , shielding them in assemblies. Not only IbpB is incapable of such strong interaction but also, paradoxically, seems to counteract these efforts as $IbpB_{Ec}$ seems to diminish $IbpA_{Ec}$ binding to the substrate [Fig. 16] which would explain why $IbpAB_{Ec}$-luc assemblies seem slightly bigger than $IbpA_{Ec}$-luc [Figs. 12, 14].

8.6. IbpB restores IbpA-inhibited Hsp70 binding to aggregated substrate

When conditions become favourable for disaggregation, preformed sHSPs-substrate assemblies have to be 'disassembled' in order to recover and refold the trapped substrate. The very first step of this process is Hsp70 binding to the assembly surface, which has to outcompete the shielding sHSPs (Żwirowski et al. 2017). sHSPs ability to tightly bind the aggregate seems to be in opposition to that process. In order to assess not only sHSPs stability on the aggregate but also how much they interfere with subsequent Hsp70 binding, I decided to modify the previously used BLI setup [Fig. 16A] for more in-depth *E. coli* sHSPs-Hsp70 interplay.

After the already presented sHSP binding step, instead of washing in plain buffer, I introduced the sensor to the buffer with the Hsp70 system in order to observe the

expected sHSP-Hsp70 transition on the aggregate [Fig. 17A]. Since BLI is unable to differentiate the proteins being bound to the sensor, I took advantage of the resulting layer thickness generated by different proteins. In this setup, sHSPs were able to generate up to 2.2 nm thickness, however, in their absence, limiting and saturating Hsp70 system generates the thickness of 3.5 and 5.9 nm respectively [Fig. 17B, dashed traces]. Therefore, each signal increase above the sHPSs binding plateau can be interpreted as Hsp70 binding to the sensor.

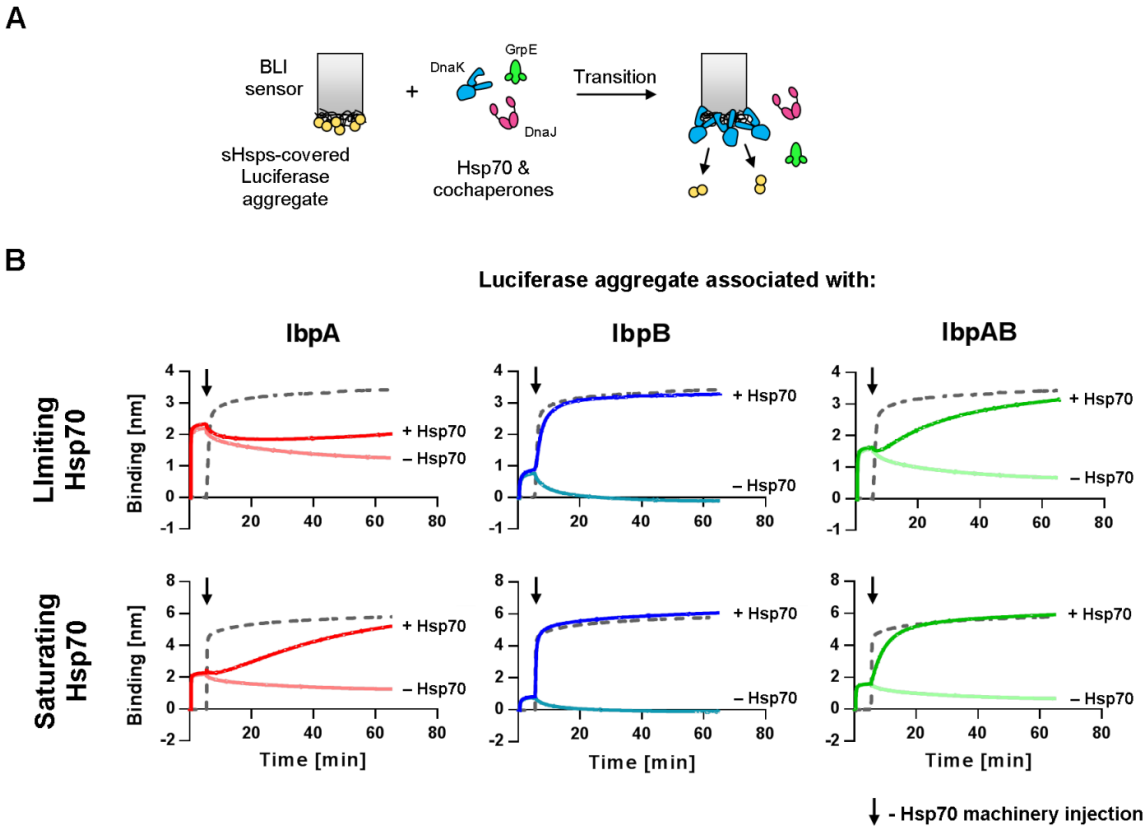

Fig. 17. Aggregate-bound sHSPs differently inhibit Hsp70 binding. (A) Experimental scheme. (B) Aggregated luciferase immobilized on the BLI sensor was associated with sHSPs as in Fig. 16 and subsequently introduced to buffer containing limiting (DnaK 0.7 μM; DnaJ 0.28 μM; GrpE 0.21 μM) (upper panels) or saturating (DnaK 3.5 μM; DnaJ 1.4 μM; GrpE 1.05 μM) (lower panels) Hsp70 machinery concentrations (dark traces). Light traces represent spontaneous sHSPs dissociation curves (as shown in Fig. 16), grey dashed traces show Hsp70 machinery binding to immobilized luciferase aggregates in the absence of any sHSPs. The experiments presented in panel B were repeated at least twice, giving essentially the same traces.

Each chart contains 3 traces: dashed trace that represents Hsp70 binding in the absence of sHSPs (upper panels limited Hsp70 concentration, lower panels saturating Hsp70 concentration); light-coloured trace of sHSPs binding and dissociation as in Fig. 16B and dark-coloured trace, where sHSPs binding is followed by Hsp70 addition. Strikingly, the

presence of IbpA$_{Ec}$ almost totally inhibits Hsp70 binding when its concentration is limiting. On the contrary, IbpB$_{Ec}$ shows hardly any impact on this process. When both IbpA$_{Ec}$ and IbpB$_{Ec}$ are present, Hsp70 binding is moderately slowed, however, almost reaches the Hsp70 saturation plateau by the end of the run. When Hsp70 system is saturating, Hsp70 binding inhibition from IbpA$_{Ec}$ is less evident and inhibition from IbpAB$_{Ec}$ is almost completely relieved [Fig. 17B], explaining superior disaggregation [Fig. 10].

