Multiple chaperonins in bacteria—novel functions and non-canonical behaviors

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Abstract Chaperonins are a class of molecular chaperones that assemble into a large double ring architecture with each ring constituting seven to nine subunits and enclosing a cavity for substrate encapsulation. The well-studied *Escherichia coli* chaperonin GroEL binds non-native substrates and encapsulates them in the cavity thereby sequestering the substrates from unfavorable conditions and allowing the substrates to fold. Using this mechanism, GroEL assists folding of about 10–15 % of cellular proteins. Surprisingly, about 30 % of the bacteria express multiple chaperonin genes. The presence of multiple chaperonins raises questions on whether they increase general chaperoning ability in the cell or have developed specific novel cellular roles. Although the latter view is widely supported, evidence for the former is beginning to appear. Some of these chaperonins can functionally replace GroEL in *E. coli* and are generally indispensable, while others are ineffective and likewise are dispensable. Additionally, moonlighting functions for several chaperonins have been demonstrated, indicating a functional diversity among the chaperonins. Furthermore, proteomic studies have identified diverse substrate pools for multiple chaperonins. We review the current perception on multiple chaperonins and their physiological and functional specificities.

Keywords Chaperonins · GroEL · Moonlighting · Heat shock

Introduction

One of the central processes in biology concerns with the conversion of genetic information encoded by DNA into proteins that finally carry out the genetic program. The genetic information from nucleotide bases is translated into polypeptides, which are required to fold into proteins in order to perform biochemical functions. Crowded cellular milieu and intermolecular interactions potentially lead to misfolding of the polypeptides, which either limits the protein availability for respective canonical function or results in forming toxic aggregates as observed in several neurodegenerative disorders. To overcome these problems, a sophisticated system of about 20 ubiquitous proteins works in the cell. These proteins, known as molecular chaperones, sequester substrate proteins during unfavorable conditions and thereby prevent misfolding. The substrate proteins are released upon resumption of favorable conditions, allowing them to refold to their native form (Lindquist and Craig 1988; Georgopoulos and Welch 1993). The recognition between chaperones and substrates is via surface-exposed hydrophobic patches that are otherwise buried in the folded state. Apart from their role as folding catalysts, molecular chaperones are involved in a multitude of biological processes, including disaggregation of protein aggregates, assembling of multisubunit proteins, polypeptide transport across biological membranes, and proteolysis (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). Since many of these proteins have been initially identified as the abundant proteins during heat shock, they are called heat-shock proteins (Hsps) and are classified according to molecular weight (Fig. 1). In addition to heat shock, genes encoding Hsps have been demonstrated to get induced, following various stresses including carbon, nitrogen, or phosphate starvation, in several prokaryotes (Volker et al. 1994; Rince et al. 2000; Svensater et al. 2000; Teixeira-Gomes et al. 2000).

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Chaperone Family	Topology of Binding	Co-chaperone	Known Function
Hsp100		ClpP, SspB, Hsp70, Hsp40	<ul style="list-style-type: none"> • Works with DnaK in ATP-dependent disaggregation and proteolysis • Prevents aggregation, degradation and turnover of unassembled mitochondrial proteins • Reactivates heat-damaged proteins • Establishes and maintains prion phenotype in yeast
Hsp90		Hop, Hip, Hsp70, Immunophilins, Grp78	<ul style="list-style-type: none"> • Refolds proteins in stressed cells. Probable secretory chaperone in prokaryotes • Major cytosolic chaperone in eukaryotes. • Cytoprotection and intracellular signaling • In ER, controls protein homeostasis, folding and assembly of secretory proteins
Hsp70		Hsp40, GrpE	<ul style="list-style-type: none"> • Ubiquitous Principal folding chaperone • Works with ClpB as disaggregase • Folding of newly synthesized proteins • Protein transport into ER and mitochondria
Hsp60 (Group I)		Hsp10	<ul style="list-style-type: none"> • Major chaperone for protein folding in prokaryotes • Stabilizes proteins during heat stress • Promotes folding of over-produced proteins • Major chaperone in mitochondria and chloroplast
Hsp60 (Group II)		Prefoldin/GimC	<ul style="list-style-type: none"> • Promotes folding of a cytosolic proteins in eukaryotes • Refolding of unfolded polypeptides in vitro
sHsps		-	<ul style="list-style-type: none"> • Stabilizes unfolded polypeptides • Prevents aggregation • Works with Hsp70 in protein refolding • Structural protein of eye lens

Fig. 1 Characteristics of different chaperone machines: molecular chaperones are classified according to their sizes and presented in the order of decreasing size: Hsp100—small Hsp. Hsp100 forms large hexameric assemblies and principally acts on misfolded protein aggregates, which, in coordination with other chaperones like Hsp70, are disassembled and either released to refold or pulled through the protease segment ClpP, where the client proteins are cleaved into peptides, thus preventing the buildup of toxic aggregates in the cell. Hsp90 exists as a dimer. With the help of ATP-induced conformational changes, the unfolded substrate is bound and released in favorable conformation. Several co-chaperones are identified for Hsp90 and several immunological roles have been demonstrated. Hsp70 acts with the help

of its co-chaperones DnaJ and GrpE. Extended unfolded polypeptides bind the substrate-binding domain (SBD) followed by the nucleotide-induced conformational changes at the nucleotide-binding domain which are channeled to SBD, releasing the substrate in a possibly folded conformation. Hsp60 exists in two forms, group I and group II, and forms large toroidal cavities for the substrate polypeptide to bind. ATP-induced conformational changes release the substrate from the cavity in a possibly folded conformation. Small Hsps are small proteins that oligomerize on the substrate and prevent its misfolding and aggregation. Release often follows return of the favorable conditions or action of ATP-dependent chaperones

The chaperones are classified functionally into three fundamental classes based on their mode of action: (1) foldases, (2) holdases, and (3) disaggregases (Richter et al. 2010; Kim et al. 2013). Foldases are the chaperones that actively refold unfolded proteins, generally with an ATP-dependent mechanism. Examples for this class include the Hsp70 and Hsp60 chaperones (Fig. 1). Holdases are the class of chaperones that

are capable of binding the folding intermediates and prevent their aggregation. The substrates are generally “handed over” to the holdases for refolding. Examples for this class include the small heat-shock proteins (sHsps) and the co-chaperone Hsp40 (Fig. 1). The third class of chaperones, the disaggregases, primarily acts on the protein aggregates and actively digests the toxic aggregates into small

peptide fragments (Parsell et al. 1994; Mogk and Bukau 2004). Examples for this class include the members of AAA+ ATPase superfamily, the Hsp100 chaperones (Fig. 1). Although this classification generally holds true, exceptions with functional overlap have been observed with Hsp33 (Hoffmann et al. 2004) and Hsp70 (Groemping and Reinstein 2001). The functional overlap for Hsp70 is notable since it switches between the holdase and foldase states canonically in response to the nucleotide exchange factor (Groemping and Reinstein 2001) but additionally assists the disaggregases (Mogk et al. 1999).

The two best-studied families of molecular chaperones are the ATP-dependent Hsp70 (KJE) and Hsp60 (chaperonin or GroEL/S) chaperones that recognize the exposed hydrophobic surfaces on the extended and the collapsed polypeptides, respectively (Bukau and Horwich 1998). Consequently, the majority of the newly synthesized polypeptides reach their functional conformation using these chaperone machines. The nascent or extended polypeptides bind Hsp70 followed by an ATP-dependent mechanism of binding and release, when the substrates are allowed to fold. Partially folded substrates from Hsp70 are handed over to the Hsp60 chaperones, which additionally sequester the substrates upon encapsulation and allow the substrates to fold. The substrate proteins that remain unfolded are destined for degradation by proteases. The Hsp60 class of molecular chaperones have been termed separately as chaperonins (Hemmingsen et al. 1988) to distinguish these ring-forming molecular chaperones from the other molecular chaperones and to simplify the complex nomenclature for similar molecular chaperones that were discovered around the same time (Ellis 1996).

