Protein homeostasis - more than resisting a hot bath

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Short title

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Abstract

Maintenance of protein homeostasis is essential for survival of all organisms. In bacteria, the protein quality control system has a broad physiological impact beyond heat shock resistance, being involved in virulence, antibiotic resistance, as well as protection against environmental stresses. Its contribution to rejuvenation and growth arrest suggests interference with protein quality control to be a novel antimicrobial strategy. Remarkably, a protein quality control module originating from environmental strains has been found to be horizontally transferred to predominant clonal groups of bacteria providing exquisite thermotolerance to recently emerged global pathogens suggesting that novel features related to protein homeostasis contribute to the transition to new environments.

Introduction

Proteins are key catalysts of physiological processes and fulfill important structural functions, while their dysfunctionality is counteracting cell replication and is at most cytotoxic. Therefore, control of protein quality (protein homeostasis) is essential for all living organisms, from bacteria to humans [1,2]. Not only de novo folding of the protein chain derived from the ribosome requires immediate assistance (Fig. 1A), but also endurance during environmental stress such as temperature changes and solvent exposure, which usually leads to protein aggregation, requires systems to maintain the protein integrity. Also other deleterious protein modifications caused by e.g. intrinsic or extrinsic reactive compounds, such as oxygen and nitrogen-based radicals, lead to protein damage as well as aggregation. Thus, sophisticated networks of chaperone proteins exist in organisms, which fulfill diverse roles such as to prevent aggregation, disaggregate aggregated proteins and aid folding, which are eventually coupled to downstream proteolysis. Not to forget,

though, controlled aggregation can also be a physiologically controlled process leading to the formation of functional amyloids [3].

To date, the protein homeostasis systems have been well characterized in bacteria, mainly on the biochemical level. In the model organism *Escherichia coli*, holding, folding and disaggregating chaperones form a functional triade (Fig. 1B). Small heat shock proteins ((sHsps); Table 1) are typically holdases serving as a transient reservoir to prevent irreversible aggregation and to facilitate the resolubilization of aggregated protein via disaggregating chaperones in an ATP independent manner [4]. Although all sHsp monomers possess a core alpha crystalline domain, sHsps form diverse higher order oligomeric structures with oligomers being monodisperse or polydisperse, dependent on the affiliation to either major class A or B sHsps, respectively [5]. A structural shift between low and high substrate affinity states occurs upon signal induction, such as heat shock [4]. Deletion of sHsp(s) frequently conveys a mild or no phenotype suggesting the existence of functional redundance or backup systems.

In the ATP dependent disaggregating bi-chaperone system, DnaK/Hsp70 and its co-chaperone DnaJ/Hsp40 in cooperation with the nucleotide exchange factor GrpE, interact with protein aggregates and subsequently guide substrates to the ClpB/Hsp100 disaggregase (Fig. 1B, [3,6]). DnaK binds to the coiled-coil structured middle domain (M-domain), which is N- and C-terminal flanked by two AAA+ ATPase domains of hexameric ClpB to modulate ClpB ATPase and disaggregating activity (Fig. 2; [6]). ATP hydrolysis generates the threading power to translocate the substrate through the ClpB hexamer. This coordinated action can be enhanced by sHsps [7]. Subsequently, the unfolded proteins are refolded by the folding chaperones, DnaK/DnaJ and GroEL/GroES, in an ATP-dependent manner [8]. The DnaK/DnaJ and GroEL/GroES chaperone systems have a dual role as they also inhibit premature misfolding of newly translated proteins and prevent aggregation of nascent polypeptides (Fig. 1A, [1]). Although chaperone systems are

highly abundant and extensively studied in many bacteria, the precise role of individual components in bacterial physiology is less understood.

In this review, we describe the impact of maintaining protein integrity on bacterial physiology with a major focus on holding and disaggregating chaperones. In addition, we discuss the biological role of modules dedicated to protein quality control present on a horizontally transferred genomic island or plasmid that provides a survival advantage against heat shock to world-wide prevalent *Pseudomonas aeruginosa* clone C and other bacterial clones of diverse species.

Role of chaperones in response to various stresses

Disfunctionalization of the heat shock regulon by deletion of the determinative sigma factor σ^{32} leads to unphysiological protein aggregation under non-stress conditions highlighting the physiological impact to aid protein folding by chaperones [9]. Heat resistance is the most prominent phenotype of members of the protein quality control system relevant for bacteria in the environment and the host. Thus, promotion of survival and virulence in the host depends on the major disaggregating chaperone ClpB in several bacterial pathogens [10-12]. Consequently, also expression of various chaperones including sHsp, DnaK and ClpB is upregulated in interaction with host immune cells such as macrophages [13-15]. As an additional phenotype, *clpB* deletion in a *F. tularensis* live vaccine strain provokes a robust early host immune response with altered cytokine production suggesting that ClpB interferes with antigen expression and/or processing [16]. Interestingly, a recent study showed that the sHsps IbpA/IbpB are upregulated during experimental colitis and protect specifically non-pathogenic *E. coli* strains from reactive oxygen species produced by macrophages [15]. As the presence of elevated number of *E. coli* contributes to the etiopathogenesis of inflammatory bowel diseases. IbpA/IbpB can be considered novel

context-dependent virulence factors of *E. coli*. As a conclusion, exposure to elevated temperature and reactive radicals are major stress factors *in vivo* leading to thermal aggregation in combination with protein oxidation.