As an additional control, I was able to strip all the proteins from the sensor prior to and after Hsp70 addition, proving the occurrence of sHSP-Hsp70 transition with SDS-PAGE sample analysis [Fig. 18]. Both IbpA$_{Ec}$ and IbpB$_{Ec}$ were found on the sensor after the initial sHSPs binding as assessed by Western blot. After incubation with limiting and saturating Hsp70 system, only DnaK rather than sHSPs was found on the sensors previously containing IbpB$_{Ec}$ and IbpAB$_{Ec}$. For the sensor containing IbpA$_{Ec}$ only incubation with saturating Hsp70 system was enough to replace IbpA$_{Ec}$ with DnaK, however, after incubation with limiting Hsp70 system, IbpA$_{Ec}$ was found partially stripped [Fig. 18].

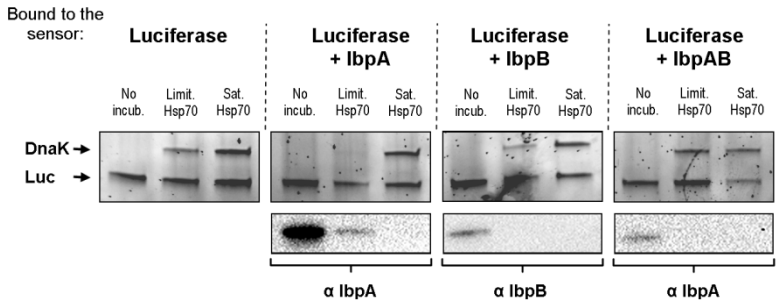

Fig. 18. BLI sensor protein content analysis. Sensor-bound proteins were analysed before and after the action of Hsp70 machinery (Fig. 17B) with SDS-PAGE followed by Western blot with IbpA or IbpB antibodies and Oriole staining for DnaK and luciferase. No incubation lanes refer to proteins associated with the sensor prior to incubation with either limiting or saturating Hsp70 concentration. SDS-PAGE (Oriole stained) and Western blot analyses were performed once.

To conclude, IbpA$_{Ec}$ presence on the aggregate is highly detrimental for subsequent Hsp70 binding. This effect can be substantially decreased in the presence of both IbpB$_{Ec}$ and IbpA$_{Ec}$, which allows both small size assemblies generation [Figs. 12, 14] and their

further potent disaggregation [Fig. 10]. It might be suspected that this novel $IbpB_{Ec}$ activity might have been the necessary fitness provider, allowing *ibpB* gene persistence in *E. coli* genome.

8.7. IbpB activity is conserved across *Enterobacteriaceae*

Having traced *E. coli* IbpB activity I decided to include additional sHSPs from related species to reinforce the already received results and try to bring them from single protein system studies to a more general evolutional conclusion. I chose a distantly related two protein IbpA and IbpB chaperone system form *Cedecea neteri* ($IbpA_{Cn}$ and $IbpB_{Cn}$) and additional, more distantly related single sHSP from *Aeromonas hydrophila* ($IbpA_{Ah}$) to supplement my analysis. Up till now, these proteins have never been a subject of biochemical studies and sHSP gene names were annotated only based on their high similarity to *E. coli ibpA* and *ibpB*. The respective genes were *de novo* commercially synthesized (Genscript), and cloned into the expression vector. Next, $IbpA_{Cn}$, $IbpB_{Cn}$ and $IbpA_{Ah}$ were overproduced and purified for further analysis.

Using this additional protein set I repeated the key experiments to examine if their sequence homology to *E. coli* sHSPs comes along with biochemical activity homology. As in Fig. 12, I titrated sHSPs against a fixed luciferase concentration, followed by heat treatment and particle size distribution measurement *via* DLS. All titrated IbpA proteins managed to provide small-size assemblies with luciferase. Similarly to *E. coli* sHSPs [Fig. 12], $IbpA_{Cn}$ caused the formation of smaller assemblies when compared to corresponding $IbpAB_{Cn}$ concentration. Also, as in the case of $IbpB_{Ec}$, $IbpB_{Cn}$ was unable to direct luciferase aggregation towards small size assemblies [Fig. 19], therefore showing very similar trends.

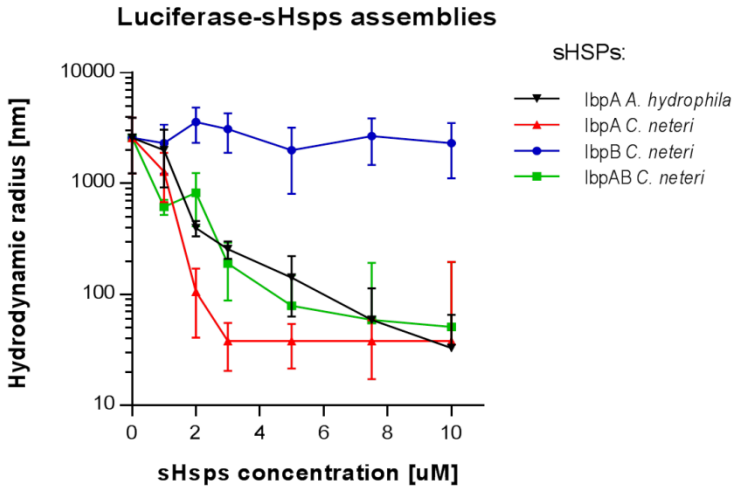

Fig. 19. DLSmeasurements of *A. hydrophila* and *C. neteri* sHSPs-luciferase assemblies. Average hydrodynamic radius (±SD) of the most occupied peak (min. 80% of total volume) from DLS size distributions of sHSPs-luciferase assemblies is presented. Experimental conditions as in Fig. 12. When $IbpA_{Cn}$ and $IbpB_{Cn}$ were tested together ($IbpAB_{Cn}$), the 1:2 stoichiometry was used (e.g. 10 μM $IbpAB_{Cn}$ is 3.33 μM $IbpA_{Cn}$ and 6.67 μM $IbpB_{Cn}$).

To assess the impact of respective sHSPs on luciferase recovery after aggregation, I performed a disaggregation experiment in conditions already exploited in Fig. 10. In this experiment, both $IbpA_{Ah}$ and $IbpA_{Cn}$ show a very pronounced inhibitory effect that is not overcome even in saturating Hsp70 system concentration. Similarly to $IbpB_{Ec}$, $IbpB_{Cn}$ presence during luciferase aggregation provides a slight disaggregation boost, which eventually becomes very evident when present alongside their $IbpA_{Cn}$ partner. It should be noted that it is only observed in saturating Hsp70, where luciferase assembled with $IbpAB_{Cn}$ is disaggregated and refolded almost as efficiently as when assembled with $IbpAB_{Ec}$ [Figs. 10, 20].