The chaperonins

Chaperonins are the Hsp60 family of molecular chaperones that are characterized by the formation of isologous seven- to nine-membered ring enclosing two cavities to encapsulate misfolded substrate proteins. The encapsulated substrate proteins are driven to productive folding with ATP-dependent cycles of binding and release. Chaperonins are classified into two groups based on their phylogenetic distribution and the requirement of a co-chaperone. Group I constitutes the members present in the cytosol of prokaryotes and the endosymbiotically related membrane-bound eukaryotic organelle, mitochondria, and chloroplast. These require the co-chaperone, GroES or Cpn10, that forms a heptamer and acts as a lid to the cavity (Fig. 2a). Examples include the GroEL/GroES system from *Escherichia coli* and several eubacteria (Bukau and Horwich 1998; Horwich et al. 2001). Group II constitutes the chaperonins localized to the archaeal and eukaryotic cytosols. They possess a built-in lid for encapsulation and thus act

independent of a co-chaperonin (Fig. 2b). TCP-1 ring complex (TriC)/chaperonin containing TCP-1 complex (CCT) and the well-studied thermosome from archaea are the members of this class (Gutsche et al. 1999; TCP-1 stands for T-complex 1). Although the cellular roles played by the two groups of chaperonins are similar, differences in the tertiary and quaternary structures of these groups of chaperonins suggest distinct mechanisms of encapsulation and action. While the group II chaperonins are assisted by prefoldin (Iizuka et al. 2004) and Hsp70 homologues (Cuellar et al. 2008) for substrate capture, group I acts independently. In addition, inter-ring allosteric movements also differ between the two groups; while the group I chaperonins show sequential movement, the group II chaperonins exhibit concerted movement. Due to the presence of multiple classes of chaperonins, a consensus for the nomenclature was developed. The group I chaperonins from bacteria, mitochondria, and chloroplast have been named as GroEL, Hsp60, and Cpn60, respectively, and the group II chaperonins from archaea, fungi, and mammals as thermosome, TriC, and CCT, respectively (Kampinga et al. 2009).

Fig. 2 Structural architecture of group I and II chaperonins: crystallographic models of group I (a) and group II (b) chaperonins are presented. The *cis* and *trans* rings in the double toroidal architecture and individual domains in one subunit each of group I (GroEL) and group II (thermosome) chaperonins are color coded. *Api* apical domain, *Int* intermediate domain, and *Equ* equatorial domain. GroES binds on the *cis* ring and caps the bound substrate proteins. ATP-induced conformational transitions in GroEL monomers are presented as *ribbon diagrams*. Coordinates for the molecules were obtained from the structures deposited in PDB with the following ID: 1AON and 1A6D for group I and group II chaperonin, respectively; the molecular illustrations were prepared in the PyMol Molecular Graphics System version 1.3

Structural architecture of chaperonins

Initial genetic studies followed by biochemical and structural studies have portrayed the three-domain architecture of *E. coli* GroEL monomers and the GroES–GroEL interactions (Braig et al. 1994; Xu et al. 1997). The central region of the GroEL polypeptide constitutes the apical domain that is rich in hydrophobic residues and is a three-layer ($\beta\beta\alpha$) sandwich which binds the non-native substrates and GroES (Chen et al. 1994; Fenton et al. 1994; Lin et al. 1995). Equatorial ATPase domain spanning two extremities of the GroEL polypeptide is responsible for the ATPase activity and the bulk of inter-subunit and inter-ring interactions (Mayhew et al. 1996; Roseman et al. 1996). The equatorial domain is structurally an alpha helical orthogonal bundle and harbors a pseudo-Walker motif where the ATP binds. Hinge-forming intermediate domain is structurally a sandwich of two alpha–beta layers. The intermediate domain spans two regions of the polypeptide and connects the equatorial and apical domains in sequence and structure (Saibil et al. 1993; Ma et al. 2000). The conformational changes resulting from ATP binding and hydrolysis at the equatorial domain are transmitted to the apical domain via this region (Hayer-Hartl et al. 1995; Weissman et al. 1995; Ueno et al. 2004). ATP binding at the equatorial domain induces a rotation along the hinge region near the apical domain, whereby the apical domain is twisted up releasing the substrate into the cavity and exposing the hydrophobic patches for GroES to bind (Fig. 2a). Conformational changes induced by the nucleotide binding result in the opening and closing of the substrate-binding cavity (Meyer et al. 2003).

GroEL binds a wide range of unfolded or partially unfolded proteins via hydrophobic interactions in the parallel α -helical groove formed by H and L helices of the apical domain (Fenton et al. 1994; Braig et al. 1994). Co-operative binding of ATP at the equatorial domain triggers inward movement of the intermediate domain resulting in a 60° upward movement and a 90° counter clockwise rotation of the apical domain, which moves the substrate-binding patches away and upward, thereby releasing the substrates and allowing GroES to bind (Xu et al. 1997; Kumar and Mande 2011).

Mechanism of action of GroEL

Much of the present understanding about the mechanism of action of chaperonins has been derived from the biochemical, biophysical, and genetic studies on *E. coli* GroEL. GroEL function is known to be mediated by an interplay between its interaction with and encapsulation of the substrate proteins, which are accompanied by conformational changes induced by ATP binding, hydrolysis, and GroES binding (Hayer-Hartl et al. 1995; Weissman et al. 1995; Ueno et al. 2004). Current understanding about GroEL–GroES-mediated protein folding

suggests two mechanisms: the so called *cis* and *trans* mechanisms that differ in the cavity of the GroEL where the substrate and the co-chaperonin bind.

The *cis* mechanism

The majority of the GroEL substrates follow this mechanism. GroES binds to the same side of the GroEL ring as that of the substrate polypeptide, and hence this mechanism is termed as the *cis* mechanism. GroEL binds to the unfolded or kinetically trapped substrate protein intermediates, by virtue of exposed hydrophobic patches. ATP binding to the pseudo-Walker motif at the equatorial domain in the *cis* ring induces a conformational change in the apical domain leading to two consequences: (a) release of the substrate into the hydrophilic cavity and (b) capping of the substrate-bound cavity by GroES upon its interaction with the hydrophobic patches on GroEL, which earlier were bound by the substrate (Fig. 3). The substrate protein is thus sequestered from the crowded cellular milieu and allowed to fold independently in the hydrophilic cavity (Weissman et al. 1995; Mayhew et al. 1996). The protein stays in the cavity till ATP is hydrolyzed to ADP, which usually takes about 10–15 s. Consequently, binding of ATP to the *trans* ring induces a further conformational change which facilitates the release of GroES and, subsequently, the substrate protein. The substrate protein is released either in a completely folded form or in a kinetically trapped form, which can further enter another cycle of binding and release (Rye et al. 1999).

The *trans* mechanism

The usual size limit for the substrate proteins, as shown by both in vitro and in vivo studies, is around 57 kDa although the *cis* cavity is reported to theoretically accommodate larger proteins of the order of 104 kDa (Houry et al. 1999; Sakikawa et al. 1999; Lin and Rye 2004). Productive in vivo folding of the proteins larger than the usual size limit has also been reported for the 86-kDa maltose-binding protein fusion (Huang and Chuang 1999) and 82-kDa mitochondrial aconitase (Chaudhuri et al. 2001). Since such large substrates are difficult to accommodate in the central cavity, it has been suggested that their productive folding might occur outside the *cis* cavity. These studies therefore indicate that the substrate recognition patterns of GroEL may be more diverse than initially thought. Binding a large substrate protein by GroEL would prevent GroES binding in the *cis* cavity. Therefore, a reaction cycle with GroES binding to the *trans* cavity for folding large protein substrates has been demonstrated by Horwich and colleagues for an 82-kDa iron–sulphur protein, yeast mitochondrial aconitase (Chaudhuri et al. 2001). Unlike the *cis* mechanism, where the entire protein is allowed to fold, only a part of the substrate protein is allowed to fold here. The iron–sulphur center of the aconitase was shown to be folded