Treatment with antibiotics is another stress condition with chaperones induced [17-19]. For instance, the expression of IbpA is induced by tobramycin up to 90-fold in *P. aeruginosa* [17]. Indeed, heat-treatment of *A. baumannii* promotes enhanced resistance to a subsequent streptomycin treatment [18]. In *M. tuberculosis*, deficiency of ClpB impairs response to antibiotics [12]. As ClpB has been shown to sequester irreversibly oxidized proteins in *M. tuberculosis* [12], upregulation of the protein quality control system possibly protects the cells from the subsequent redox stress upon antibiotic treatment [20]. Protein oxidation and disulfide stress also occurs upon starvation with induction of the heat shock response [21,22].

Dramatic temperature intervals are probably the most common adverse condition for all living organisms including bacteria. The role of protein homeostasis at low temperature was highlighted by demonstrating expression of the GroEL chaperone from an antarctic bacterium to lower the minimal growth temperature of *E. coli* by more than 20°C [23]. Upregulation of genes related to protein homeostasis, such as *groESL*, *dnaK* and *clpB*, under cold shock in *Sphingopyxis alaskensis* also highlights the role of chaperones in cold adaptation [24].

Organic solvents, i.e. aromatic compounds such as phenol and toluene, are stressors due to their membrane-damaging effect and denaturation of proteins. Upregulation of stress-response genes including various chaperones, such as Hsp90 family protein HtpG and ClpB, occurs in the presence of aromatic solvents [25]. A recent report notes the contribution of a tricistronic sHsp gene cluster of *Pseudomonas putida* to tolerance against organic solvents [26]. This tricistronic gene cluster is found exclusively in environmental bacteria with high metabolic versatility suggesting a specialized role in protection against aromatic compounds in polluted soils. Besides prevention of protein

aggregation, sHsps as dimers might maintain membrane integrity as a second mechanism of protection (Fig. 1B, [27,28]).

Desiccation and ultraviolet responses are common stresses mainly to environmental microorganisms to cause DNA damage and protein denaturation with the involvement of small heat shock proteins [29,30]. As another phenotype, chaperones such as the sHsps IbpA/IbpB as well as specialized GroEL affect biofilm formation in *E. coli* and *Mycobacterium smegmatis*, respectively [31,32]. A general role of protein homeostasis in biofilm formation, though, needs to be further investigated. In conclusion, a response to plethora of stresses requires elevated activity to maintain protein homeostasis.

Regulation of protein homeostasis

The protein homeostasis regulon is directed by the heat shock sigma factor σ^{32} which is efficiently degraded upon interaction with DnaK [33]. Under permissible conditions, elevated temperature and upon oxidative stress induced glutathionylation disrupt this interaction and allows the heat shock response [34]. Intriguingly, in *Caulobacter crescentus*, aggregated proteins serve as signals to allosterically regulate the activity of the Lon protease to degrade DnaA, the universal replication initiator thereby regulating proliferation [35]. Thus, aggregated proteins delicately regulated by chaperones might be general intrinsic signals to regulate proteostasis. These recent studies on the regulation of protein homeostasis put a glimpse on regulatory mechanisms to be discovered.

A genetic island encodes protein quality control modules in world-wide *P. aeruginosa* clone C

Surprisingly, protein homeostasis in the environmental organism and opportunistic pathogen *P. aeruginosa* has hardly been characterized. Genome analysis though, revealed that *P. aeruginosa* harbors all essential chaperone components as described in *E. coli* (Table 1, Fig. 1). Recently, comparative genome analysis identified a clone C specific island (PACGI-1) in *P. aeruginosa* clone C strains. Interestingly, the PACGI-1 contains genes involved in protein quality control such as proteins scavenging oxygen radicals and second copies of core genome encoded protein homeostasis gene products. Clone C, a cluster of closely related strains, is the most prevalent clone in patients with acute and chronic diseases, predominantly in cystic fibrosis and chronic obstructive pulmonary disease (COPD) [36,37]. On the other hand, clone C strains are often recovered from environmental and technical aquatic habitats [38-40]. However, it has been largely unclear what factors determine the success of clone C strains in the host and environment.

A novel system for protein quality control: sHsp20_{GI} and ClpG_{GI} proteins

On PACGI-1, most prominent is the *dna-shsp20c-clpG_{GI}* gene cluster with this gene arrangement to be phylogenetically most conserved (Table 2). Indeed, *dna-shsp20c-clpG_{GI}* mediates heat shock resistance to the host strain and genetically unrelated *P. aeruginosa* strains [41]. Structural and biochemical characterization revealed that sHsp20c (renamed sHsp20_{GI}) contains an α -crystallin domain, forms a monodisperse 24-meric sphere-like oligomeric structure (Fig. 3A) and exhibits holding chaperone activity characteristic for bacterial class B small heat shock protein [41]. Indeed, sHsp20_{GI} is the founding member of a novel class B subclass consisting exclusively of highly homologous horizontally transferred *shsp20_{GI}* homologues. The core genome encoded small heat shock protein lbpA, a class A heat shock protein, is only distantly related to sHsp20_{GI}. Consistent with class A proteins, *P. aeruginosa* lbpA has a polydisperse oligomeric structure (Fig. 3B) as lbpB of *E. coli* [42]. In contrast, *E. coli* lbpA is prone to form filaments *in vitro* [42], which probably is a

purification artifact (A. Mogk, personal communication).