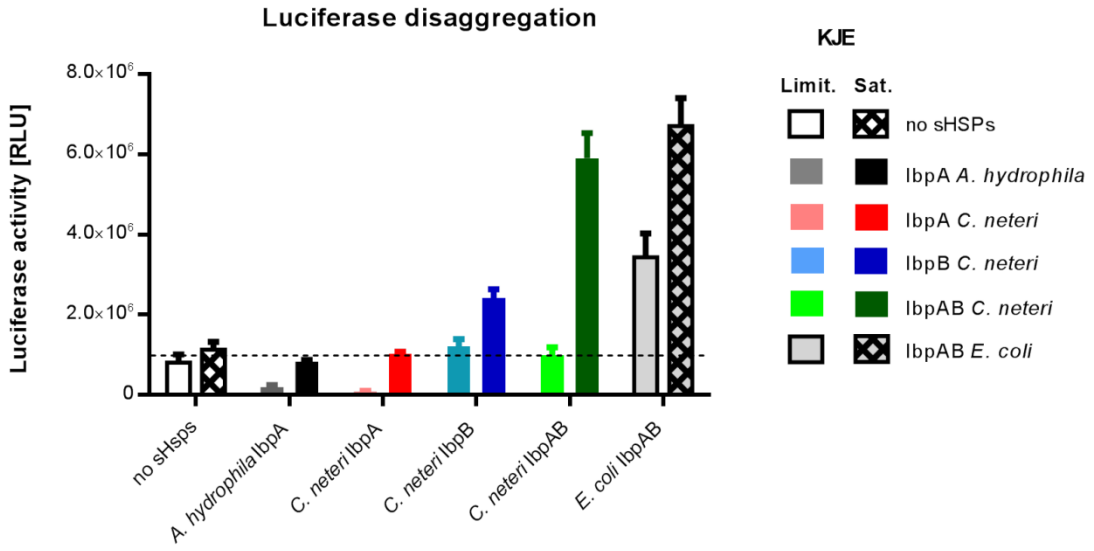

Fig. 20. Refolding potential of *A. hydrophila* and *C. neteri* sHSPs-substrate assemblies. Luciferase (1.5 μM) was denatured and refolded as in Fig. 10 at limiting (DnaK 0.7 μM; DnaJ 0.28 μM; GrpE 0.21 μM) or 5x higher saturating (DnaK 3.5 μM; DnaJ 1.4 μM; GrpE 1.05 μM) Hsp70 machinery concentrations and ClpB at 2 μM concentration for 40 min. Data is presented as mean ±SD from at least 3 independent experiments. Dashed line represents the mean activity of refolded Luciferase aggregates.

Both *E.coli* and *C. neteri* IbpAB systems seem to act very similarly, subdividing sHSP roles between two distinct proteins. IbpAs are dedicated to stable binding to aggregating polypetides, driving the assembly process. IbpBs partner them, subsequently allowing easier Hsp70-dependent substrate recovery. Since *E. coli* and *C. neteri* IbpAB systems seem to form one of the most distantly related sHSP system pairs within *Enterobacteriaceae* species and act in the very same manner, it is rational to infer that their subdivided functional roles are conserved among all *Enterobacteriaceae*.

8.8. IbpB reduces the demand for Hsp70 to outcompete IbpA from aggregated substrate

Since sHSPs highly interfere with Hsp70 binding, it is reasonable to trace this process not only by analysing Hsp70 [Figs. 17, 18], but also from the perspective of sHSPs being dissociated in the process. To achieve that, I adapted the methodology of glycerol gradient centrifugation published by Żwirowski et al. (2017). I isolated sHSPs-luciferase assemblies formed in the presence of the already exploited sHPSs test set ($IbpA_{Ec}$, $IbpAB_{Ec}$, $IbpA_{Cn}$, $IbpAB_{Cn}$, $IbpA_{Vh}$ and $IbpA_{Ah}$) [Fig. 21] and incubated them in the

presence of the Hsp70 system. Samples where then subjected to the second round of glycerol centrifugation to separate liberated sHSPs from the assemblies. Top fractions containing liberated sHSPs and middle fractions characteristic for the assemblies [Fig. 22A] were then pooled and precipitated prior to Western blot analysis of sHSP content [Fig. 22B].

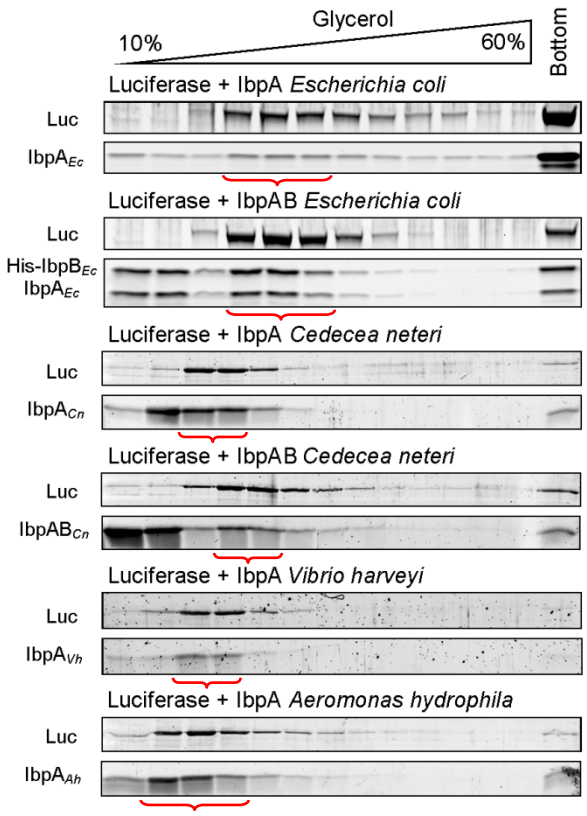

Fig. 21. sHSPs-substrate assemblies isolation. Luciferase (1.5 µM) was aggregated in presence of either IbpA or IbpAB sHSPs (10 µM) and applied for sedimentation in glycerol gradient (see 'Methods'). Luciferase + IbpA$_{Ec}$/IbpAB$_{Ec}$ samples were denatured at 3 and 20 µM proteins concentrations respectively. After SDS-PAGE fractions analysis (Oriole staining), middle fractions containing assemblies (indicated with red brackets) were pooled and stored in -80°C for further experiments.