Fig. 3 Mechanism of chaperonin-mediated protein folding: naïve unfolded proteins (*U*) emerging from the ribosomes or extended non-native protein bound by other chaperones like DnaK and released as kinetically trapped intermediates (*I_{kt}*) are channeled to GroEL. The proteins bind the asymmetric GroEL by hydrophobic patches (red). ATP binding to the *cis* cavity induces conformational changes, which switches the GroEL–substrate complex into either *cis* or *trans* folding pathway depending on the size and structure of the substrate polypeptide. GroEL molecules bound to smaller substrates that can be encapsulated in the cavity assume the *cis* pathway while those binding to large substrate proteins that protrude outside the cavity and thus prevent GroES binding enter the *trans* pathway. ATP-induced conformational changes release the substrate into the cavity and facilitate GroES binding (blue). Substrate protein gets folded with the virtue of hydrophilic cavity (green) in a span

of about 15 s when the ATP is hydrolyzed to ADP. Binding of another set of ATP molecules to the *trans* cavity releases the substrate in either native (*N*) or folding-committed intermediate (*I_{fc}*) form, which can enter another cycle of GroEL-mediated folding till it reaches native stage. The *trans* pathway operates on proteins which require only a portion of the protein to be folded. Unfolded aconitase was proposed to bind the open cavity of the GroEL/GroES/ADP complex. The portion of the polypeptide in a hydrophilic environment is allowed to fold forming a folding nucleus, which further facilitates the folding of the rest of the protein, followed by ATP binding in the polypeptide-bound ring (*trans*) and consequent release of GroES from the *cis* ring. Aconitase is released during the formation of iron–sulphur cluster to produce a holo-enzyme, and binding of another set of ATP and GroES at the *cis* ring releases the mature holo-enzyme

by the GroEL/GroES machinery, suggesting a nucleation step that triggers the folding of the rest of the protein outside the chaperonin (Fig. 3).

On the contrary, electron microscopic studies on a single-ring version of GroEL, SR1, demonstrated housing of an 86-kDa mitochondrial branched-chain α -ketoacid dehydrogenase by expanding the cavity by 80 % more than that shown in crystal structure (Chen et al. 2006). Notably, productive release of the large encapsulated proteins was not demonstrated in the *cis* cavity. These two mechanisms of action of GroEL, therefore, emphasize the requirement of the tetradecameric ring for the functioning of GroEL as a chaperonin.

Although studies on structure and mechanism present a detailed understanding on how GroEL/S system functions as a molecular chaperonin, the conundrum on how chaperonin system promotes folding of the client proteins has been difficult to resolve. Based on several biochemical and biophysical studies on various model substrate proteins, three models have been proposed to explain how GroEL promotes folding of the substrate proteins. The first model known as the Anfinsen cage or passive caging model popularized by Arthur Horwich

and colleagues advocates that GroEL merely provides an environment that prevents aggregation of the substrate proteins and the substrate proteins are allowed to fold unconstrained in such an environment (Park et al. 2005; Apetri and Horwich 2008; Horwich et al. 2009). This model also implies that the substrate protein is capable of reaching native state in a biologically relevant timescale provided that the aggregation is prevented and that the folding energy landscape of the substrate protein remains unaltered in the presence of GroEL. However, the second model, known as active caging model proposed by Ulrich Hartl and colleagues, contradicts passive caging model by proposing that encapsulation by chaperonin accelerates folding of the substrate proteins in addition to preventing aggregation (Brinker et al. 2001; Tang et al. 2006; Chakraborty et al. 2010; Gupta et al. 2014). This model suggests that by accelerating folding of certain substrate proteins, GroEL can modulate their folding energy landscape. Active caging model, therefore, particularly advocates the folding of certain proteins whose folding pathways are kinetically frustrated and therefore require GroEL to fold at a biologically relevant speed. The third model, however, considers

encapsulation as a dispensable step in chaperonin action (Shtilerman et al. 1999). This model, known as iterative annealing or forced unfolding model proposed by George Lorimer and colleagues, states that GroEL/S system exerts catalyzed unfolding action on kinetically trapped folding intermediates through iterative binding and release cycles, with the subsequent folding occurring either inside (Yang et al. 2013) or outside the cage (Thirumalai and Lorimer 2001). Since the three models proposed have been supported by substantial experimental evidence (Ellis and Hartl 1999), we believe that GroEL might adopt all the models depending either on the substrate protein or on its folded state. Moreover, having the two mechanisms and three ways of action established in their place hints at a diversification in chaperonins, which might extend from distribution of substrates to a diversification of chaperonins that might take up different mechanisms. Additionally, variations in the levels of these chaperonins hint at the divergence in the ways of regulation of these chaperonins.

Multiple ways of regulation of heat-shock response

Response to heat or other stresses comprises a transient but enhanced expression of a distinct set of about twenty heat-shock genes which encode the heat-shock proteins (Narberhaus et al. 2006). This evolutionarily conserved cellular protection mechanism is primarily regulated at the level of transcription. Several mechanisms have been described in bacteria that regulate the heat-shock response including the activation of σ^{32} factor in Gram-negative bacteria and repression of the *groE* operon by HrcA in Gram-positive bacteria. Although several repressors act in controlling the expression of different heat-shock genes, we limit our discussion to the regulation of *groE* operon.

Regulation of heat-shock response in Gram-negative bacteria

Heat-shock response in Gram-negative bacteria is largely mediated by the group II alternative sigma factor, RpoH (σ^{32}), which induces the expression of the heat-shock genes (Grossman et al. 1984, 1987; Arsene et al. 2000). During normal growth conditions, the levels of σ^{32} are controlled by two mechanisms: first, *rpoH* mRNA assumes a complex secondary structure preventing its own translation, and second, the cellular chaperones sequester σ^{32} and possibly deliver it to the heat-induced metalloprotease FtsH for degradation (Tilly et al. 1983; Straus et al. 1990; Tomoyasu et al. 1998; Tatsuta et al. 1998; Morita et al. 1999). However, under heat-shock conditions, the envelope stress response sigma factor, σ^E , enhances the expression of σ^{32} and the secondary structure in *rpoH* mRNA is released, thereby increasing its expression.

Concomitantly, the chaperones, which are now sequestered by other cellular unfolded proteins, contribute to the increase in availability of σ^{32} by releasing it. The increased levels of σ^{32} thereby induce the expression of chaperone genes (Straus et al. 1987; Yura and Nakahigashi 1999; Arsene et al. 2000). After the normal growth conditions are restored, the amount of active σ^{32} is rapidly brought down via the negative feedback mechanism (Fig. 4). In addition, SRP-mediated targeting of σ^{32} to the inner membrane established a novel mode of regulation of the heat-shock response (Lim et al. 2013). This elegant study implicates the possibilities of novel regulation mechanisms at different stages of the heat-shock response and several novel interactions among the players (Lim et al. 2013). Therefore, in the light of compartmentalization of bacterial cell (Rudner and Losick 2010; Amster-Choder 2011), sub-cellular localization patterns of mRNA (Nevo-Dinur et al. 2011), and the resultant proteins including the Hsps (Shapiro et al. 2009), mapping the intracellular interactions of different players of heat-shock response by employing the modern imaging techniques will aid in understanding the complexity of the heat-shock response.

Regulation of heat-shock response in Gram-positive bacteria

Distinct regulatory mechanisms that direct the expression of specific heat-shock genes have been characterized in other bacteria. For example, regulation of *groE* operon in *Bacillus subtilis* was shown to be regulated by the HrcA repressor and the *dnaK* operon in *Streptomyces coelicolor* by HspR (Schumann 2003). HrcA repressor binds to conserved Controlling Inverted Repeat of Chaperone Expression (CIRCE) operator elements that are located upstream of *groE* operon (Baird et al. 1989; Zuber and Schumann 1994; Yura and Nakahigashi 1999). The CIRCE/HrcA system is one of the best-characterized operator–repressor pairs in heat-shock response and has a widespread occurrence in the bacterial kingdom with its presence in more than 40 different species (Hecker et al. 1996; Narberhaus et al. 1992; Narberhaus and Bahl 1992; Wetzstein et al. 1992). Activity of HrcA is modulated by the GroES/L system, probably by facilitating its folding and assembly (Fig. 4). In response to increased protein damage upon heat shock, the GroES/L folding machinery is occupied by the unfolded proteins and folding of the HrcA repressor is stalled, thereby releasing the repression, providing a direct sensing mechanism for protein misfolding (Mogk et al. 1997).

