Chapter 3
Prokaryotic Multiple Chaperonins: The Mediators of Functional and Evolutionary Diversity

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Abstract Chaperonins are a class of molecular chaperones that form large multimeric assemblies for encapsulation of substrate proteins. Surprisingly, 30% of newly sequenced bacterial genomes encode multiple copies of the chaperonins. The distribution of these multiple copies appears to follow a phylum-specific pattern. Functional and structural studies on several of these chaperonins have delineated how these *extra* chaperonins evolved functional diversity and contributed towards the biological adaptation of the hosting organisms. Since several of these bacteria are either pathogenic or economically important, and the chaperonins regulate the pathogenic processes in these organisms, it is important to understand their biology. This chapter is aimed to act as a primer for the subsequent chapters that describe different examples of multiple chaperonins and the plethora of their functional diversity.

3.1 Introduction

Advancements in genomic technologies have yielded wealth of information from completely sequenced genomes. The startling revelation of the presence of several eukaryotic-like features in bacteria, such as the protein kinases (Kumar et al. 2009; Perez et al. 2008), different classes of intronic regions (Ferat and Michel 1993; Hausner et al. 2014; Martinez-Abarca and Toro 2000) and protein-protein interaction mediating ankyrins (Price et al. 2010), has provided interesting insights into understanding the biology of these organisms. Likewise, the presence of multiple copies of genes encoding chaperonins in 30% of the bacterial genomes (Barreiro et al. 2005; Fischer et al. 1993; Karunakaran et al. 2003; Kong et al. 1993), another well-known eukaryotic feature, encoding 2–3 copies of chaperonin genes (Nishio

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et al. 1999; Vitlin Gruber et al. 2013), has gained a lot of interest in recent times. Interestingly, many of the bacteria that possess multiple copies of chaperonin genes are either pathogenic to human, livestock and crops or economically important. In addition, these excess chaperonin copies have been demonstrated to be involved in the pathogenic or economically important biological functions in those bacteria. These observations, therefore, have propelled intense investigations to unravel the functional diversity of these chaperonins, thereby aiming to provide tools for either curbing the pathogens or tuning beneficial bacteria towards human well-being.

3.2 Distribution of Multiple Chaperonins

Comprehensive phylogenetic analyses on the multiple chaperonins have revealed that their distribution follows a phylum-specific pattern (Kumar et al. 2015; Lund 2009). While many bacterial phyla possess a single copy of the chaperonin gene, the presence of multiple copies of chaperonin genes predominates in five phyla: (a) phylum *Actinobacteria* that constitutes high-G + C Gram-positive species, (b) phylum *Firmicute*s that constitutes low-G + C Gram-positive species, (c) phylum *Cyanobacteria* that constitutes photosynthetic bacteria, (d) phylum *Chlamydia* that constitutes obligate intracellular pathogens and (e) alpha subdivision of phylum *Proteobacteria* that constitutes root-nodulating symbionts (Table 3.1). I will briefly review below the current understanding of the salient features of the multiple chaperonins, such as gene organisation, regulation, essentiality, sequence and functional diversity and the possible modes of evolution in the following sections. For detailed description, the readers are advised to read a comprehensive review by Peter Lund (Lund 2009).

3.2.1 Functional Diversity Among the Chaperonins of Actinobacteria

Actinobacteria constitutes a phylum of Gram-positive bacteria that are characterised by high-G + C content genomes, such as *Mycobacterium tuberculosis*, *M. leprae*, *Streptomyces albus* and *Bifidobacterium longum*. The presence of multiple chaperonins was first reported in *Actinobacteria*, in the genome of *M. tuberculosis* (Kong et al. 1993). About 70% of the sequenced actinobacterial genomes possess two copies of GroEL genes, with instances of three or four copies occurring at a lower frequency (Table 3.1). While the first copy is in operonic arrangement with the co-chaperonin gene, the second and subsequent copies exist singly (Kong et al. 1993; Rinke de Wit et al. 1992). Interestingly, the major difference between these copies lies at their carboxy-terminal segments (CTS). While the chaperonin encoded by the copy in operonic arrangement bears a non-canonical histidine-rich carboxy terminus (Ferreyra et al. 1993; Kumar and Mande 2011; Mande et al. 2013), the other copy bears characteristic glycine-methionine-rich carboxy terminus, probably