ClpG_{GI} is an ATP-dependent ClpB-like Hsp100 family protein (previously designated ClpBc) encoded downstream of *shsp20_{GI}*. ClpG_{GI} has unique characteristics, an extended N- and C-terminal region and a shorter M-domain [41] (Fig. 2). In *E. coli*, the M-domain of ClpB is involved in interaction with DnaK. Noticeably, three conserved cysteines and a single histidine in the N-terminal region possibly forming a zinc finger motif. These unique features suggest a distinct role compared to the disaggregating chaperone ClpB of the core genome. Also, compared to *E. coli*, the chaperone network is extended in *P. aeruginosa* with a ClpG_{GI}-like protein with 76.3% similarity, named ClpG, encoded on the core genome (Table 1). The horizontally transferred back-up copy of ClpG_{GI}, though, is functionally coupled to sHsp20_{GI} as it elevates the heat resistance phenotype mediated by sHsp20_{GI} [41].

ClpK, a ClpG_{GI} homologue with 96% sequence identity, located on a conjugative plasmid, contributes to thermotolerance in the clinical isolate *Klebsiella pneumoniae* C132-98 [43] (Table 2). The frequent acquisition of an additional small heat shock protein downstream of the *dna-shsp20_{GI}-clpG_{GI}* gene cluster (Table 2), though, suggests promiscuity of ClpG_{GI}/ClpK functionality in interaction with sHsps. Of note, thermotolerance mediated by ClpK partially required the ClpP protease in the heterologous host *E. coli* [44] suggesting that ClpK possesses unfoldase functionality homologous to ClpA and ClpX. Of note though, in contrast to ClpA and ClpX, a ClpP-interacting loop is not present in ClpG_{GI}/ClpK (Fig. 2). Thus, at least two functional modes of ClpG_{GI}/ClpK seem to exist, assistance in ClpP-dependent proteolysis and disaggregating chaperone activity (Fig. 1). If these activities are proven *in vitro*, further question such as characterization of substrate specificity of ClpG-like proteins and interactions with substrate and ClpP arise.

A horizontally transferred chaperone gene cluster

The *P. aeruginosa* clone C specific genomic island (PACGI-1) harbors more than 100 genes in addition to the *dna-shsp20_{GI}-clpG_{GI}* gene cluster [41]. The composite island structure is modular with several additional genes involved in protein quality control. The *dna-shsp20_{GI}-clpG_{GI}* gene cluster is frequently part of a longer ~18 kbp Transmissible Locus for Protein Quality Control-1 (TLPQC-1), flanked by mobile element genes and encoding additional protein homeostasis genes, such as FtsH_{GI}, a second copy of the essential membrane-bound ATP-dependent protease and Trx_{GI}, another thioredoxin homologue ([41], Table 2). Islands most closely related to PACGI-1 are found in environmental isolates, such as *Ralstonia pickettii* 12D and *Methylobacillus flagellates* KT isolated from lake sediment and sewer, respectively (Table 2, [41,45]).

Of note, the protein homeostasis module with *dna-shsp20_{GI}-clpG_{GI}* as the core unit is variably present in clinically relevant strains of species such as *Klebsiella pneumoniae* [43], *Cronobacter sakazakii* [45] and food born *E. coli* [46] (examples in Table 2; [46]). Analysis of thermotolerance in *K. pneumoniae* and other species suggests that the module genes are determinative for an elevated heat resistance phenotype, which is subsequently enhanced by downstream gene products [43,45,46]. Indeed, *K. pneumoniae* C132-98, which harbors at least four additional TLPQC-1 genes shows high thermotolerance, while MGH78578 with only the *dna-shsp20_{GI}-clpG_{GI}* module shows moderate thermotolerance (Table 2). *dna-shsp20_{GI}-clpG_{GI}* might have a distinguished role in starvation stress, since the gene products are expressed at temperatures between 20 and 42°C in stationary phase in *P. aeruginosa* and potentially other bacteria [41,44].

Thermotolerance might contribute to successful survival of common clonal isolates

P. aeruginosa clone C strains with a sHsp20_{GI} protein are significantly more heat resistant than non-sHsp20_{GI} bearing non-clone C strains [41]. Of note, the PACGI-1 island is also present in the

fourth most common *P. aeruginosa* clone J. Indeed, the *dna-shsp20_{GI}-clpG_{GI}* module is frequently found in isolates of *K. pneumoniae* clones, which cause significant global and local outbreaks, such as sequence types ST258 and ST16, but also in other sequence types on outbreak-related plasmids [47,48]. As outbreak plasmids of *K. pneumoniae* otherwise almost exclusively code for elements related to acquisition or transposition of antibiotic and heavy metal resistance, the thermotolerance conferred by the module may aid successful spread and infection of certain common clonal strains.