Multiple GroELs in bacteria

Recent genome annotation studies on various bacteria have revealed that a few bacterial genomes possess multiple copies

Fig. 4 Regulation of chaperonin genes in Gram-negative and Gram-positive bacteria: stress responses in Gram-negative bacteria begin with the enhancement in the level of envelope stress response sigma factor σ^E , which in turn enhances the level of σ^{32} mRNA. Heat-shock response activates the otherwise inactive RpoH mRNA by denaturing it and facilitating its translation to produce σ^{32} , which is further stabilized by the cellular chaperones. Stable σ^{32} in turn induces the expression of heat-shock genes leading to the production of chaperones and proteases that either prevent cellular proteins from stress-induced unfolding or cleave the unfolded proteins and prevent accumulation of toxic aggregates. Either subsequent buildup of the chaperones or recommencement of normalcy brings about a negative feedback control, wherein the chaperones and the heat-induced proteolytic enzymes that are free from substrate proteins bind and either sequester or degrade σ^{32} , thereby limiting its

activity. Chaperone-bound σ^{32} is released upon heat shock while the chaperones attend to the unfolded proteins. Stress response in Gram-positive bacteria is complicated with additional repressors. Expression of GroES/L operon is regulated by a repressor known as HrcA. Under normal conditions, GroEL stabilizes HrcA by facilitating its assembly into an active dimer, which in turn binds the upstream CIRCE elements and represses the expression of its own and *groE* genes, thereby preventing the unwanted buildup of chaperones. Upon heat shock, HrcA is destabilized, released from the CIRCE element, allowing the production of heat-shock proteins, which in turn attend to the cellular stressed proteins till normalcy is resumed. Upon recommencement of normalcy, GroEL, free from cellular stressed proteins, facilitates the folding and assembly of HrcA and the repression of the downstream genes

of *groEL* genes (Kong et al. 1993; Fischer et al. 1993; Karunakaran et al. 2003; Barreiro et al. 2005). The existence of multiple *groEL* genes opens up several hypotheses on their function and evolution and on the distribution of the substrates. The organism expressing multiple GroELs might benefit either from the dosage effect of multiple chaperonins (Kondrashov and Kondrashov 2006) or from the functional divergence of different chaperonins (Goyal et al. 2006). The former appears unlikely since the levels of chaperonins are always high in the cell. Moreover, multiple GroELs have been largely observed in organisms with complex lifestyle, suggesting plausibility of the latter scenario although evidence for the former is beginning to appear. We present the current understanding on multiple chaperonins, reviewing the fascinating examples of bacteria with multiple chaperonin genes. We begin the review with the case of myxobacterial GroELs, wherein two dispensable GroELs are encoded that distribute the substrates in a lifestyle-specific manner. We then move to the GroELs from the pathogenic genus *Mycobacteria*, where one GroEL is indispensable and believed to act as a generalist chaperonin, while the others are supposed to have diversified in sequence, thereby function, and thus are responsible for several stages in pathogenesis. We then review the most diverse situation of chaperonins in the root-nodulating rhizobia that harbor the highest number of GroELs, wherein at least one of the GroELs is dedicated to fold proteins involved in nitrogen fixation. Consistently, such GroEL is located in close proximity to the genome and regulated similar to the *niF* genes. Finally, we review the fascinating and unique co-existence of group I and group II chaperonins in the mesophilic methanogens, methanosarcina, wherein the two classes of chaperonins have been demonstrated to share the substrates and have evolved to suit the co-existence and shared substrate spectrum. The current understanding reveals a species-dependent requirement of multiple GroELs and leaves several paths to contemplate in gaining comprehension.

Multiple GroELs in myxobacteria—living in relation

Myxobacteria are δ -proteobacteria characterized by a unique and complex multicellular social behavior (Rosenberg et al. 1973; Dworkin and Kaiser 1985; Kaiser 1998; Whitworth and Cock 2008) that includes multiple life stages with swarming on solid surfaces (Kaiser et al. 1979), predating on microbes (Varon et al. 1984; Berleman and Kirby 2009; Wenzel and Muller 2009; Weissman and Muller 2009; 2010) and forming multicellular fruiting body structures for containing myxospores during starvation (Shimkets 1990). Understandably, myxobacteria contain the largest genome size (9.14 Mb) among bacteria (Chen et al. 1990), encompassing several duplicated genes that are supposed to support the multifaceted lifestyle (Goldman et al. 2006; Schneiker et al. 2007). Interestingly, major duplication occurs with the genes encoding

molecular chaperones, although some of the duplicated paralogs are not heat induced (Otani et al. 2001). As observed in the pulse-chase experiments with ^{35}S -labeled cells during vegetative growth and spore formation stages, heat-shock genes follow social phase-specific expression patterns (Nelson and Killeen 1986; Killeen and Nelson 1988) and probably support the sociality of these organisms (Weimer et al. 1998; Yang et al. 1998).

Myxobacteria encode two *groEL* paralogs: *groEL1* and *groEL2*, which are 83 and 79 % identical at the gene and protein levels, respectively (Jiang et al. 2008). While *groEL1* exists in the conventional bicistronic operon with an upstream *groES* gene, *groEL2* exists separately (Li et al. 2010). Post heat-shock proteomic analysis of *Myxococcus xanthus* at different time intervals identified about 30 proteins as heat-shock proteins. Moreover, the two *groEL* paralogs are induced at different time points of heat shock, with *groEL1* inducing early, suggesting that the two paralogs might have different roles in adaptation to heat shock (Otani et al. 2001). Therefore, the quest to understand differences in functional roles of the *groEL* paralogs was followed with deletion studies (Li et al. 2010). Although both the *groEL* copies are independently dispensable for general survival of these organisms, depletion of both the copies is lethal (Li et al. 2010). Moreover, cell-viability studies following heat treatment of the individual mutant strains demonstrated that while GroEL1 is vital for cell viability, GroEL2 depletion has a minor effect. In addition, expression studies showed that while expression of *groEL1* is at high levels and it fluctuates with growth phase, *groEL2* expresses stably but at low levels (Wang et al. 2013). However, *groEL1* depletion entails higher expression of *groEL2*, but not vice versa for *groEL2* depletion, suggesting that the intrinsic high levels of *groEL1* are sufficient to compensate *groEL2* loss (Li et al. 2010; Wang et al. 2013). Furthermore, GroEL1 has been involved in developmental processes and sporulation, while GroEL2 is involved in the bactericidal predation (Li et al. 2010). Compared to the wild type, *M. xanthus groEL2* mutant strain exhibited four times lower predation ability on live *E. coli* lawn (Li et al. 2010). Consistently, GroEL2 has been shown to be required for the biosynthesis of myxovirescin, an essential secondary metabolite for bactericidal predation, and insertion of C-terminal equatorial domain of GroEL2 can make GroEL1 predation competent (Wang et al. 2013). In order to understand the fundamental reason for these distinct physiological roles, the substrates interacting with the two GroELs were mapped using proteomic studies on the GroEL mutant strains (Wang et al. 2013). In this study, GroEL1 and GroEL2 bind 151 and 144 substrates, respectively, with 68 substrates that interact with both GroELs, which leaves 83 and 46 substrates that are specific to GroEL1 and GroEL2, respectively. Interestingly, the shared 68 substrates have been associated with fundamental cellular metabolism, while GroEL1- or GroEL2-specific substrates

have been associated with the stages in the social behavior, constituting swarming, predation, sporulation, and fruiting body formation. These are affected by either of the *groEL* depletions, i.e., development/sporulation and predation-related proteins bind specifically with GroEL1 and GroEL2, respectively. Notably, the average size of the substrates specific to GroEL1 has been similar to *E. coli* GroEL, while that specific to GroEL2 has been larger, suggesting a different mechanism of action between the two GroELs (Wang et al. 2013).

Taken together, genetic and proteomic studies have elegantly demonstrated the distinct physiological roles for the mycobacterial GroELs and attributed these differences to the interacting substrate proteins (Otani et al. 2001; Li et al. 2010; Wang et al. 2013). Furthermore, distinctions in the substrate spectrum are attributed to the differences in the primary sequences of these GroELs (Wang et al. 2013). Since the primary function of GroEL is to recognize, encapsulate, and assist folding of the substrate proteins, the differences in the substrate spectrum between the two GroELs could occur at any of the said stages. Therefore, to understand the basis for the differences in substrate recognition and folding, and thereby differences in the physiological roles of these chaperonins, comprehensive biochemical and structural studies are required.