Table 3.1 Phylum-wide distribution of multiple chaperonin genes among completely sequenced bacteria

	Number of chaperonin homologues						
Phyla	1	2	3	4	5	6	7
Actinobacteria	34	119	10	6	0	0	0
Aquificae	11	0	0	0	0	0	0
Bacteroidetes/Chlorobi group	92	2	1	0	0	0	0
Caldiserica	1	0	0	0	0	0	0
Chlamydiae/Verrucomicrobia group	3	3	10	0	0	0	0
Chloroflexi	5	7	0	0	0	0	0
Chrysiogenetes	1	0	0	0	0	0	0
Cyanobacteria	1	49	3	0	0	0	0
Deferribacteres	4	0	0	0	0	0	0
Deinococcus-Thermus	16	0	0	0	0	0	0
Dictyoglomi	2	0	0	0	0	0	0
Elusimicrobia	2	0	0	0	0	0	0
Fibrobacteres/Acidobacteria group	8	0	0	0	0	0	0
Firmicutes	243	7	2	0	1	0	0
Fusobacteria	5	0	0	0	0	0	0
Gemmatimonadetes	1	1	0	0	0	0	0
Nitrospirae	4	0	0	0	0	0	0
Planctomycetes	0	0	6	0	0	0	0
Proteobacteria	470	79	25	8	6	2	1
Spirochaetes	35	0	0	0	0	0	0
Synergistetes	4	0	0	0	0	0	0
Tenericutes	9	0	0	0	0	0	0
Thermodesulfobacteria	2	0	0	0	0	0	0
Thermotogae	14	1	0	0	0	0	0

The table is adopted from Kumar et al. (2015), with permission

conferring essentiality to this copy (Mazodier et al. 1991; Rinke de Wit et al. 1992). Interestingly, in the organisms with more than two chaperonin genes, the third and subsequent copies possess a pattern-free CTS (Fig. 3.1). Since all these bacteria are fast-growing, these chaperonin copies are implicated in enhancing the growth rate of those organisms (Kumar et al. 2015). Surprisingly, in the organisms where only one copy of chaperonin is present, such as *B. breve*, *B. longum* and *B. animalis lactis*, the chaperonin and co-chaperonin genes exist separately on genome (Maiwald et al. 2003; Ventura et al. 2004). Notably, such a situation is observed in 20–22% of the *Actinobacteria*, and interestingly in these organisms, in addition to the loss of operonic arrangement, the expression of chaperonin and co-chaperonin is differentially regulated. Generally, the expression of actinobacterial chaperonin genes is regulated via repression by HrcA (de Leon et al. 1997; Duchene et al. 1994; Grandvalet et al. 1998) or, in rare cases, by HspR (Barreiro et al. 2005), which bind the upstream inverted repeat elements, CIRCE and HAIR, respectively. Functionally, while the essential chaperonin copy has been proposed to act as the generalist chaperonin, the dispensable copy has been demonstrated to have diverged to attain

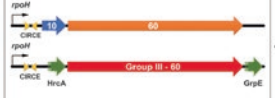
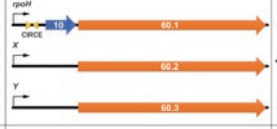
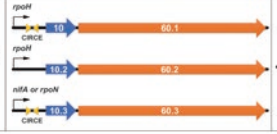
Phylum	Gene Organization	Essentiality	Moonlighting Functions	CTS	Modes of Evolution
Actinobacteria		• Cpn60.2 is essential • Cpn60.1 in Mtb - required for Granuloma Formation	• Mtb Cpns – Immunogenic	• Operonic Cpn60 – His-rich • Essential Cpn60 – GGM-like • Third and fourth copies – Pattern-less tail	• Gene Duplication at the LUCA
Firmicutes		• Not determined	• Group III Cpn60 - Folds proteins involved in anaerobic carbon monoxide oxidation	• Pattern-less tail	• Horizontal Transfer
Cyanobacteria		• Cpn60.1 is essential	• Cpn60.2 – Provides thermo-protection for photosynthesis related proteins	• Cpn60.1 – Smaller GGM-like tail • Cpn60.2 – Longer GGM-like tail	• Gene Duplication
Chlamydia		• Cpn60.1 is essential	• Cpn60.2 and Cpn60.3 – regulate Host's molecular events during infection.	• Cpn60.1 – smaller GGM-like tail • Other copies – pattern-less tail	• Successive Gene Duplication Events
α-Proteobacteria (Rhizobia)		• Cpn60.1 is essential	• NifA regulated Cpn – Dedicated for folding nitrogen fixation related proteins	• Generally GGM-like tail • Some have pattern-less tail	• Gene Duplication at the LUCA • NifA regulated Cpn – Horizontal Transfer