Although elevation in temperature is one of the most significant changes during the transition from the environment to the host, PACGI-1 is present in only 20% of genetically unrelated clinical strains of *P. aeruginosa*, compared to 56% of environmental isolates [41]. In the same line, although TLPQC-1 confers heat resistance to a food isolate of *E. coli*, only 2% of all sequenced isolates harbor the module [46]. This suggests that TLPQC-1/PACGI-1 confers only an advantage in certain strain backgrounds, or is deleterious under certain circumstances. Indeed, although being one of the most highly expressed proteins of *P. aeruginosa* clone C strains [41], sHsp20_{GI} is deleterious when overexpressed in the native host and in *E. coli* [41].

Ecological disadvantage may be conferred as chaperones can serve as antigens. For example, sHsp Acr2 of *M. tuberculosis* is a strong immunogen in both cattle and humans [49]. Recently, ClpB of commensal gut bacteria *E. coli* was identified as a conformational antigen mimetic of α -melanocyte-stimulating hormone (α -MSH), influencing food intake, satiety, body weight and anxiety [50,51]. This suggests that ClpB-expressing gut microorganisms are involved in the feeding and emotional behaviour in humans with eating disorders.

Interference with protein homeostasis, a successful approach to treat bacterial infections?

Bacterial cell division is only superficially symmetric. Upon stress, aggregated and irreversibly oxidized proteins accumulate at the old cell pole sequestered by chaperones, which leads to decreased replication proficiency, impaired recovery [12,52] and ultimately growth arrest [35]. Thus, drugs that interfere with protein quality control components might aid the treatment of bacteria including *M. tuberculosis* [12]. In an alternative approach, the cytotoxic effect of protein aggregation was utilized for a novel antibacterial strategy [53]. Cell-penetrable peptides designed based on aggregation prone regions in bacterial proteins specifically lead to fast cell death. As mammalian cells are not affected, this approach can be a therapeutic alternative to conventional antibiotics. On the other hand, the horizontal transfer of protein quality control modules, as described above, already indicates that also against such novel antimicrobial approaches, resistance mechanisms may arise.

Conclusion

Protein homeostasis is required for bacteria to adapt to various niches and for virulence. Of note, horizontal transmission of protein quality control modules is found in world-wide prevalent niche-spanning clones of *P. aeruginosa*, outbreak strains of *K. pneumoniae* and other clinically relevant isolates of diverse genera suggesting heat tolerance to be a novel virulence, persistence and resistance factor probably promoted by modern human life style and the advanced medical care systems. On the other hand, protein homeostasis and aggregation are novel targets for antibacterial interference strategies. To date, several chemical compounds inhibiting the activity of chaperone systems have been discovered, such as pyrrolicorin targeting DnaK [54,55] and several small molecules targeting ClpB [56,57]. However, the protein quality control system can be extendedly targeted to develop antimicrobial agents. Moreover, since several chaperone proteins act as highly expressed immunogens which penetrate the host, they are potential bacterial

250 markers in diagnostics.

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258 **References**

259 * Special interest

260 ** Outstanding interest

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262 1. Hartl FU, Bracher A, Hayer-Hartl M: **Molecular chaperones in protein folding and proteostasis.**
263 *Nature* 2011, **475**:324-332.

264 2. Valastyan JS, Lindquist S: **Mechanisms of protein-folding diseases at a glance.** *Dis Model*
265 *Mech* 2014, **7**:9-14.

266 3. Tyedmers J, Mogk A, Bukau B: **Cellular strategies for controlling protein aggregation.** *Nat Rev*
267 *Mol Cell Biol* 2010, **11**:777-788.

268 4. Haslbeck M, Franzmann T, Weinfurter D, Buchner J: **Some like it hot: the structure and**
269 **function of small heat-shock proteins.** *Nat Struct Mol Biol* 2005, **12**:842-846.

270 5. Fu X, Jiao W, Chang Z: **Phylogenetic and biochemical studies reveal a potential evolutionary**

271 **origin of small heat shock proteins of animals from bacterial class A.** *J Mol Evol* 2006,
272 **62:**257-266.

273 6. Mogk A, Kummer E, Bukau B: **Cooperation of Hsp70 and Hsp100 chaperone machines in**
274 **protein disaggregation.** *Front Mol Biosci* 2015, **2:**22.

275 7. Mogk A, Deuerling E, Vorderwulbecke S, Vierling E, Bukau B: **Small heat shock proteins, ClpB**
276 **and the DnaK system form a functional triade in reversing protein aggregation.** *Mol*
277 *Microbiol* 2003, **50:**585-595.

278 8. Saibil H: **Chaperone machines for protein folding, unfolding and disaggregation.** *Nat Rev*
279 *Mol Cell Biol* 2013, **14:**630-642.

280 9. Tomoyasu T, Mogk A, Langen H, Goloubinoff P, Bukau B: **Genetic dissection of the roles of**
281 **chaperones and proteases in protein folding and degradation in the *Escherichia coli***
282 **cytosol.** *Mol Microbiol* 2001, **40:**397-413.