Multiple GroELs in mycobacteria

Mycobacteria include several causative agents of tubercular diseases, such as *Mycobacterium tuberculosis* (Mtb), *Mycobacterium leprae*, *Mycobacterium bovis*, and *Mycobacterium avium*. Occurrence of multiple GroELs had been first identified in Mtb (Kong et al. 1993). Although several members of this genus encode multiple copies of chaperone genes, owing to the scope of this review, we limit the discussion to Mtb *groEL* genes. Since several heat-shock proteins of Mtb were identified as antigens (Young et al. 1987; Shinnick et al. 1988; 1989), initial studies were targeted to understand the heat-shock response of Mtb at temperatures ranging from 37 to 48 °C for varying lengths of time (Patel et al. 1991; Young and Garbe 1991). These studies have identified two major regulons for heat-shock genes, the HrcA and HspR regulons, which have been demonstrated to control the expression of *groE* and *dnaKJE* operons, respectively (Stewart et al. 2002). Mtb encodes two copies of *groEL* genes, *groEL1* (*cpn60.1*) and *groEL2* (*cpn60.2*), and one *groES* (*cpn10*) gene, all regulated by HrcA (Rao and Lund 2010). As per the convention, *groEL* gene that is in operonic arrangement with *groES* has been termed *groEL1*, while that that exists separately on the genome has been termed *groEL2* (Kong et al. 1993; Cole et al. 1998). Despite high sequence similarity to other chaperonins, functional studies on GroEL2 initially identified it as the immunodominant antigen in the mycobacterial culture filtrates, leading to the search for its

immunological roles (Lamb et al. 1989; Cehovin et al. 2010; Henderson et al. 2010, 2013). Therefore, studies that followed focused on understanding the immunological tasks of the Mtb GroELs, which eventually demonstrated that all the chaperonins, GroEL1 (Lewthwaite et al. 2001), GroEL2 (Hickey et al. 2010), and GroES (Sonnenberg and Belisle 1997), are secreted, with GroEL1 being the more immunopotent (Lewthwaite et al. 2001). Immunological function is driven by a polypeptide segment in the equatorial domain of GroEL1 (Hu et al. 2013).

Around the same time, structural and biochemical studies were initiated on these chaperonins. Analogous to the immunological studies, structural studies began with determination of GroEL2 structures (Qamra and Mande 2004; Shahar et al. 2011) while structural information on GroEL1 is limited to its apical domain (Sielaß et al. 2011). Interestingly, biochemical studies on the recombinant mycobacterial GroELs observed significant deviations in chaperonin characteristics, with the foremost being their inability to form canonical large double toroidal structures (Qamra et al. 2004; Qamra and Mande 2004). Both the chaperonins rather existed as lower oligomers (dimers) irrespective of the presence of cofactors such as the ATP or the cognate GroES. Interestingly, the co-chaperonin GroES readily assembles as a heptamer (Taneja and Mande 2001; Taneja and Mande 2002). Consequent to their hampered oligomeric nature, mycobacterial GroELs failed as chaperones: displayed weak ATPase activities and failed in either refolding or preventing aggregation of the denatured polypeptides. Furthermore, evolutionary studies on Mtb *groEL* sequences have suggested rapid evolution of the *groEL1* gene (Goyal et al. 2006) and that the difference in the rates of evolution between GroEL1 and GroEL2 has been proposed to be due to differential interaction of these chaperonins with the host immune system (Hughes 1993). Although these studies have principally employed biochemical tools on purified proteins and convincingly demonstrated the inability of Mtb GroELs to act as chaperones, further genetic studies on these chaperonin genes have provided evidence towards the hypothesis that Mtb GroELs are inactive as chaperonins (Kumar et al. 2009; Henderson et al. 2010; Kumar and Mande 2011). Phenotypic investigations on the Mtb GroELs in different *E. coli groEL* mutant strains established that Mtb GroELs are ineffective in complementing *E. coli* GroEL (Kumar et al. 2009). Furthermore, directed evolution studies employing gene shuffling and domain swapping to derive functional GroELs from Mtb GroELs established that oligomerization is the prelude to the formation of an active GroEL chaperonin, and therefore, impaired oligomerization of Mtb GroELs results in their inactivity. Furthermore, attempts to understand the cause of the inactivity led to the discovery that Mtb GroEL1 follows facilitated oligomerization to the tetradecameric state mediated by a phosphorylation switch, suggesting that determinants of oligomerization

for Mtb GroEL1 are distinct from their *E. coli* counterpart (Kumar et al. 2009). Such regulated oligomerization of GroEL has been postulated to help nutrient-deprived and slow-growing Mtb to cope up with GroEL that, if perpetually active, would consume the ATP pools (Kumar and Mande 2011; Haslbeck et al. 2005). Moreover, the observation that GroEL1 knockout strains failed to form biofilms (Ojha et al. 2005) and granulomas (Hu et al. 2008) suggested GroEL1's involvement in disease establishment or progression, probably via folding proteins that are responsible for pathogenesis. Based on structural (Sielaff et al. 2010) and functional (Sielaff et al. 2011) studies, GroEL1 has been proposed to possess the canonical chaperonin activity. Further, promoter probe studies on heat-shock promoters in mycobacteria have shown that the promoter regulating expression of the bicistronic *groES/L1* operon is stronger than that of *groEL2* and is stress inducible (Aravindhan et al. 2009). In addition, expression of the essential chaperonin, *groEL2*, has been shown to be repressed by the nitric oxide-responsive transcription factor, WhiB, by occluding *groEL2* promoter to the activating CRP-family transcription factor Cmr (Stapleton et al. 2012). These studies therefore indicate that although both the chaperonins are inducible, the mechanisms of regulation are different, suggesting that the two chaperonins might be playing roles that differ either temporally in the life of the pathogen or by location.

While GroEL1 has been functionally characterized in these studies, the ability of GroEL2 in complementing *E. coli* GroEL was studied independently by two groups, which resulted in an understanding that GroEL2 can functionally replace *E. coli* GroEL in vivo and in vitro only upon abundance (Hu et al. 2008; Kumar et al. 2009; Fan et al. 2012). Moreover, GroEL2 has been shown to localize at the cell wall (Hickey et al. 2009) as a multimer and upon cleavage by a serine protease, Hip1, separates into the secretion-competent monomeric form. This GroEL2–Hip1 interaction and further cleavage have been demonstrated to pacify macrophage response (Naffin-Olivos et al. 2014). The unusual nature of mycobacterial GroELs has triggered curiosity to identify any moonlighting function (Mande et al. 2013) to these chaperones and led to the discovery that dimeric GroEL1 interacts with DNA in a sequence-independent manner (Basu et al. 2009).

Although the said studies underlined biochemical features of these chaperone molecules, several questions on their in vivo roles are still unsolved. The precise distribution of the substrate pools shared between the two GroELs is yet to be established. Marked difference between the two GroELs is apparently the repeat sequence at the C-terminus, which, in *E. coli* GroEL, has been demonstrated to enhance substrate folding (Fan et al. 2012). While GroEL1 has a His-rich sequence, GroEL2 has a Gly–Met-rich sequence at the C-terminus, homologous to the *E. coli* counterpart. Thus, probably, GroEL2 is essential and GroEL1 is dispensable (Stewart et al. 2002; Colaco and MacDougall 2013). Taken together,

GroEL2, being the essential chaperonin and bearing the C-terminus homologous to *E. coli* GroEL, might be functioning as a general chaperonin for mycobacterium, while GroEL1, being a dispensable chaperonin but involved in critical steps in disease progression such as granuloma and biofilm formation, appears to be involved in folding special class of proteins such as those involved in pathogenicity and perhaps metal-binding proteins, owing to its His-rich C-terminus, via *trans* mechanism (Chaudhuri et al. 2001).