Fig. 3.1 Salient features of the multiple chaperonins in different phyla. Numbers 10 and 60 represent Cpn10 and Cpn60 homologues. *CTS* stands for carboxy-terminal segments and *LUCA* stands for last universal common ancestor. *CIRCE* stands for controlling inverted repeat of chaperone expression. In the cartoon depicting cyanobacterial chaperonins, H and K represent the upstream enhancing elements; H-box and K-box are induced by heat and light, respectively. In the cartoon depicting *Chlamydia* chaperonins, X and Y represent the two uncharacterised promoter elements

atypical functions to assist the organism during specific life stages, principally, the pathogenic stages (Basu et al. 2009; Ojha et al. 2005). This argument is supported by the evolutionary studies where a faster rate of evolution was observed for the dispensable copy (Goyal et al. 2006; Hughes 1993; Kumar et al. 2015). In addition, phylogenetic studies have shown that the modes of origin of multiple chaperonins in actinobacterial species have resulted due to a gene duplication event at the last common ancestor of *Actinobacteria* (Goyal et al. 2006; Hughes 1993; Kumar et al. 2015; Mande et al. 2013). A detailed description on the current advances in mycobacterial chaperonins is given in Chap. 5. Surprisingly, in *S. lividans* the second chaperonin copy can function independent of a co-chaperonin (de Leon et al. 1997). This observation provided a probable explanation for non-operonic location and independent regulation of the second chaperonin gene and suggested that this copy might play a different cellular role. Taken together, *Actinomycetes* provide a fascinating picture of genetic and functional diversity among the multiple chaperonins.

3.2.2 *Unique Chaperonins in* Firmicutes

Firmicutes constitute several Gram-positive bacteria, such as *Carboxydothermus hydrogenoformans*, *Staphylococcus aureus* and *Desulfitobacterium dehalogenans*, which are characterised by a low-G + C content genome. Surprisingly, in addition to the classical group I chaperonin genes, unlike their high-G + C phylogenetic neighbours, some of the *Firmicutes* encode archaeal-like chaperonins that are classified as group III chaperonins owing to their primary, tertiary and quaternary structural features, peculiar genomic location alongside the *dnaK* operon and unique mode of regulation (Techtmann and Robb 2010). Majority of the *Firmicutes* encode multiple copies of chaperonins (Table 3.1). Apparently, the group I and group III chaperonin genes are regulated by HrcA-mediated heat shock response. Surprisingly, all the chaperonin copies possess pattern-free CTS (Fig. 3.1). Since the Gly-Met-rich tail is supposed to determine the substrate pool, this observation suggests that the substrate pool of *Firmicutes* chaperonins is different from the other bacteria. Moreover, since these bacteria dwell in carbon monoxide-rich environments and thus rely on anaerobic oxidation of CO, the extra chaperonin copy is believed to fold the proteins involved in this pathway. In addition, in several *Firmicutes*, the location of chaperonin genes is peculiarly in operonic arrangements with either the Hsp70 system or with the gene encoding trigger factor (Smidt et al. 2000), suggesting a unified and temporal mode of regulation for the genes encoding different chaperone systems. Owing to such genomic organisation, phylogenetic analysis, therefore, proposed that the group III chaperonins might have been acquired horizontally from ancient archaea. Since the two phylogenetically diverse chaperonins coexist and share substrate pools, *Firmicutes*, therefore, present a unique coitus among chaperonin groups. There is therefore a need for comprehensive structural and functional studies to delineate their functional and phylogenetic diversity.