When resedimenting each type of the assemblies without Hsp70 addition, sHSPs are found exclusively in the middle fractions, pointing to the high stability of the assemblies during the procedure. Comparing single IbpA-luc assemblies with respective IbpAB-luc assemblies in both *E. coli* and *C. neteri* systems show that sHSPs are found liberated at a lower Hsp70 concentration when the IbpB partner is present. IbpAB$_{Ec}$ are already being liberated from the assemblies in limiting Hsp70 concentration, whereas IbpA$_{Ec}$ in the absence of IbpB$_{Ec}$ is found only partially dissociated in the top fractions after the action of saturating Hsp70 system. *C. neteri* sHSP equilibrium is shifted towards higher Hsp70 doses, only showing some dissociation from IbpAB$_{Cn}$-luc assemblies at saturating Hsp70 system, where IbpA$_{Cn}$-luc assemblies remain intact. Single sHSPs require either

saturating Hsp70 system - to sHSPs dissociation from IbpA$_{Vh}$-luc assemblies to be observed or remain intact in the middle fraction (IbpA$_{Ah}$-luc) regardless of any incubation [Fig. 22].

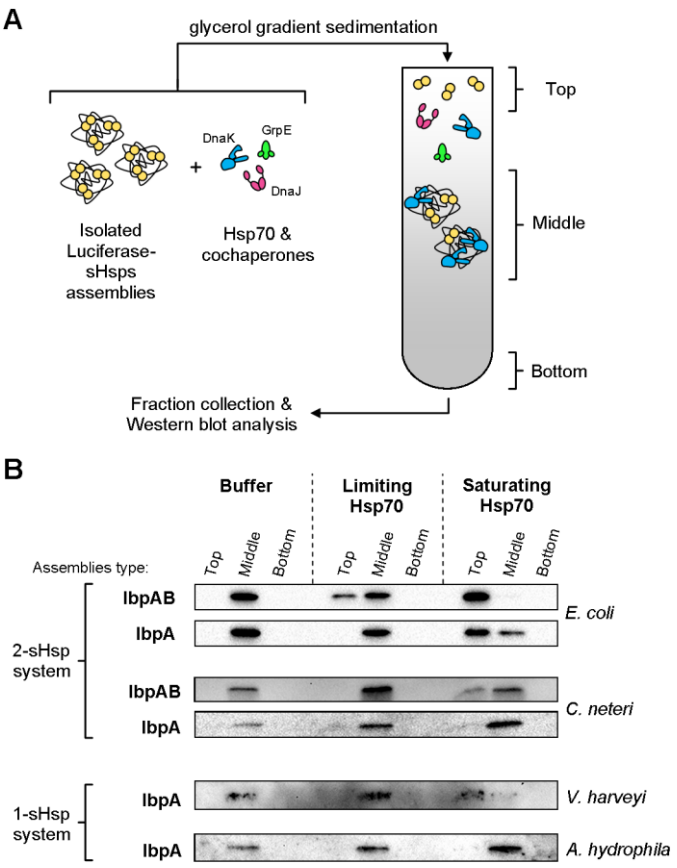

Fig. 22. IbpB presence in IbpAB–substrate assemblies allows for efficient Hsp70-dependent dissociation of IbpA from assemblies. (A) Experimental scheme. Purified IbpA- and IbpAB-luciferase assemblies were incubated with indicated components and resubjected to glycerol gradient sedimentation. (B) Isolated sHSPs-luciferase assemblies were incubated with buffer or limiting (DnaK 0.7 μM; DnaJ 0.28 μM; GrpE 0.21 μM) or saturating (DnaK 3.5 μM; DnaJ 1.4 μM; GrpE 1.05 μM) Hsp70 machinery concentration followed by glycerol gradient sedimentation. Fractions were collected from the top, pooled (top - fractions containing free sHSPs; middle - fractions (as in Fig. 21) containing sHSPs-luciferase assemblies; bottom - material recovered from the bottom of the centrifugation tube) and analysed by Western blot with αIbpA antibodies following SDS-PAGE. The experiments were repeated at least twice.

Presenting different patterns of dissociation, the analysed two-sHSP system showed the same trend of enhanced dissociation when the IbpB component was present alongside IbpA. To strengthen this conclusion, I repeated the experiment for *E. coli* sHSPs using an alternative substrate - citrate synthase. Similarly, to the luciferase assemblies, IbpB also enhanced dissociation of IbpA from the CS assemblies both at the limiting and saturating concentrations of Hsp70 [Fig. 23B]. Strikingly for all sHSPs, the Hsp70 conditions allowing sHSPs dissociation are also precisely the conditions of effective refolding of the trapped luciferase shown in Fig. 10 and Fig. 20. Thus, sHSPs dissociation is a highly restraining bottleneck for this process, that also underlines the importance of the IbpB component for making the substrate supply available for disaggregation.

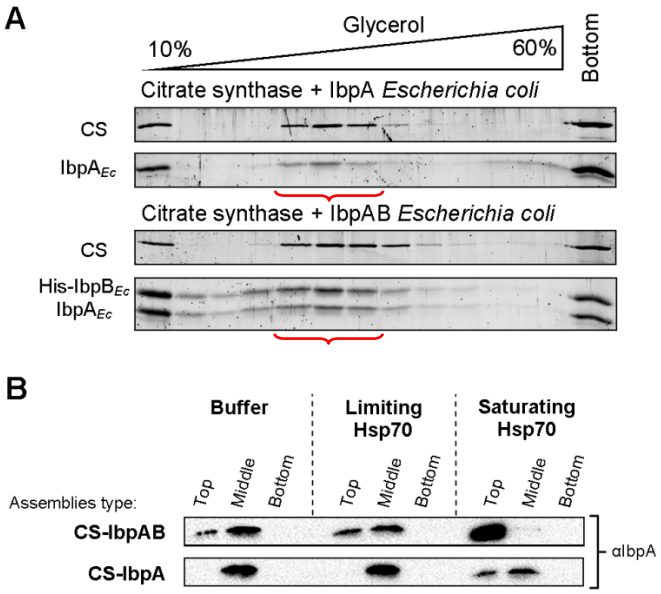

Fig. 23. IbpB presence in CS-IbpAB assemblies allows for efficient Hsp70-dependent dissociation of IbpA from assemblies. (A) Isolated by sedimentation CS-sHSPs assemblies (indicated with red brackets) (B) were incubated with buffer or limiting (DnaK 0.7 μM; DnaJ 0.28 μM; GrpE 0.21 μM) or saturating (DnaK 3.5 μM; DnaJ 1.4 μM; GrpE 1.05 μM) Hsp70 machinery concentration followed by glycerol gradient sedimentation. Fractions were collected from the top, pooled (top - fractions containing free sHSPs; middle - fractions containing sHSPs-luciferase assemblies; bottom – material recovered from the bottom of the centrifugation tube) and analysed by Western blot with αIbpA antibodies following SDS-PAGE.