Multiple chaperonins in *Rhizobium*—the division of labor situation

The genus *Rhizobium* belongs to the phylum proteobacteria and constitutes bacteria with distinct biological pathways of symbiosis with the plant partners and nitrogen fixation. These bacteria offer an interesting picture for multiple chaperonin genes (Rusanganwa and Gupta 1993; Wallington and Lund 1994), boasting the highest number of chaperonin genes among bacteria. *Bradyrhizobium japonicum* hosts seven *groEL* genes with five of them in operonic arrangement with *groES* homologues (Fischer et al. 1993; Kaneko et al. 2000, 2002), and at least one of the multiple GroELs has been dedicated for folding proteins involved in nitrogen fixation. Interestingly, several non-rhizobial nitrogen-fixing bacteria bear three to four copies of chaperonin genes (Moulin et al. 2001; Normand et al. 2007), while non-nitrogen-fixing rhizobia have only one copy (Wood et al. 2001), suggesting a correlation between the presence of multiple chaperonins and nitrogen fixation pathway. The quest to understand the biology of these multiple GroELs has led to understanding the biology and regulation of nitrogen fixation (Fisher et al. 1988; Ogawa and Long 1995; Ivic et al. 1997; Gould et al. 2007a, b). The well-studied *Rhizobium leguminosarum* have three copies of GroELs. Unlike the actinobacteria, all the GroEL genes in *R. leguminosarum* are in operonic arrangement with the GroES genes. Two groups pioneered the studies on rhizobial GroELs: Sharon Long's group at Stanford University, USA, and Peter Lund's group at University of Birmingham, UK. While Long's group largely focused on understanding the relation of GroEL with the nitrogen fixation genes *nif*s and *nods* (Fisher et al. 1988; Ogawa and Long 1995), Lund's group was involved in understanding the genetic and biochemical features of all the three chaperonins (Ivic et al. 1997; Gould et al. 2007a, b).

Attempts to purify the transcriptional regulator for nitrogen fixation genes, NodD, have identified co-purifying chaperonin GroEL3 (Fisher et al. 1988; Ogawa and Long 1995). Further, purified GroEL3 has been demonstrated to assist assembly of the NodD complex from its constituents Nod1, 2, and 3 (Ogawa and Long 1995), and co-expression of GroEL3 in *E. coli* improved folding and assembly of the NodD (Yeh et al. 2002), analogous to the GroEL-dependent

functional regulation of LuxR of *Vibrio fischeri* (Dolan and Greenberg 1992). Moreover, expression of *nod* genes and *groEL3* has been shown to be co-regulated with HrcA-like mechanism (Gould et al. 2007a, b). In addition, extensive genetic studies revealed that *groEL3* is regulated by σ^{54} -based *niF* regulon in addition to moderate repression by HrcA (Gould et al. 2007a, b). Also, search for mutations defective in Nod gene expression has mapped to the GroEL3 locus, suggesting a direct relation between GroEL3 and nitrogen fixation (Ogawa and Long 1995). These results therefore identified GroEL3 as the dedicated chaperone responsible for the folding and assembly of the proteins involved in nitrogen fixation.

Although GroEL3 has been essentially involved in nitrogen fixation, the presence of two more chaperone copies is intriguing. Since each GroEL gene is associated with a GroES gene, it will be interesting to identify which of the two GroELs acts as a generalist chaperonin and what is the function for other non-generalist chaperonins. Attempts to understand the biological functions of GroEL1 and GroEL2 continued with genetic and biochemical tools. Among the three GroELs, GroEL1 is essential while GroEL2 and GroEL3 are dispensable individually and in combination (Rodriguez-Quinones et al. 2005) although double mutants in other rhizobia are lethal as observed in the case of *B. japonicum* (Fischer et al. 1999). The presence of multiple copies of chaperonins immediately suggests differences in the regulation of expression or differences in function. The situation with *R. leguminosarum* apparently exhibits both the possibilities; the three chaperonin genes are regulated in a complex manner (George et al. 2004; Gould et al. 2007b), and the gene products exhibit divergent behavior in vivo (Ogawa and Long 1995; Ivic et al. 1997; Gould et al. 2007b) and in vitro (George et al. 2004; Yeh et al. 2002) as reviewed below. Expression studies on these chaperonin operons, however, revealed a complex picture. Although all the three operons are heat inducible and mediated through the CIRCE elements, differential levels of expression have been demonstrated; the essential chaperonin *groEL1* shows the highest induction followed by *groEL2* (Rodriguez-Quinones et al. 2005). On the other hand, induction of *groEL3* is very low following heat shock, but higher levels are observed under anaerobic conditions and in the presence of NifA (Rodriguez-Quinones et al. 2005; Yeh et al. 2002). Sequence analysis followed by detailed promoter probe assays revealed that the *groEL1* operon is regulated by RpoH- and HrcA-dependent mechanisms while *groEL2* appears to be solely regulated by RpoH. Expectedly, *groEL3* is induced by NifA and moderately repressed by HrcA (Rodriguez-Quinones et al. 2005; Gould et al. 2007b). These results therefore indicate that the essential chaperonin is expressed at the highest levels and the expression is tightly controlled by RpoH and HrcA. The dispensable chaperonins, though, are expressed at

low levels, thus contributing less to the total GroEL pool in the cell.

Complementation studies with *R. leguminosarum* GroELs showed that GroEL1 and GroEL3 but not GroEL2 could rescue an *E. coli groELts* mutant at 43 °C, when co-expressed with cognate *groES* (Gould et al. 2007a, b). Interestingly, when *groEL1* was co-expressed with *E. coli groES*, it could support growth only up to 37 °C, but not at 43 °C, suggesting the differences in the co-chaperonin compatibility (Ivic et al. 1997). Interestingly, GroEL3 has been demonstrated to perform additional moonlighting function by inducing CD14-mediated cytokine production in human monocytes, possibly by an epitope located in its C-terminal hapten (Lewthwaite et al. 2002), analogous to the reported epitope in Mtb GroEL1 (Hu et al. 2013). Moonlighting is defined as the ability of a protein to perform multiple unrelated functions (Kumar and Mande 2011). In addition, the GroEL homologues have revealed distinct biochemical properties such as thermal stability, ATP hydrolysability, substrate interaction, and ATP-dependent substrate folding (George et al. 2004). These chaperonin homologues were less robust biochemically when compared with their *E. coli* counterpart, GroEL (George et al. 2004). Studies on the structural stability of the rhizobial chaperonins to temperature and denaturant, guanidinium hydrochloride, demonstrated that although all the homologues are unstable when compared to their *E. coli* counterpart, among rhizobial chaperonins, GroEL2 is the most labile while GroEL3 is the most stable chaperonin (George et al. 2004). In addition, unlike the Mtb GroELs (Qamra et al. 2004), all the rhizobial chaperonins existed as canonical tetradecamers. Although all the three homologues efficiently bind the substrate, the essential chaperonin, GroEL1, has been the most and GroEL3 the least effective in refolding LDH, suggesting that GroEL1 might be the generalist chaperonin in the cell. Interestingly, GroEL2 showed higher ATPase activity. In addition, co-expression followed by interaction studies showed that although individual chaperonins tend to form homo-oligomers, a small amount of the hetero-chaperone is detected, suggesting chaperone inter-dependence on folding each other (Gould et al. 2007a, b). These studies therefore established that the essential chaperonin, GroEL1, is the principal chaperonin of the cell while GroEL2 and GroEL3 have diversified on their regulation and function to aid the complex cellular processes in the root nodulating rhizobia.

Although the function of the three GroELs in *R. leguminosarum* is understood, the roles of multiple copies of *groEL* in the organism having higher number of *groEL* copies, such as seven copies in *B. japonicum*, are still unknown. Thus, comprehensive biochemical and genetic studies to correlate the function of each chaperonin copy with the cellular metabolism or growth stage are required. In addition, functional studies aimed at studying complementarity of individual GroEL and GroES are essential to understand the need

for multiple GroES copies and the extent of inter-dependencies between the chaperonin and co-chaperonin. Moreover, studies aimed at substrate selection among the chaperonins and those aimed at identifying the significance and structural aspects of the possible GroEL hetero-oligomer will enhance the understanding of the biology of multiple chaperones.