3.2.3 Functional Distribution Among the Chlamydial Chaperonins

Chlamydiae phylum constitutes several obligate intracellular pathogens such as *Chlamydia trachomatis*, *C. psittaci* and *C. pneumoniae* that are characterised by complex developmental cycles through different host cell types. *Chlamydiae* portray an extremely complex and unique scenario of chaperonins (Table 3.1). While majority of *Actinobacteria* possess 2 copies of chaperonin genes, majority of chlamydial species (10 out of 16 completely sequenced species) possess 3 chaperonin genes (Kumar et al. 2015; McNally and Fares 2007). However, similar to *Actinobacteria*, only one of the chaperonin genes is in operonic arrangement with the co-chaperonin gene (Fig. 3.1). This chaperonin bears the characteristic Gly-Met-rich CTS, which is essential and thus believed to function as the generalist chaperonin (Fig. 3.1). The other two chaperonin copies deviate from characteristic features, such as unusual ATP-binding site and lack of Gly-Met-rich CTS, and thus are believed to have diverged to acquire different non-canonical functions. Such a notion is further supported by the complex lifestyle-specific expression patterns of these chaperonins. Intriguingly, the expression of only the first copy is heat shock regulated and is thus repressed by the HrcA-CIRCE system (Karunakaran et al. 2003). However, the second copy is induced when the bacterium is in pathogenic mode, either inside a monocyte for a persistent infection or in synovial macrophages during reactive arthritis (Kol et al. 1999). On the other hand, the expression of the third copy is induced when the bacterium is in Hep-2 cells (Gerard et al. 2004). These observations suggest a life-cycle-specific expression patterns for these chaperonins. Additionally, low sequence identity among these chaperonins and the observation that the second and third copy deviate further in sequence from the first copy (Karunakaran et al. 2003) suggested the possibility of two independent gene duplication events during the evolution of chlamydial chaperonins (McNally and Fares 2007). Taken together, the chlamydial chaperonins present a complex interplay with sequence divergence, differential expression patterns and genome locations that have aided these chaperonin copies to perform specific functions during different life stages of chlamydia.

3.2.4 Rhizobial Chaperonins: The Aristocrats of Chaperonin Biology

Alphaproteobacteria constitute several legume symbionts that engage in nitrogen fixation in root nodules. This class of bacteria, called the rhizobia, harbours the highest number of copies for chaperonins, with the *Bradyrhizobium japonicum* hosting seven genes. Rhizobia, therefore, present a perfect division of labour among the chaperonins (Fischer et al. 1993). In the most well-characterised example, *Rhizobium leguminosarum*, the bacteria harbour three copies of chaperonin genes with all of

them forming separate operons along with the respective co-chaperonin genes (George et al. 2004; Gould et al. 2007). Interestingly, one among the three operons exhibits unique features; it is located in a genomic island that hosts nitrogen fixation genes, unlike the regular heat shock, it is regulated by NiF that regulates expression of nitrogen fixation gene, and as a chaperone it assists the folding and assembly of several Nod proteins (Ogawa and Long 1995). These observations added credence to the notion that one copy of chaperonin in rhizobia is dedicated to fold the proteins involved in nitrogen fixation (Kumar et al. 2015). Among the other two operon copies, one of them is essential, regulated by HrcA and thus is believed to act as a generalist chaperonin (Gould et al. 2007). Although considerable literature on the second copy is not available, this copy is demonstrated to act as a chaperone in folding several model substrates albeit possessing a pattern-free CTS. A detailed description on the rhizobial chaperonins is given in Chap. 6.