8.9. IbpB enhances IbpA clearance in aggregated protein fraction *in vivo*

All performed *in vitro* experiments indicate that IbpB has a strong, positive impact on protein disaggregation, allowing Hsp70 to penetrate the IbpA layer that shields scavenged polypeptides from cellular environment. Thus, the IbpB function alongside IbpA seems very important to the whole-cell protein quality control. Still, I was unable to observe any growth rescue from IbpB presence in drop test experiment [Fig. 8]. Therefore, I decided to design yet another, more targeted assay. In order to assess my hypothesis *in vivo*, I constructed Δ*ibpA* and Δ*ibpB E. coli* strains by introducing single stop codons into each gene on the chromosome with CRISPR technique (Reisch and Prather 2015). I chose this approach to minimize any potential effects on mRNA structure, as it is involved in regulation of *ibpAB* operon expression in *E. coli* (Waldminghaus et al. 2009) and deleting each gene was shown to induce overexpression of the other one (Tao et al. 2015). Still, in both of these knock-out strains sHSPs were overexpressed compared to the wild type strain [Fig. 24A].

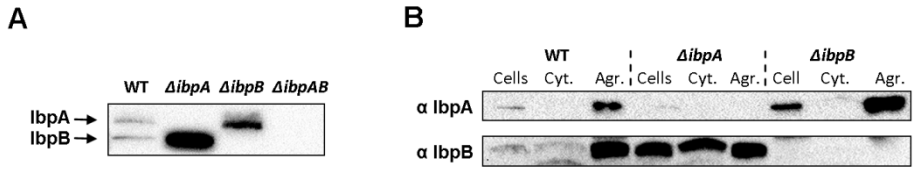

Fig. 24. Expression of IbpA and IbpB in constructed strains. (A) *E. coli* MC4100 WT, Δ*ibpA*, Δ*ibpB* and Δ*ibpAB* cultures were grown at 30˚C then subjected to mild heat shock (42˚C, 10 min and 48˚C, 5 min) and later analysed by SDS-PAGE in the presence of 6M urea (to separate IbpA from IbpB) and western blot against IbpA and IbpB to assess their expression. (B) Localization of IbpA and IbpB in soluble and aggregated protein fractions. WT, Δ*ibpA* and Δ*ibpB* strains were heat-shocked as in A, followed by isolation of soluble and aggregated protein fractions, see 'Methods'. Obtained fractions were then analysed by SDS-PAGE in presence of 6M urea and Western blot against IbpA and IbpB.

Analogically to *in vitro* aggregate binding [Fig. 16], the abundance of sHSPs *in vivo* in the aggregated protein fraction after heat shock should demonstrate their affinity to aggregates. To investigate this, I fractionated cells after heat shock into soluble and aggregated protein fractions, which I later analysed against IbpA or IbpB presence by Western blot [Fig. 24B]. In the wild type strain both sHSPs are found mainly in aggregated fraction. In agreement with its high binding potential, IbpA in the *ΔibpB* strain was exclusively present in the aggregated protein fraction, while IbpB in the *ΔibpA* strain was detected both in the soluble (approx. 90% of total IbpB) and in the aggregated fraction (approx. 10%) [Fig. 24B]. Thus, also *in vivo* IbpA shows much greater affinity to aggregates than IbpB.

Knowing the high affinity of IbpA to aggregates, I wanted to trace its removal from these and how it is influenced by IbpB presence *in vivo*. For that I designed the experiment in which I traced the clearance of both aggregates and aggregate-associated sHSPs along cells recovery after heat shock. I optimized the conditions to represent mild, sub-lethal heat shock, avoiding potential bias from dead and therefore non-disaggregating bacteria. Along recovery, I took several culture aliquots to isolate the aggregated protein fraction, which was later analysed *via* SDS-PAGE and Western blot [Fig. 25]. In the wild type strain and in all the knock-out mutants, both the proportion of aggregated proteins (approx. 2.8% of the total protein content) and the kinetics of aggregate removal were similar. In the wild type strain both IbpA and IbpB are removed simultaneously and slightly faster than the total aggregate volume. This is in agreement with the recently

proposed disaggregation mechanism, where sHSPs removal is the first step of disaggregation (Żwirowski et al. 2017). In *ΔibpA* strain, IbpB co-disappears with the aggregates and in *ΔibpB* strain, IbpA disappears noticeably slower, being selectively maintained in the aggregated fraction [Fig. 25]. Thus, IbpB presence is important for IbpA release from aggregates *in vivo*. Still, IbpB absence alongside IbpA does not seem to slow the clearance of the aggregates in the conditions tested, which might be due to overall gentleness of the heat shock.

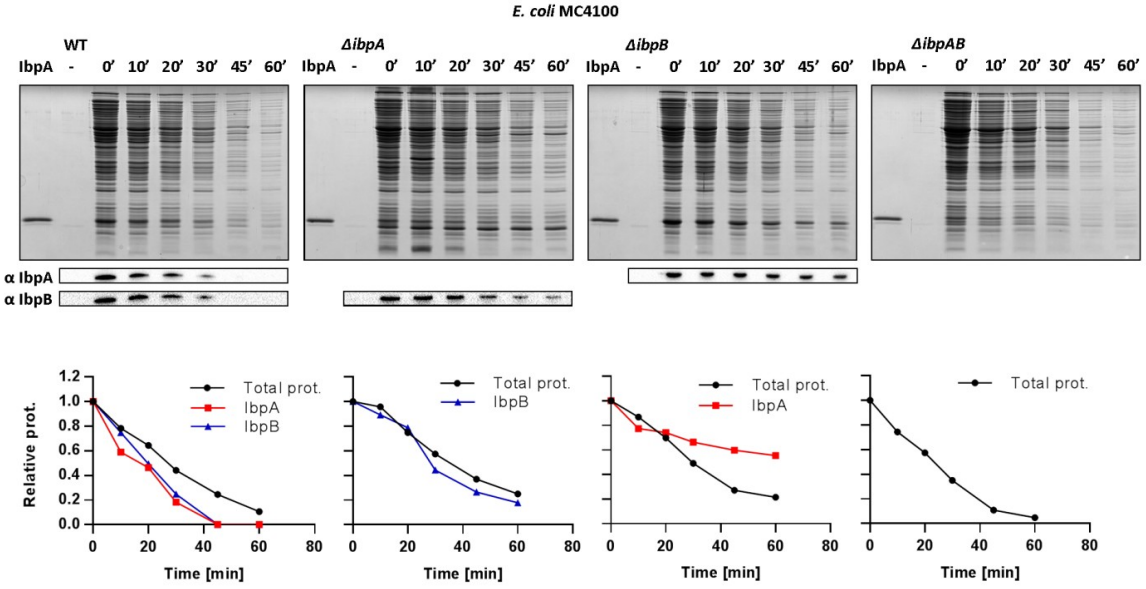

Fig. 25. IbpB presence allows for IbpA removal from aggregates. Indicated bacterial strains were heat-shocked as in Fig. 24 and allowed to recover at 30˚C. Aggregated and soluble protein fractions were isolated from culture aliquots sampled at indicated time points along the recovery period and further analysed by SDS-PAGE + Coomassie staining and SDS-PAGE in the presence of 6M urea + Western blot using antibodies against IbpA or IbpB. Quantifications of total aggregated proteins (Total. prot.) and sHSPs were plotted against recovery time.