Co-existence of group I and group II chaperonins in mesophilic methanogen—life of Pi

Methanosarcina constitutes a genus of mesophilic prokaryotes that are metabolically diverse methanogens that utilize acetate, methylamines, and methanol to produce the greenhouse gases methane and carbon dioxide (Jager et al. 2009; Williams et al. 2009a, b). The methanogenic pathways, therefore, necessitated these organisms to avoid inimical oxygen and consequently dwell in diverse anoxygenic environments during different life phases (Macario and Conway De Macario 2001; Klunker et al. 2003) and exist in unique morphologic forms involving unicellular and multicellular packets or laminal forms (Yao et al. 1992). Genome sequencing projects on different methanosarcina, *Methanosarcina mazei* (Deppenmeier et al. 2002), *Methanosarcina acetivorans* (Galagan et al. 2002), and *Methanosarcina barkeri* (Maeder et al. 2006), revealed that about 20–33 % of methanosarcina genes (~1000 genes) have been acquired horizontally, including the genes encoding group I chaperonins from bacteria. These studies therefore presented the first evidence of the co-existence of group I (GroES/L) and group II (thermosome/prefoldin) chaperonin genes (Macario et al. 1999, 2004) that are expressed concurrently (Klunker et al. 2003), and distinct substrate pools (Hirtreiter et al. 2009), thereby offering a fantastic model to study the evolution of chaperonin dependent protein folding. While the group II chaperonin genes that are exclusive to archaea and eukaryotes are understood to be inherited, group I chaperonin genes that are exclusive of bacterial cytosol and endosymbiotic organelle are believed to be acquired horizontally, indicating probable relationship between archaea and bacteria. In addition, a few members of the clostridia and cyanobacteria have been shown to possess group II-like chaperonins, suggesting a two way transfer between archaea and bacteria (Williams et al. 2009a, b).

Methanosarcina genus is characterized by having the highest number of chaperonin genes among archaea (Macario et al. 2004). The members of this genus harbor single *groES* and single *groEL* genes arranged as operon, representing the group I chaperonin and co-chaperonins, and three genes representing α , β , and γ subunits of the group II chaperonin thermosome, which principally assumes $\alpha\alpha\beta\gamma\alpha\alpha\beta\gamma$ architecture in an eight-membered thermosome ring (Klunker et al. 2003). Surprisingly, the thermophilic methanogens, methanococci, are characterized by a single group II gene

(Furutani et al. 1998). The group I chaperonin and co-chaperonins are about 47 and 35 % identical to their bacterial counterparts while group II chaperonins share 50–80 % identity with their archaeal and eukaryotic counterparts. Striking differences have been observed in the GroEL-interacting mobile loop of methanosarcina GroES, which might reduce flexibility of the mobile loop and thereby might possibly increase affinity to and/or retention time on the cognate chaperonin (Klunker et al. 2003). Expression studies revealed that both the chaperonins exist at equimolar ratio in the cell but are moderately induced by heat stress. Co-existence of both the chaperonin groups poses interesting questions on their function and the substrate distribution.

Studies to understand the features of the co-existing chaperonins revealed interesting details. Although both the chaperonins are capable of binding model substrates, such as rhodanese (Klunker et al. 2003) and MBP (Figueiredo et al. 2004), GroES/L is capable of refolding the substrates albeit at a slower pace. Moreover, methanosarcina GroEL exhibits slower ATPase activity and cycles of substrate binding and release (Figueiredo et al. 2004), suggesting a significant divergence from the well-studied *E. coli* GroEL (Klunker et al. 2003; Williams et al. 2009a, b). Consistent with the divergence between *E. coli* and methanosarcina GroES/L system, the latter could not functionally replace the former chaperonin (Figueiredo et al. 2004). These observations therefore led to an understanding that the co-existing group I chaperonins have significantly diverged from their bacterial counterparts and that the divergence might have assisted them to adapt to the presence of group II chaperonin in sharing the substrate pool. Since about 20 % of methanosarcina proteins have bacterial origin, whether the two chaperonin systems fold the origin-specific proteins or they exhibit overlapping substrate distribution is essential to investigate in order to understand the evolution of chaperone-dependent protein folding in the cell.

Proteomic studies on the isolated chaperonin–substrate complexes have identified 333 chaperone interactors among about 2500 soluble proteins (Hirtreiter et al. 2009). Among these 333 chaperone-bound substrates, group I and II chaperonins are bound by 183 and 252 substrates, respectively, with an overlap of about 100 proteins that are bound by both the chaperonin systems. Interestingly, about 75 proteins of archaeal origin were found to bind to GroES/L, while several metabolic enzymes with bacterial origin were observed to bind to the thermosome, indicating that the chaperonin selectivity is phylogeny independent but dependent on the intrinsic structural features of the individual substrates (Hirtreiter et al. 2009; Williams et al. 2009a, b). These results further indicate that the chaperonin–substrate interactions have diverged to facilitate substrate-specific folding pathways. Although these studies elucidated the functional differences between the co-existing chaperonins, in light of slow-acting group I

chaperonins, comprehensive structural and biochemical studies would highlight the mechanism of different chaperonin systems especially from the slow-growing organisms.

Distribution of multiple GroELs

As discussed previously, about 30 % of the sequenced genomes have been found to possess multiple genes encoding the chaperonins (Lund 2009). In the light of new genomic tools and consequently several new bacterial genomes being available, we wished to understand the consequence of the presence of multiple GroEL genes in the new genomes and update the existing list (Lund 2009). InterPro (<http://www.ebi.ac.uk/interpro/>) and UniProt (<http://www.uniprot.org/>) were mined for GroEL paralogs from completely sequenced 1318 bacterial genomes (NCBI Genome—<http://www.ncbi.nlm.nih.gov/genome/>), and the retrieved entries were pruned to remove repetitions. Distribution of multiple GroELs in different phyla (Fig. 5a) and the frequencies of multiple GroELs per genome (Fig. 5b) are presented. It is observed that the trend of multiple GroEL genes sustained despite several newly sequenced genomes, suggesting that new cases of multiple GroEL genes continued to emerge and surprise with novel capabilities. Furthermore, to understand whether the multiplicity has been a result of horizontal gene transfer or a gradual evolutionary inheritance, the 1775 polypeptide sequences for GroELs represented above were retrieved and evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Following alignment of the polypeptide sequences using MUSCLE (Edgar 2004), a phylogenetic tree was inferred using the neighbor-joining method with 500 rounds of boot strapping, and the branches belonging to individual phyla are colored using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Since the tree depicts divergence of GroEL homologues, branching of GroELs with homologues from phylogenetically related species would suggest that the multiple copies have diverged during an ancestral era by means of gene duplication, and the variations have accumulated over the course of evolution (Mande et al. 2013). On the other hand, if GroEL homologues from phylogenetically distant bacteria branch together, it would suggest that although bacteria are phylogenetically diverged, their chaperonins are similar, thus suggesting a horizontal acquisition. Horizontally acquired genes are sustained if they facilitate the organism in adapting a particular niche or environmental conditions; else, they are turned into pseudogenes and eliminated.

We therefore attempted to identify the source of multiple GroELs in bacteria. Tree representing GroEL divergence revealed a few interesting features on the evolution of GroEL copies (Fig. 5c). The top panel depicts a sub-tree of proteobacteria representing five GroELs from *B. japonicum*

in red. Although this organism hosts seven GroEL copies, two of them branched to the other side of the main tree (Fig. 5c). The five GroEL copies branch with GroELs from other rhizobia, suggesting that these five GroELs might have diverged at a common ancestor and acquired variations gradually as observed in gene duplication events. However, the two copies branch with GroELs from phylogenetically distant species, suggesting horizontal transfer of these two copies. The precise roles of these two copies and the advantage they offer, if any, to the organism need to be comprehended to understand the evolutionary significance of their acquisition. However, GroELs from myxobacteria, wherein both GroELs can compensate for the loss of the other, occupied a very distinct small branch with GroELs from other myxobacteria, suggesting that gene duplication event occurred possibly just at the time of myxobacterial divergence followed by characteristic variations, which might support myxobacterial lifestyle. The third example we probed is of the GroELs belonging to mycobacteria. Mycobacteria pose an interesting distribution of their GroELs. GroEL1 and GroEL2 cluster at two different places on actinobacterial sub-tree, suggesting an ancient gene duplication. However, mycobacteria hosting more than two GroELs, such as *Mycobacterium chubuense* and *Mycobacterium smegmatis*, exhibit a different phenomenon as observed in rhizobial GroELs. The third GroELs in *M. chubuense* and *M. smegmatis* occupy an entirely different place on this actinobacterial sub-tree, suggesting a possible horizontal gene transfer. Considering that *M. chubuense* and *M. smegmatis* are faster-growing mycobacteria and that GroEL overproduction enhances growth rate, we are tempted to speculate that the third GroEL in these organisms might be contributing to their faster growth rate. We therefore are trying to understand the correlation between chaperone expression and growth enhancement. Understandably, co-existing group I and group II chaperones apparently followed horizontal transfer. Taken together, the evolution of multiple GroELs appears case specific: (a) gene duplication as observed with myxobacterial GroELs, with mycobacterial first and second GroELs, and in a few cases of rhizobial GroELs and (b) horizontal transfer as observed with the mycobacterial third GroEL, a few rhizobial GroELs, and the obvious methanosarcinal chaperonins. Interestingly, although GroEL1 and GroEL2 of *M. smegmatis* are homologous to the respective GroELs from other mycobacteria and cluster with them in the tree, GroEL3 occupies a different place on the tree, highlighting the striking difference with a patternless C-terminus (Fig. 5c).