3.2.5 *Multiple Chaperonins in* Cyanobacteria*: One Copy is Green!*

Cyanobacteria phylum largely constitutes photosynthetic bacteria such as *Synechococcus platensis*, *Synechocystis* sp., *Anabaena variabilis* and *Prochlorococcus marinus*. About 90% of the currently available cyanobacterial genomes encode two chaperonin genes (Table 3.1), with one copy in operonic arrangement with the co-chaperonin and the other located separately (Kumar et al. 2015). Although this situation appears similar to that of *Actinobacteria*, the difference shows up in the species with three chaperonin genes (Fig. 3.1), where two of the chaperonins are in operonic arrangement with their co-chaperonin genes, while one is independent (Lund 2009). In contrast to the *Actinobacteria*, in *Cyanobacteria* the chaperonin(s) in operonic arrangement is (are) essential, while the individual one is dispensable (Sato et al. 2008). Interestingly, both chaperonins bear a Gly-Met-rich CTS, although CTS of the independent and dispensable chaperonin is very long (Lund 2009). Interestingly, since *Cyanobacteria* is photosynthetic, the extra copy is believed to offer thermo-tolerance to the photosynthetic system during heat shock. This notion is strongly supported by the way the chaperonin genes are regulated. Although both copies are regulated positively by RpoH and negatively by HrcA, the expression of the operon is rapidly induced upon heat shock due to the presence of the upstream enhancer elements known as the H, K and N boxes, while the expression of the second gene is induced gradually (Kojima and Nakamoto 2007; Rajaram and Apte 2010). In addition, the observation that even upon heat shock the second gene remains repressed during several photosynthesis-diminishing circumstances, such as when the bacteria are cultured in dark, when the photosystem's electron transfer is obstructed or when intracellular nitrate levels are increased (Kojima and Nakamoto 2007; Rajaram and Apte 2010), suggested that this chaperonin might have a direct connection with photosynthesis, probably by providing thermo-protection to the proteins involved in the light reaction.

Notably, similar dual copies of chaperonins are observed in chloroplasts of higher organisms, such as plants, suggesting ancient connections between the chaperonins and the evolution of photosynthesis (Nishio et al. 1999). Moreover, phylogenetic studies observed that the extra copies might have emerged by a single gene duplication event at the LUCA of cyanobacteria (Goyal et al. 2006). Moreover, functional studies on these chaperonins lead to interesting insights on the role of CTS in chaperonin function. While the copy in operonic arrangement that has optimal CTS could complement readily, the second gene albeit with a longer Gly-Met-rich CTS failed to complement *E. coli* GroEL (Furuki et al. 1996; Kovacs et al. 1992; Tanaka et al. 1997). Since a longer CTS has been shown to fill the chaperonin cavity, limit encapsulation to only smaller proteins and consequently decrease the client repertoire (Tang et al. 2006), the inability of the second chaperonin to complement *E. coli* GroEL could be due to its longer CTS and consequent smaller cavity. However, this limitation might have been evolutionarily driven to sequester only the photosynthesis-related proteins that are populated by smaller-sized proteins (Nakamura et al. 1998). A comprehensive chaperonin-client interaction studies are therefore required to comprehend the functional diversity in these chaperonins. Taken together, although the current understanding indicates that the cyanobacterial chaperonins have diverse functions and that the second chaperonin is linked to the photosynthesis, the precise characterisation of these chaperonins is required to delineate their functional diversity. Comprehensive description of cyanobacterial chaperonin system is presented in Chap. 7.

3.3 Why Multiple Chaperonins: Specific Examples

The existence of multiple genes for chaperonin has led to several hypotheses:

(a) Functional diversity: if all the copies work as intracellular chaperonins or have diverged to perform different functions.
(b) Evolutionary lineage: if these copies have resulted by horizontal acquisition from niche neighbours or due to gene duplication within the organism and do these multiple copies have any phylogenetic signature.
(c) Substrate spectrum: do the multiple chaperonins share the substrates or they have distinct substrate pools?
Primarily it was proposed that the organisms with multiple chaperonins might benefit either from the dosage effect (Kondrashov and Kondrashov 2006) or from the functional divergence of different chaperonins (Goyal et al. 2006). The former seems unlikely as the intracellular levels of chaperonins are always high. Moreover, as elaborated in the following chapters, multiple GroELs have been characteristic of organisms with complex lifestyle, suggesting the plausibility of the latter scenario. The following chapters will, therefore, review the current advances in understanding on the functional dictum of multiple chaperonins by presenting fascinating examples of bacteria and archaea with multiple