Presented *in vivo* studies are in a good agreement with preceding *in vitro* experiments, all supporting the hypothesis of IbpA being the strong polypeptide binder and IbpB being the factor allowing easier dissociation of sHSPs from aggregates at the initiation of disaggregation.

Most of the data presented in this chapter was published in 'Duplicate divergence of two bacterial small heat shock proteins reduces the demand for Hsp70 in refolding of substrates' (Obuchowski et al. 2019).

9. Discussion

sHSPs act as the first line of defence during proteotoxic stress, scavenging misfolding proteins to form safe-storage sHSPs-substrate assemblies. Later, sHSPs-substrate assemblies serve as reservoirs for substrate disaggregation and refolding by Hsp70 and Hsp100 chaperones. The majority of γ-proteobacteria possess only one sHSP (IbpA), while most *Enterobacterales* possess two sHSPs (IbpA and IbpB). A series of results presented in the previous section elucidates the earlier unknown functional interplay of two sHSPs found in *Enterobacteriaceae* species. A single sHSP gene duplication in the ancestor of *Enterobacterales* gave rise to IbpA protein, which itself functions as canonical, aggregation-protecting single sHSP and its IbpB partner, which acquired different abilities - enhancing disaggregation by improving IbpA dissociation from aggregates. This scenario is conserved among *Enterobacteriaceae* species.

9.1. Mechanistic features of IbpA-IbpB cooperation

The weak ability of IbpB to associate with aggregating polypeptides suggested by presented *in vitro* experiments is in agreement with previous cellular localization studies, showing that in the absence of IbpA, IbpB is found in the cytoplasm (Kuczyńska-Wiśnik et al. 2002). Consistently, the results for the *ΔibpA* strain show that at heat shock conditions, only a fraction of IbpB is found in the aggregates. In contrast, when IbpA is expressed, the aggregated fraction contains most of the cellular IbpB. Thus, the presence of IbpA efficiently directs IbpB to aggregates. Curiously, the studies of the protein–protein interaction network in *E. coli* (Butland et al. 2005) show that the pool of interacting partners of IbpA is much bigger than that of IbpB (42 and 10, respectively), and that 9 out of 10 IbpB substrates also interact with IbpA (Butland et al. 2005). This, together with my results, suggests that IbpB interacts with misfolded substrates *via* IbpA.

The functional cooperation between IbpA and IbpB involves: (*a*) joint formation of assemblies that are more manageable by Hsp70-Hsp100 (Żwirowski et al. 2017; Ratajczak, Zietkiewicz, and Liberek 2009), (*b*) their ability to form mixed complexes *in vitro* (Ratajczak, Zietkiewicz, and Liberek 2009; Strózecka et al. 2012) and (*c*) their mutual influence on each other *in vivo* and *in vitro* degradation rates (Shi et al. 2014; Bissonnette et al. 2010). The ability of IbpA and IbpB to form mixed complexes is probably the key factor determining the formation of the refolding-potent assemblies.

However, it is not known what the basic building blocks of such IbpA-IbpB complexes are. The two most possible scenarios involve IbpA and IbpB forming either heterodimers or homodimers that further hetero-oligomerize. In both cases the formation of such complexes results in the incorporation of IbpB molecules, alongside IbpA, into the assemblies during stress. In consequence, the co-introduced IbpB, with low substrate binding potential, decreases the local IbpA-to-substrate ratio and weakens the interaction with substrates. As a consequence, sHSPs dissociation from the assemblies is easier which favours rapid initiation of disaggregation [Figs. 10, 16, 17, 20, 22, 23].

9.2. Potential benefits of utilizing an IbpAB system

The system composed of the two cooperating sHSPs (IbpA and IbpB) appears to be beneficial for the refolding process in comparison to single-sHSP (IbpA) systems. All analysed IbpA proteins, namely: IbpA from *V. harveyi, A. hydrophila* (pre-duplication), *E. amylovora* (lost *ibpB*), *E. coli* and *C. neteri* (two-sHSP systems), were similarly efficient in the formation of the assemblies with protein substrates [Figs. 12, 14, 19]. However, in both analysed two-sHSP systems (*E. coli* and *C. neteri),* the presence of IbpB in the assemblies substantially decreased the amount of Hsp70 required for the refolding [Figs. 10, 20]. This could be beneficial for the cells at harsh or long-lasting stress conditions, when the overall cellular demand for Hsp70 is high.

The presented data also suggests that the lower demand for Hsp70 in refolding of proteins from aggregates might not be the only benefit of maintaining two sHSP genes in *Eneterobacterales* genomes. All the IbpA proteins are highly specialized in binding to substrates, thus their intense production at stress conditions results in fast and efficient sequestration of aggregation-prone polypeptides in assemblies, which prevents other proteins from further co-aggregation. In *Enterobacterales,* which possess the two-sHSP system, IbpA binds to the substrates more strongly and rapidly [Fig. 16]. Thus, it provides even faster and more effective suppression of aggregation than the single-sHSP systems. At the same time, IbpB allows for easier dissociation of the tightly-bound IbpA from the assemblies [Fig. 22]. Thus, the *ibpA* duplication event at the base of *Enterobacterales* allowed the emergence of a single operon-encoded sHSPs system, which combines both the efficient holdase activity and dissociation effectiveness during stress. These are the characteristics of an ideal small heat shock protein.

Another possible gain from retaining a two-sHSP system is suggested by our results with MDH as a substrate. IbpA alone did not allow formation of small-size assemblies with MDH. Simultaneous presence of IbpB with IbpA was necessary for efficient formation of the assemblies [Fig. 15], even though IbpB itself has a low binding potential [Fig. 16]. This suggests, that for a certain pool of substrates, both IbpA and IbpB might be required to beneficially moderate the aggregation process. In consequence, the two-sHSP system's spectrum of substrates could be broadened.