283 10. Lourdault K, Cerqueira GM, Wunder EA, Jr., Picardeau M: **Inactivation of clpB in the pathogen**
284 ***Leptospira interrogans* reduces virulence and resistance to stress conditions.** *Infect*
285 *Immun* 2011, **79:**3711-3717.

286 11. de Oliveira NE, Abranches J, Gaca AO, Laport MS, Damaso CR, Bastos Mdo C, Lemos JA,
287 Giambiagi-deMarval M: **clpB, a class III heat-shock gene regulated by CtsR, is involved**
288 **in thermotolerance and virulence of *Enterococcus faecalis*.** *Microbiology* 2011, **157:**656-
289 665.

290 ** 12. Vaubourgeix J, Lin G, Dhar N, Chenouard N, Jiang X, Botella H, Lupoli T, Mariani O, Yang G,
291 Ouerfelli O, et al.: **Stressed mycobacteria use the chaperone ClpB to sequester**
292 **irreversibly oxidized proteins asymmetrically within and between cells.** *Cell Host*
293 *Microbe* 2015, **17:**178-190.

294 This study shows that in mycobacteria ClpB associates with irreversibly oxidized proteins to
295 distribute asymmetrically between progeny, eventually affecting responses to antibiotics

- and counteract infection.
13. Buchmeier NA, Heffron F: **Induction of *Salmonella* stress proteins upon infection of macrophages.** *Science* 1990, **248**:730-732.
 14. Greenberg DE, Sturdevant DE, Marshall-Batty KR, Chu J, Pettinato AM, Virtaneva K, Lane J, Geller BL, Porcella SF, Gallin JI, et al.: **Simultaneous Host-Pathogen Transcriptome Analysis during *Granulibacter bethesdensis* Infection of Neutrophils from Healthy Subjects and Patients with Chronic Granulomatous Disease.** *Infect Immun* 2015, **83**:4277-4292.
 15. Goeser L, Fan TJ, Tchaptchet S, Stasulli N, Goldman WE, Sartor RB, Hansen JJ: **Small heat-shock proteins, IbpAB, protect non-pathogenic *Escherichia coli* from killing by macrophage-derived reactive oxygen species.** *PLoS One* 2015, **10**:e0120249.
 16. Barrigan LM, Tuladhar S, Brunton JC, Woolard MD, Chen CJ, Saini D, Frothingham R, Sempowski GD, Kawula TH, Frelinger JA: **Infection with *Francisella tularensis* LVS *clpB* leads to an altered yet protective immune response.** *Infect Immun* 2013, **81**:2028-2042.
 17. Wu X, Held K, Zheng C, Staudinger BJ, Chavez JD, Weisbrod CR, Eng JK, Singh PK, Manoil C, Bruce JE: **Dynamic Proteome Response of *Pseudomonas aeruginosa* to Tobramycin Antibiotic Treatment.** *Mol Cell Proteomics* 2015, **14**:2126-2137.
 18. Cardoso K, Gandra RF, Wisniewski ES, Osaku CA, Kadowaki MK, Felipach-Neto V, Haus LF, Simao Rde C: **DnaK and GroEL are induced in response to antibiotic and heat shock in *Acinetobacter baumannii*.** *J Med Microbiol* 2010, **59**:1061-1068.
 19. Tran TD, Kwon HY, Kim EH, Kim KW, Briles DE, Pyo S, Rhee DK: **Decrease in penicillin susceptibility due to heat shock protein ClpL in *Streptococcus pneumoniae*.** *Antimicrob Agents Chemother* 2011, **55**:2714-2728.
 20. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT, Lobritz MA, Braff D, Schwarz EG, et al.: **Antibiotics induce redox-related physiological alterations as**

- part of their lethality. *Proc Natl Acad Sci U S A* 2014, **111**:E2100-2109.
21. Dukan S, Nystrom T: **Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon.** *Genes Dev* 1998, **12**:3431-3441.
22. Muller A, Hoffmann JH, Meyer HE, Narberhaus F, Jakob U, Leichert LI: **Nonnative disulfide bond formation activates the sigma32-dependent heat shock response in *Escherichia coli*.** *J Bacteriol* 2013, **195**:2807-2816.
23. Ferrer M, Chernikova TN, Yakimov MM, Golyshin PN, Timmis KN: **Chaperonins govern growth of *Escherichia coli* at low temperatures.** *Nat Biotechnol* 2003, **21**:1266-1267.
24. Ting L, Williams TJ, Cowley MJ, Lauro FM, Guilhaus M, Raftery MJ, Cavicchioli R: **Cold adaptation in the marine bacterium, *Sphingopyxis alaskensis*, assessed using quantitative proteomics.** *Environ Microbiol* 2010, **12**:2658-2676.
25. Trautwein K, Kuhner S, Wohlbrand L, Halder T, Kuchta K, Steinbuchel A, Rabus R: **Solvent stress response of the denitrifying bacterium "*Aromatoleum aromaticum*" strain EbN1.** *Appl Environ Microbiol* 2008, **74**:2267-2274.
- * 26. Krajewski SS, Joswig M, Nagel M, Narberhaus F: **A tricistronic heat shock operon is important for stress tolerance of *Pseudomonas putida* and conserved in many environmental bacteria.** *Environ Microbiol* 2014, **16**:1835-1853.
- Identification and characterization of a novel tricistronic small heat shock protein gene cluster wide spread in metabolically versatile environmental bacterial species.
27. Maitre M, Weidmann S, Dubois-Brissonnet F, David V, Coves J, Guzzo J: **Adaptation of the wine bacterium *Oenococcus oeni* to ethanol stress: role of the small heat shock protein Lo18 in membrane integrity.** *Appl Environ Microbiol* 2014, **80**:2973-2980.
28. Nakamoto H, Vigh L: **The small heat shock proteins and their clients.** *Cell Mol Life Sci* 2007,

64:294-306.