Diversity of C-terminal segment in GroELs

Recent studies on the C-terminus of GroEL have revealed a significant role of the C-terminal segment in the overall function of chaperonin (Tang et al. 2006; 2008; Farr et al. 2007),

Fig. 5 Distribution of multiple GroELs in bacteria: distribution of multiple *groEL* genes among completely sequenced 1318 bacterial genomes are presented (a) along with the phylum-specific frequencies of *cpn60* genes per genome (b). Polypeptide sequences of these GroELs were retrieved from www.uniprot.org and were aligned using MUSCLE.

Evolutionary history was inferred from the aligned sequences using the neighbor-joining method conducted in MEGA6. The un-rooted phylogenetic tree was prepared using FigTree v1.4.2. Major bacterial phyla are color-coded as indicated (c). Indicated sub-trees represent the diversity of multiple chaperonins as described in the text

including interaction with the encapsulated substrate protein (Chen et al. 2013). Moreover, genetic engineering

experiments swapping GroEL tails from organisms growing in diverse temperatures indicated that the C-terminus of

GroEL acts as a thermometer and is characteristic of the host organism (Nakamura et al. 2004). In the case of multiple chaperonins, strikingly, the C-terminus has been diverse and chaperone copy specific. While *E. coli* GroEL bears a 13-residue (GGM)₄M segment, mycobacterial GroELs show a distinct pattern. All the GroEL2 homologues show GGM-like segment, while the GroEL1 and GroEL3 homologues sport histidine-rich and patternless tails, respectively. Interestingly, GroEL2 is indispensable for regular growth, while His-rich-tail-bearing GroEL1 is dispensable for normal growth but required for pathogenesis (Colaco and MacDougall 2013). Considering that GGM tails interact with substrates in the cavity, it is tempting to speculate that the GGM-bearing GroEL2, which is an essential chaperonin, might be acting as a generalist chaperonin in the cell, while GroEL1 is specialized for folding proteins involved in pathogenesis. Additionally, with a His-rich C-terminus, GroEL1 could be involved in folding metalloproteins, possibly by *trans* mechanism. However, mycobacterial chaperonins, GroEL1 and GroEL2, bear C-terminal tails with high and low identity to GGM tail. Unlike the mycobacteria, either of the mycobacterial GroELs functions in the absence of the other, indicating that both the chaperonins interact with all the obligate GroEL substrates in the cell (Wang et al. 2013). Therefore, both the GroELs have evolved with GGM tails (Table 1). However, GroEL1, which is responsible for cellular growth and development and generally highly expressed, bears C-terminus with higher identity with *E. coli* GroEL, while GroEL2, principally involved in predation and expressed less, hosts a tail with lower identity (Wang et al. 2013), indicating a strong correlation between the C-terminal tail and GroEL function. Interestingly, the situation in the case of rhizobial chaperonins is very complex. The essential chaperonin in rhizobia, GroEL1, bears a GGM-like tail while the tail of GroEL2 is pattern-free (Table 1). On the other hand, the chaperonin involved in nitrogen fixation, GroEL3, has a less-similar GGM-like tail, which possibly is the reason for the mixed oligomers of GroEL1 and GroEL3, but not GroEL2, and low thermal stability of GroEL2 (George et al. 2004). Interestingly, expression of GroEL1 and GroEL3, but not GroEL2, is regulated by HrcA with the same proportion of identity to GGM (Table 1), indicating a possible correlation (Gould et al. 2007a, b). Additionally, since alterations in GGM have been demonstrated to perturb GroEL's ATPase activity (Farr et al. 2007) and that C-terminal functions as the thermometer (Nakamura et al. 2004), lack of GGM-like tail in GroEL2 could explain the unusually high ATPase activity, thermal instability, and inability to functionally replace *E. coli* GroEL (George et al. 2004). In addition, the observation that GroEL1 and GroEL3 possibly make hetero-oligomers adds strength to this hypothesis (Gould et al. 2007a, b). The situation with methanosarcina is even more complex. The only GroEL in methanosarcina surprisingly lacks GGM tail,

Table 1 Salient features of the chaperonins described in this review

Organism/taxon	Chaperonin	Operonic with GroES	Regulation	Essentiality	Other functions	Known substrate interaction	GGM	Possible mode of evolution
<i>M. xanthus</i>	GroEL1	Yes	ND	No	Generalist chaperone	Cellular proteins	GGM-like	Duplication
	GroEL2	No	ND	No	Bactericidal predation	Cellular proteins	GGM-like	Duplication
	GroEL1	Yes	HrcA	No	Biofilm formation Granuloma Formation DNA binding	KasA	His-rich	Duplication
<i>R. leguminosarum</i>	GroEL2	No	HrcA	Yes	Generalist chaperone	ND	GGM-like	Duplication
	GroEL1	Yes	RpoH and HrcA	Yes	Generalist chaperone	Model substrates	GGM-like	Duplication
	GroEL2	Yes	RpoH	No	ND	Model substrates	Pattern-free	Duplication
	GroEL3	Yes	NiF and HrcA	No	Folding of proteins involved in nitrogen fixation	Nod proteins	GGM-like	Horizontal transfer
<i>M. mazei</i>	GroEL	Yes	Moderately by heat stress	ND	Generalist chaperone	Cellular proteins	Pattern-free	Horizontal transfer
	Thermosome α	–	Moderately by heat stress	ND	Generalist chaperone	Cellular proteins	GGM-like	–
	Thermosome β	–	Moderately by heat stress	ND	Generalist chaperone	Cellular proteins	Pattern-free	–
	Thermosome γ	–	Moderately by heat stress	ND	Generalist chaperone	Cellular proteins	GGM-like	–

ND not detected

possibly explaining the reason for its slow ATPase activity and inability to functionally replace *E. coli* GroEL (Figueiredo et al. 2004). Additionally, the presence of GGM-like tail in α and γ subunits suggests an evolutionary mechanism that enables thermosome to fold proteins of bacterial origin. However, lack of GGM tail in the β subunit could explain its significant divergence from the other counterparts and consequent inability to oligomerize (Klunker et al. 2003). Taken together, the chaperonin paralogs with the GGM-like tail, either essential or expressed with *groES*, function as generalist chaperonins in supporting growth and development of the organism in analogy to the well-studied *E. coli* GroEL, while the non-GGM chaperonins appear to have evolved to perform specialized functions (Ojha et al. 2005; Wang et al. 2013). The general chaperonins might presumably adapt the *cis* mechanism of action, while the specialized chaperones might adapt either the *cis* or *trans* mechanism. Further studies are therefore essential to understand the biological significance of these specialized chaperonins.

Conclusions

The significance of chaperonins is by the virtue of their ability to assist folding of about 10–15 % of cellular proteins, some of which are essential. Several bacteria thus possess multiple chaperonin genes that are believed to assist the organism during different phases of life cycle. Analysis of distribution of multiple GroELs revealed a specific pattern, rather than a random distribution, suggesting strong biological correlation for the presence of multiple genes. The examples explained in this review attempt to address the necessity of these multiple GroELs in individual cases. Moreover, evolutionary analysis suggested that acquisition of multiple GroELs followed case-specific evolutionary paths: gene duplication in mycobacteria, while horizontal acquisition in methanogens. However, observing the C-terminal repeat sequences depicts a unique correlation of these repeats with the function of the chaperonin paralogs. The GroELs with GGM-like repeats have been proposed to function as generalist chaperonins while those with a different C-terminus have diverged to perform specialized functions. Comprehensive structural and functional studies on these chaperonins are therefore essential to understand the functional significance of the multiple chaperones.

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