9.3. Evolutionary view on IbpAB system emergence

It is tempting to speculate that during the evolution of γ-proteobacteria the sequestration of polypeptides during stress (holdase function of sHSPs) was important. Additionally, in the ancestor of *Enterobacterales,* fast and efficient refolding of proteins from aggregates also became critical. Thus, after duplication, IbpB acquired new properties allowing easier dissociation of sHSPs from the aggregates to initiate efficient substrate refolding. Consistently, *V. harveyi* and *A. hydrophila* (pre-duplication) IbpA show the highest holdase activity and strong inhibition of substrate refolding [Figs. 10, 20]. All the post-duplication bacteria contain sHSPs with enhanced dissociation properties. Interestingly, in *Erwiniaceae,* IbpA exhibited an IbpB-like behaviour: it showed the lowest ability to form assemblies as compared to other IbpA proteins [Figs. 12, 14] and the most effective dissociation from the assemblies among the analysed IbpA proteins [Fig. 16]. Both the holdase activity and effective dissociation in a single $IbpA_{Ea}$ resulted in efficient refolding of the luciferase from the assemblies at the limited Hsp70 concentration [Fig. 10]. Presumably, such properties of IbpA from an ancestor of *Erwiniaceae* allowed the *ibpB* gene to be lost.

Considering the dissociation-enhancing function of IbpB, it is hard to resist the impression that this feature is not actually brand new. Dissociation is the inherent fundamental consequence of non-covalent binding, including sHSP binding to the unfolding polypeptide. Thus each single sHSP is to some extend able to dissociate from the substrate. However, they are burdened with the serious trade-off as in the first place they need to effectively bind unfolding polypeptides. It appears that the investigated two-sHSP systems somehow split the function to bypass this limitation – allowing the emergence of an additional pro-dissociation component alongside the necessary pro-binding sHSP. The observed scenario could be considered as an interestingly peculiar

subfunctionalization (Stoltzfus 1999; Force et al. 1999), along which IbpB must have experienced substrate binding (ancient function) degeneration with simultaneous IbpA-IbpB interaction kept unharmed. This 'degeneration' did not corrupt the sHSPs system's ancient function (IbpA was still there to perform rapid binding), but additionally introduced a new quality to its action (improved later assemblies disaggregation). Thus, I would rather consider the analyzed case a subneofunctionalization, which is a much more flexible concept integrating both subfunctionalization (IbpA and IbpB functional split) and following neofunctionalization (two-sHSP system cooperation) of the system in a single evolutionary scenario (He and Zhang 2005). However, it would require the ancestral reconstruction and further biochemical research to judge how exactly IbpA and IbpB functions partitioned, changed and adjusted with *ibpA* and *ibpB* genes divergence.

9.4. Convergent similarities and differences between sHSPs in *Enterobacterales* and other species

The cooperation of more than one sHSP in cellular defence against protein misfolding stress is not restricted to *Enterobacterales.* In plants, there are many classes of sHSPs and several classes have multiple members, which can form heterooligomers (Kirschner et al. 2000; Basha et al. 2010). These sHSPs heterooligomers interact with aggregating substrates. Similarly in humans, sHSPs that have been reported to display chaperone activity, have also been shown to interact with at least one other sHSP (André Patrick Arrigo 2013). A model example of such behaviour are αA- and αB-crystallins in human, the products of *CRYAA* and *CRYAB* genes, which co-assemble to form α-crystallin in eye lens (Slingsby, Wistow, and Clark 2013). It seems that αA-crystallin is the result of gene duplication with a specialized function in eye lens while the product of the other gene copy, αB-crystallin is also widely expressed in other tissues. Thus, the cooperation of two sHSPs is common in different organisms and can be considered an example of convergent evolution. On the other hand, there are multiple examples showing two sHSPs working in parallel independently of each other, as reported for Hsp20.2 and Hsp17.7 from *Deinococcus radiodurans* (Bepperling et al. 2012) or Hsp26 and Hsp42 from *Saccharomyces cerevisiae* (Specht et al. 2011). Recently, it has been reported that most sHSP paralogs related to an oligomeric ancestor have evolved not to form complexes with each other, and the lack of heterooligomerization correlates with acquisition of distinct functions (Hochberg et al. 2018). IbpA and IbpB are then a contrasting example of

proteins which retained their ability to interact with each other after a gene duplication event (Ratajczak, Zietkiewicz, and Liberek 2009; Strózecka et al. 2012) and their preserved interaction seems to be of high importance for their cooperation.