29. Cocotl-Yanez M, Moreno S, Encarnacion S, Lopez-Pliego L, Castaneda M, Espin G: **A small heat-shock protein (Hsp20) regulated by RpoS is essential for cyst desiccation resistance in *Azotobacter vinelandii*.** *Microbiology* 2014, **160**:479-487.
30. Rastogi RP, Sinha RP, Moh SH, Lee TK, Kottuparambil S, Kim YJ, Rhee JS, Choi EM, Brown MT, Hader DP, et al.: **Ultraviolet radiation and cyanobacteria.** *J Photochem Photobiol B* 2014, **141**:154-169.
31. Ojha A, Anand M, Bhatt A, Kremer L, Jacobs WR, Jr., Hatfull GF: **GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria.** *Cell* 2005, **123**:861-873.
32. Kuczynska-Wisnik D, Matuszewska E, Laskowska E: ***Escherichia coli* heat-shock proteins IbpA and IbpB affect biofilm formation by influencing the level of extracellular indole.** *Microbiology* 2010, **156**:148-157.
33. Straus D, Walter W, Gross CA: **DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of sigma 32.** *Genes Dev* 1990, **4**:2202-2209.
34. Zhang H, Yang J, Wu S, Gong W, Chen C, Perrett S: **Glutathionylation of the Bacterial Hsp70 Chaperone DnaK Provides a Link Between Oxidative Stress and the Heat Shock Response.** *J Biol Chem* 2016.
35. Jonas K, Liu J, Chien P, Laub MT: **Proteotoxic stress induces a cell-cycle arrest by stimulating Lon to degrade the replication initiator DnaA.** *Cell* 2013, **154**:623-636.
36. Wiehlmann L, Cramer N, Tümmler B: **Habitat-associated skew of clone abundance in the *Pseudomonas aeruginosa* population.** *Environ Microbiol Rep* 2015.
37. Römling U, Fiedler B, Bosshammer J, Grothues D, Greipel J, von der Hardt H, Tümmler B: **Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis.** *J Infect*

Dis 1994, **170**:1616-1621.

38. Römling U, Wingender J, Muller H, Tümmler B: **A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats.** *Appl Environ Microbiol* 1994, **60**:1734-1738.

39. Selezska K, Kazmierczak M, Musken M, Garbe J, Schobert M, Haussler S, Wiehlmann L, Rohde C, Sikorski J: ***Pseudomonas aeruginosa* population structure revisited under environmental focus: impact of water quality and phage pressure.** *Environ Microbiol* 2012, **14**:1952-1967.

40. Petit SM, Lavenir R, Colinon-Dupuich C, Boukerb AM, Cholley P, Bertrand X, Frenay J, Doleans-Jordheim A, Nazaret S, Laurent F, et al.: **Lagooning of wastewaters favors dissemination of clinically relevant *Pseudomonas aeruginosa*.** *Res Microbiol* 2013, **164**:856-866.

* 41. Lee C, Wigren E, Trcek J, Peters V, Kim J, Hasni MS, Nimtz M, Lindqvist Y, Park C, Curth U, et al.: **A novel protein quality control mechanism contributes to heat shock resistance of worldwide-distributed *Pseudomonas aeruginosa* clone C strains.** *Environ Microbiol* 2015.

This study characterizes the novel heat shock protein sHsp20_{GI}, from worldwide prevalent *P. aeruginosa* clone C and shows that the horizontally transferred gene cluster *dna-shsp20_{GI}-clpG_{GI}* contributes to heat shock resistance in *P. aeruginosa* strains.

42. Ratajczak E, Strozecka J, Matuszewska M, Zietkiewicz S, Kuczynska-Wisnik D, Laskowska E, Liberek K: **IbpA the small heat shock protein from *Escherichia coli* forms fibrils in the absence of its cochaperone IbpB.** *FEBS Lett* 2010, **584**:2253-2257.

43. Bojer MS, Struve C, Ingmer H, Hansen DS, Krogfelt KA: **Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*.** *PLoS One* 2010, **5**:e15467.

First report on the molecular mechanism of thermotolerance in *Klebsiella pneumoniae* clinical

isolates identifying the ClpB-like disaggregating chaperone ClpK (ClpG) as the major mediator of thermotolerance.

44. Bojer MS, Struve C, Ingmer H, Krogfelt KA: **ClpP-dependent and -independent activities encoded by the polycistronic clpK-encoding locus contribute to heat shock survival in *Klebsiella pneumoniae*.** *Res Microbiol* 2013, **164**:205-210.

This study suggests that the thermotolerance of *K. pneumoniae* derived ClpK (ClpG) is partially mediated via the protease ClpP in the *E. coli* heterologous host.