chaperonin genes. Chapter 4 will review the functional redundancy observed in chaperonins of myxobacteria and how the two dispensable chaperonins distribute their substrates and functions in life-stage-specific fashion (Chap. 4). Chapter 5 presents the current understanding in the functional diversity of mycobacterial chaperonin paralogues, where only one copy is essential and thus might function as the generalist chaperonin (Chap. 5). The other copies, on the other hand, have diverged in sequence and have been demonstrated to play important roles in the establishment and progression of the pathogenesis. Chapter 6 reviews the fascinating division of labour among the rhizobial chaperonins, where one set of chaperonins functions exclusively to fold the proteins involved in nitrogen fixation (Chap. 6). Likewise, Chap. 7 illustrates how one copy of chaperonin is dedicated to photosynthesis (Chap. 7). Notably, rhizobia are the bacteria which harbour the highest number of chaperonins. Chapter 8 reviews the situation of multiple chaperonins in thermoresistant archaea (Chap. 8) and reviews how the coexistence of evolutionarily diverse group I and group II chaperonins shaped the proteomes of the mesophilic methanogens (Chap. 8) and how this understanding can be translated to therapeutic approaches. The final chapter will review probable means of evolution of the multiple chaperonins (Chap. 9). These chapters are scientifically scintillating and reveal how the multiple chaperonin copies have been tuned according to the species-dependent requirement.

3.4 A Note on Chaperonin Nomenclature

Apart from the functional diversity that multiple chaperonins display, diversity prevails even in their nomenclature, leading to a conundrum. The purpose of this note is to explain the basis of the conundrum and try to unify different ways the chaperonins are referred to. Molecular chaperones are classified according to their molecular masses as Hsp100, Hsp90, Hsp70, Hsp60 and small Hsps (Kumar et al. 2015). Thus, the 60 kD chaperones are named as Hsp60 chaperones. Further, since they form rings, they were called chaperonins and thus were abbreviated as Cpn60 (Hemmingsen et al. 1988). Incidentally, since the chaperonin homologue of *E. coli* was identified as a gene required for the growth of bacteriophage lambda (Georgopoulos et al. 1973), it was named as GroEL (or GroL). Therefore, the same protein has been given in different names by different researchers as Hsp60, Cpn60 and GroEL. Likewise, the 10 kD co-chaperonins are called Hsp10, Cpn60 and GroES, respectively. The situation with multiple chaperonins is even more complicated. The copies of the chaperonins are named either as GroEL1, GroEL2 and so on or as Cpn60.1, cpn60.2 and so on. The Hsp60-type nomenclature, Hsp60_1 and Hsp60_2, is less common in multiple chaperonins. Peculiarly, some researchers prefer to name the chaperonin copy that forms an operon with its co-chaperonin as GroEL while the independent copy as Cpn60, as seen with a few cyanobacteria

(Lehel et al. 1993). Such a diversity in the nomenclature, obviously, leads to confusion to the readers, and a unified code for naming chaperonins, especially in the case of multiple chaperonins, has been proposed (Coates et al. 1993). According to this proposal, the GroEL name should be limited to the *E. coli* GroEL since this implicates a function in bacteriophage maturation, and since the chaperonins in other bacteria have not been demonstrated a bacteriophage maturation role, they should be termed as Cpn60 (Coates et al. 1993; Lund 2009). Hsp60 type of naming, however, is generally used for the mitochondrial chaperonins. The diversity still remains, since the researchers tend to continue to follow the names they are comfortable with. Therefore, while editing this book, we have acknowledged the nomenclature styles that the respective authors are comfortable with. Therefore, the purpose of this note is to make the readers familiar with the variety in chaperonin nomenclature that can be encountered in the subsequent chapters and thus have a lucid reading.

3.5 Conclusions

Multiple chaperonins are becoming common in prokaryotes that go through either several growth stages or hosts during their life cycle. In several organisms, these chaperonins have been demonstrated to assist either a particular life phase or a process (Fig. 3.1). Examples for the former appear in the chlamydial chaperonins, where the different chaperonins conquest as the bacterium passes through different host cells. Examples for the latter, however, appear in the rhizobia, mycobacteria and cyanobacteria where one of the copies of chaperonins is dedicated to assist the nitrogen fixation, pathogenesis and photosynthesis, respectively (Fig. 3.1). Taken together, such observations suggest a strong correlation to the biological significance for the existence of these multiple chaperonin copies and therefore compel a need for comprehensive investigations to unravel the biology of these fascinating molecules.

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