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11. Supplementary figures

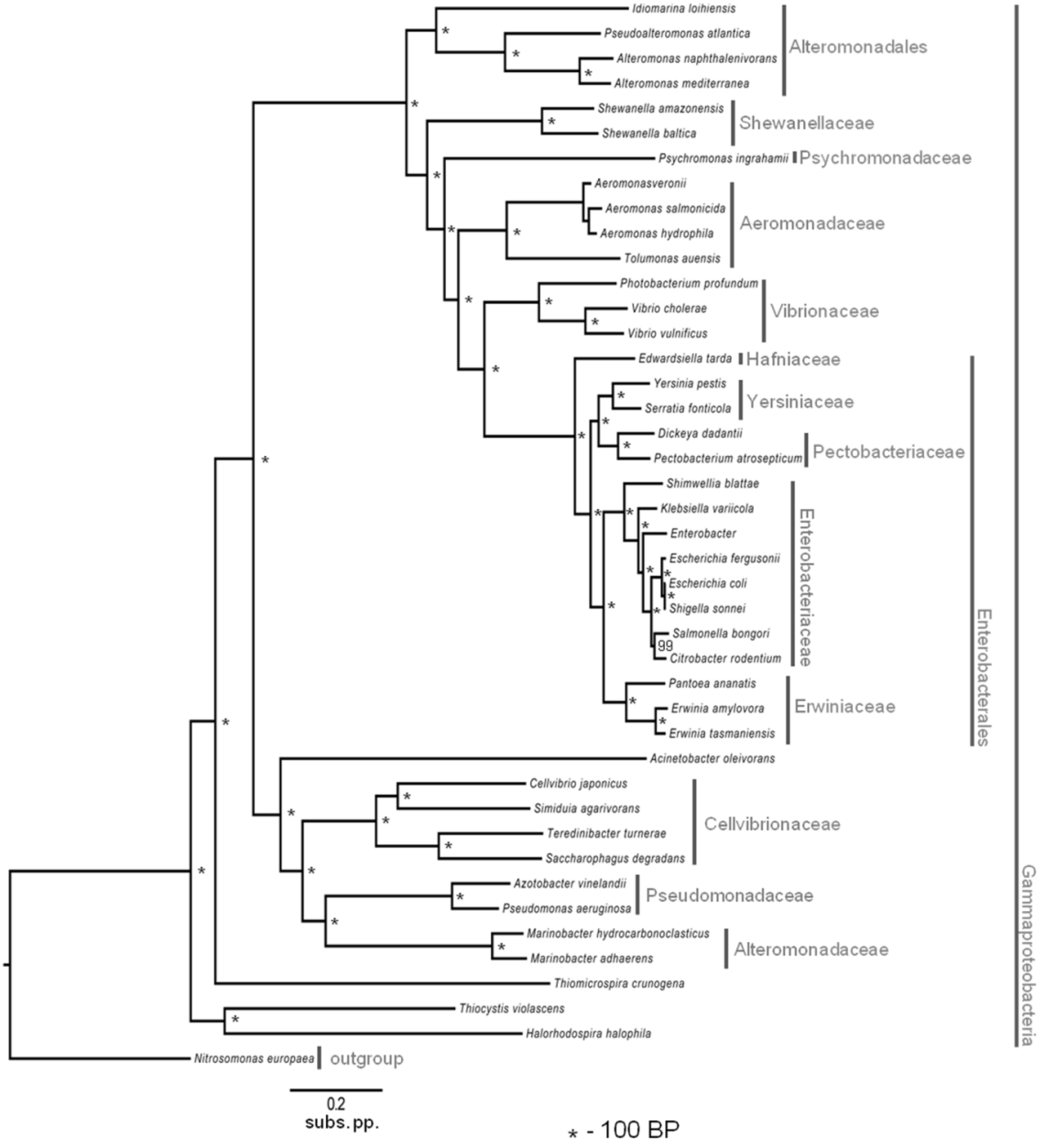

Fig. S1. Phylogeny of 48 Gammaproteobacteria species based on 1,489 genes. Analysis of 163,081 unambiguously aligned positions with 10% missing data. Tree was reconstructed using Γ+GTR model under a Maximum Likelihood analysis. Monophyly of *Enterobacterales* is supported. *Erwiniacea* is a sister group to *Enterobacteriaceae*. *Vibionaceae* and *Enterobacterales* are sister groups. Nodes with BP =100 are marked with a star. Scale bar, substitutions per position. Calculations performed by M. Stolarska and B. Tomiczek.

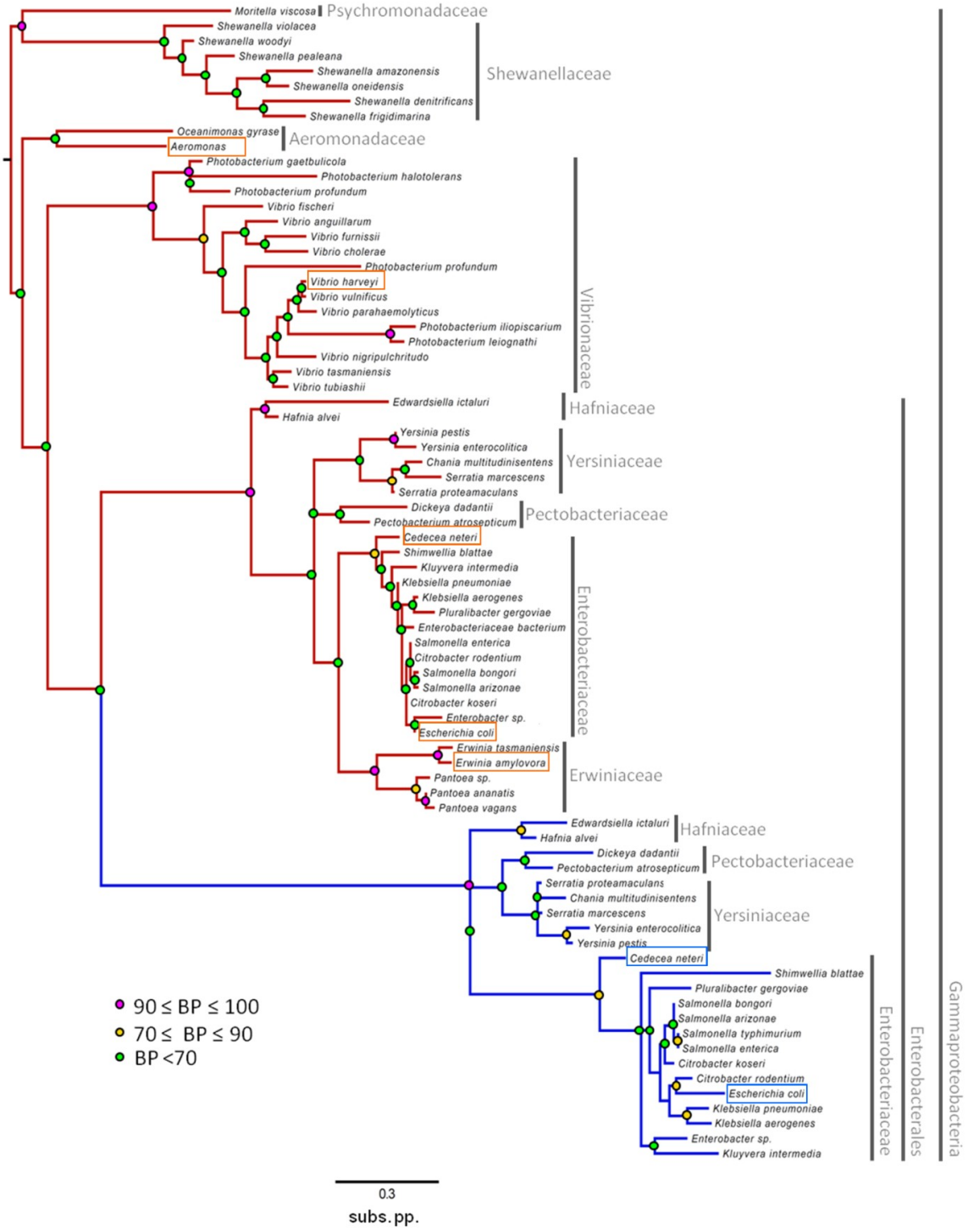

Fig. S2. sHSPs phylogeny in Gammaproteobacteria. Full Maximum Likelihood tree of 93 sHSPs amino acid sequences from Gammaproteobacteria, calculated with LG + I + G model in RAxML based on 82 amino acid positions. Scale is in expected amino acid substitutions per site. Level of bootstrap support is indicated with dots, bootstrap > 90 in magenta, bootstrap ≤ 90 and > 70 in yellow, bootstrap ≤ 70 in green. IbpA sequences are in red and IbpB sequences are in blue. Proteins used in *in vitro/vivo* experiments are marked with brackets. Calculations performed by M. Stolarska and B. Tomiczek.