45. Gajdosova J, Benedikovicova K, Kamodyova N, Tothova L, Kaclikova E, Stuchlik S, Turna J, Drahovska H: **Analysis of the DNA region mediating increased thermotolerance at 58 degrees C in *Cronobacter* sp. and other enterobacterial strains.** *Antonie Van Leeuwenhoek* 2011, **100**:279-289.

46. Mercer RG, Zheng J, Garcia-Hernandez R, Ruan L, Ganzle MG, McMullen LM: **Genetic determinants of heat resistance in *Escherichia coli*.** *Front Microbiol* 2015, **6**:932.

47. Lohr IH, Hultner N, Bernhoff E, Johnsen PJ, Sundsfjord A, Naseer U: **Persistence of a pKPN3-like CTX-M-15-encoding IncFIIK plasmid in a *Klebsiella pneumonia* ST17 host during two years of intestinal colonization.** *PLoS One* 2015, **10**:e0116516.

48. Sandegren L, Linkevicius M, Lytsy B, Melhus A, Andersson DI: **Transfer of an *Escherichia coli* ST131 multiresistance cassette has created a *Klebsiella pneumoniae*-specific plasmid associated with a major nosocomial outbreak.** *J Antimicrob Chemother* 2012, **67**:74-83.

49. Wilkinson KA, Stewart GR, Newton SM, Vordermeier HM, Wain JR, Murphy HN, Horner K, Young DB, Wilkinson RJ: **Infection biology of a novel alpha-crystallin of *Mycobacterium tuberculosis*. Acr2.** *J Immunol* 2005, **174**:4237-4243.

* 50. Tennoune N, Chan P, Breton J, Legrand R, Chabane YN, Akkermann K, Jarv A, Ouelaa W, Takagi K, Ghousali I, et al.: **Bacterial ClpB heat-shock protein, an antigen-mimetic of the**

anorexigenic peptide alpha-MSH, at the origin of eating disorders. *Transl Psychiatry* 2014, **4**:e458.

* 51. Breton J, Tennoune N, Lucas N, Francois M, Legrand R, Jacquemot J, Goichon A, Guerin C, Peltier J, Pestel-Caron M, et al.: **Gut Commensal *E. coli* Proteins Activate Host Satiety Pathways following Nutrient-Induced Bacterial Growth.** *Cell Metab* 2015.

These two recent studies, 50 and 51, show that ClpB is an antigen mimetic affecting human eating disorder and also satiety pathways.

52. Lindner AB, Madden R, Demarez A, Stewart EJ, Taddei F: **Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation.** *Proc Natl Acad Sci U S A* 2008, **105**:3076-3081.

** 53. Bednarska NG, van Eldere J, Gallardo R, Ganesan A, Ramakers M, Vogel I, Baatsen P, Staes A, Goethals M, Hammarstrom P, et al.: **Protein aggregation as an antibiotic design strategy.** *Mol Microbiol* 2015.

This study shows an intriguing novel antimicrobial strategy by using aggregation prone peptides to disrupt protein homeostasis.

54. Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos L, Jr.: **The antibacterial peptide pyrrocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding.** *Biochemistry* 2001, **40**:3016-3026.

55. Otvos L, Jr., O I, Rogers ME, Consolvo PJ, Condie BA, Lovas S, Bulet P, Blaszczyk-Thurin M: **Interaction between heat shock proteins and antimicrobial peptides.** *Biochemistry* 2000, **39**:14150-14159.

56. Martin I, Underhaug J, Celaya G, Moro F, Teigen K, Martinez A, Muga A: **Screening and evaluation of small organic molecules as ClpB inhibitors and potential antimicrobials.** *J Med Chem* 2013, **56**:7177-7189.

442 57. Torrente MP, Castellano LM, Shorter J: **Suramin inhibits Hsp104 ATPase and disaggregase**
443 **activity**. *PLoS One* 2014, **9**:e110115.

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446 **Table 1. Chaperones and AAA+ proteases in *E. coli* and *P. aeruginosa***

Chaperone /protease	<i>E. coli</i> K-12 MG1655	<i>P. aeruginosa</i>		
		PAO1	PA14	SG17M (clone C)
sHsp20	IbpA IbpB	PA3126	PA14_23680	EW28912.1 sHsp20 _{GI} (EW27924.1)
Hsp70/40 GrpE	DnaK DnaJ GrpE	PA4761 PA4760 PA4762	PA14_62970 PA14_62960 PA14_62990	EW24242.1 EW24241.1 EW24243.1
ClpB	ClpB	PA4542	PA14_60190	EW24017.1
ClpG	-	PA0459	PA14_06000	EW25562.1 ClpG _{GI} (EW27925.1)
ClpP¹ proteolysis complex	ClpA ClpX ClpP	PA2620 PA1802 PA1801	PA14_30230 PA14_41230 PA14_41240	EW28279.1 EW27217.1 EW27216.1
AAA+ Protease	FtsH Lon	PA4751 PA1803	PA14_62860 PA14_41220	(EW24232.1 FtsH _{GI} (EW27927.1) EW27218.1

447 ¹ ClpP protease is not an AAA+ family protein but forms a proteolytic complex with AAA+
448 proteins ClpA and ClpX.

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Table 2 TLPQC-1 in various clinical and environmental isolates

Strains	Genetic map of TLPQC-1 ¹	Origin/source	Location of TLPQC-1	Reference
<i>P. aeruginosa</i> 8277 (clone C)		Urine, human	Genome	Unpublished
Environmental isolates				
<i>P. aeruginosa</i> SG17M (clone C)		River, Germany	Genome	[41]
<i>R. pickettii</i> 12D		Lake sediment	Genome	[45]
<i>M. flagellates</i> KT		Sewer	Genome	[45]
Clinical isolates				
<i>K. pneumoniae</i> C132-98 ²		Blood, human	Plasmid: pMB58	[43]
<i>K. pneumoniae</i> MGH78578		Sputum, human	Plasmid: pKPN3	[43]
<i>C. sakazakii</i> ATCC 29544		Throat, human	Unknown	[45]
<i>E. coli</i> AW1.7		Beef	Genome	[46]

<i>E. cloacae</i> ATCC 13047		Cerebrospinal fluid, human	Genome	[45]
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¹ Open reading frames (ORF) of TLPQC-1 were marked. *dna*, MerR-like transcriptional regulator; *shsp20_{Gh}*, small heat shock protein; *clpG_{Gh}*/*clpK*, ATPase chaperone; cardiolipid synthase, phospholipase; *ftsH_{Gh}*, metalloprotease; *shsp_{Gh}*, small heat shock protein; *yfdX_{Gh}1;2*, function related to thermal; osmotic and desiccation stress; *hdeD_{Gh}*, transmembrane protein with acid tolerance; *trx_{Gh}*, thioredoxin; *kefC_{Gh}*, glutathione-dependent potassium-efflux system and methylglyoxal detoxification; *htpX_{Gh}*, peptidase controlling quality of inner membrane proteins; *degP_{Gh}*, periplasmic protein with chaperone and protease activity. ORF16 encodes a putative HTH-type transcriptional regulator/D-alanyl-D-alanine endopeptidase. ORF10, 13, and 17 encode hypothetical proteins. ORFs with grey color indicate mobile elements associated genes encoding e.g. transposases and integrases. Exceptionally, the *cas1*, a multifunctional nuclease in CRISPR-Cas system is located at the 3' region of TLPQC-1 in SG17M. ORFs with no homology to TLPQC-1 encoded genes are shown as open arrows.

² Plasmid TLPQC-1 of pMB58 is not completely sequenced.

Figure legends

Figure 1. Demonstrated and suggested role of novel chaperones, sHsp20_{GI} and ClpG_{GI}, in protein homeostasis in cooperation with core genome proteins (suggested for *P. aeruginosa* based on data from *E. coli*). (A) DnaK/DnaJ/GrpE and GroEL/GroES chaperone systems stabilize newly synthesized polypeptides on ribosomes and aid folding into the native conformation (N: native protein). (B) The core functional triad to maintain protein integrity consists of IbpA, ClpB and DnaK/DnaJ/GrpE (suggested in *P. aeruginosa*/adapted from *E. coli*). As IbpA of the core genome, sHsp20_{GI} functions as a holding chaperone. IbpA and sHsp20_{GI} might also be involved in maintenance of membrane integrity as dimers. ClpG/G_{GI} is predicted to have two functional modes, disaggregating activity and cooperation for ClpP-mediated proteolysis [44]. Suggested functionality/phenotypes in gray.

Figure 2. Domain structures and characteristic motifs of Hsp100 family proteins of *E. coli* and *P. aeruginosa*. AAA+: ATPases associated with a wide variety of cellular activities superfamily domain (cd00009), M domain: coiled-coil structure; Clp_N: Clp amino terminal domain (pfam02861); ClpB_D2-small: C-terminal, D2-small domain of ClpB protein (pfam10431); ClpP-interacting loop: a loop containing the conserved tripeptide sequence IGF; Zf-C4_ClpX: ClpX C4-type zinc finger (pfam06689); 3Cys-His: a potential zing finger motif with three conserved cysteines and a single histidine.

Figure 3. Oligomeric structure of (A) sHsp20_{GI} and (B) IbpA of *P. aeruginosa* SG17M clone C strain displayed by transmission electron microscopy. sHsp20_{GI} is homogenous in size with a particle diameter of roughly 14.5 nm. IbpA appears heterogenous in morphology and often has an ellipsoidal shape, ~33 nm in length and ~22 nm in diameter. (Samples have been diluted to 50 µg/mL and adsorbed to a 30 nm carbon film - supported by a C-collodium microgrid - which was blotted and shortly stained with saturated uranylacetate, pH 4.5. After blotting and air-drying the

sample was analyzed in the elastic bright field-mode with an integrated energy-filter transmission electron microscopy (EF-TEM) at 120 kV (Libra 120plus, Zeiss, Oberkochen, Germany) and the energy-selecting slit set to 10 eV. Images were recorded with a bottom-mounted cooled 2048x2048 CCD-camera (SharpEye; Tröndle, Moorenwies, Germany) at nominal x40000. Scale bars: 100 nm).

Figure 1

Figure 2

Figure 3