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The Role of GroE Chaperonins in Developing Biocatalysts for Biofuel and Chemical Production

Peng-Fei Xia[1], Timothy Lee Turner[2] and Lahiru N Jayakody[3]*

[1]School of Environmental Sciences and Engineering, Shandong University, 27 Shanda Nanlu, Jinan, 250100, P.R. China
[2]Department of Food Science and Human Nutrition, University of Illinois, Urbana-Champaign, Urbana, IL, 61801, USA
[3]National Bioenergy Center, National Renewable Energy Lab, 15013 Denver West Parkway, Golden, CO, 80401, USA

Abstract

As the need and interest for producing renewable biofuels and biochemical has grown, new avenues to improve product yields and productivity have been explored. Specifically, improving the tolerance of host microbes towards stressors, such as heat shock or the presence of harmful solvents, has been an especially important route to improve industrial-scale chemical production. In this review, we discuss recent advances in microbial engineering for renewable chemical production through the introduction and expression of chaperonins, especially the bacterial GroE complex. The GroE complex provides a closed-off environment and allows vital proteins to enter and engage in post-translational folding or refolding in a more-ideal environment, allowing the microbe to possess increased survival rates in low/high temperatures or in high concentrations of otherwise harmful end-products. Overall, we highlighted how chaperonin systems such as the GroE complex could have many industrially-relevant uses in the coming years.

Keywords: Chaperones; GroE; Biofuel; Biochemical; Engineered microbes

Introduction

Metabolic engineering and synthetic biology have been applied for the discovery and redesign of the potentials of microorganisms for numerous desired purposes. Both model hosting strains and microorganisms with highly-specific functions have been engineered to improve feedstock utilization, target fuel and chemical production, as well as regulate cellular physiology [1-6]. For instance, the baker's yeast, *Saccharomyces cerevisiae,* which was first used by the human society thousands of years ago, has been genetically engineered to ferment otherwise non-fermentable carbon sources. Indeed, C5 sugars such as xylose cannot natively be catabolized by *S. cerevisiae*. However, the engineered *S. cerevisiae* strains are able to metabolize xylose efficiently and to produce ethanol. The engineered microbes could simultatneously co-ferment carbon in the hydrolysate of lignocellulosic biomass such as hemicellulose- and cellulose-derived C5/C6 sugars and lignin-derived aromatics [5,7-9] and produce fuels and value-added chemicals such as ethanol, *n*-butanol [10-12], sesquiterpenes, polyhydroxyalkanoates (PHA), and fatty acid ethyl esters [5,11]. These advances are not limited to model hosts, such as *S. cerevisiae* and *Escherichia coli*, but have also been demonstrated in *Clostridium acetobutylicum*, *Bacillus subtilis*, *Pseudomonas putida*, and *Synechococcus elongatus* [13-17].

However, many chemicals are toxic to the microbial producers. As reported, *n*-butanol is toxic to both the innate producer *C. acetobutylicum* as well as the engineered host, *E. coli*, with a 1% *n*-butanol concentration severely inhibiting the growth of *E. coli* [18-20]. Besides the product toxicity, microorganisms may suffer from temperature stress [21] and inhibitors from the feedstock, such as the hydrolysate of lignocellulosic biomass [22,23]. Therefore, one of the major challenges for the further development in industrial bioconversions is to improve the tolerance of microorganisms against various stressors in engineered industrial systems.

It has been well documented that post-translational modification machinery plays a vital role in quality control of proteins and enhances the robustness of biocatalysts in stress conditions [24,25]. Indeed, ubiquitination and sumoylation play key roles for enhancing tolerance of *S. cerevisiae* towards the toxicity of lignocellulose hydrolysates [22,26]. Thus, engineering post-translation machinery of biocatalysts has emerged as an essential tool for enhancing the functions of enzymes and tolerance of microorganisms for bio-manufacturing processes [23,27].

Besides the advanced eukaryotic ubiquitin-dependent protein quality control system, both eukaryotes and prokaryotes have evolved molecular chaperone systems to assist protein folding and re-folding in protein quality control and damage recovery under natural and stressed environments [28]. Chaperones perform their functions via allosteric machinery and they are mainly driven by cycles of ATP binding and hydrolysis [28-30]. Unlike other proteins with highly specific targets, chaperones have a broad range of substrates. For instance, the bacterial HSP60 chaperone, GroEL and its co-chaperone, GroES, which together assemble the GroE complex, work on numerous proteins [29,31,32], and GroE increases *E. coli* tolerance towards heat shock extreme or sudden changes in temperature. Previous studies reported that the overexpression of prokaryotic GroE can alleviate the inhibitory effects of various end-products on engineered strains [27]. Thus, co-expression of chaperone systems together with homologous or heterologous pathways leading to the production of target chemicals would be a promising strategy for improving strain tolerance towards these value-added chemicals.

Moreover, the chaperone systems not only assist with protein folding and re-folding but also are essential for some proteins to achieve

***Corresponding author:** Lahiru N Jayakody, National Bioenergy Center, National Renewable Energy Lab,15013 Denver West Parkway, Golden, CO, 80401 USA, Tel: 3033847696; E-mail: Lahiru.Jayakody@nrel.gov

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their functional conformations. A recent study systematically analyzed the GroE dependent proteins in *E. coli*, and found that 57 proteins could not be active in their native host *E. coli* without a well-functioning GroE chaperone system [31,32]. Moreover, it has been demonstrated that the *E. coli* xylose isomerase and arabinose isomerase, both previously reported as being non-functional in yeast [33,34], can work well in *S. cerevisiae* via co-expression of *E. coli* GroE [35]. Thus, the chaperone systems can also help with the expression of heterologous enzymes and pathways.

In this review, we focus on the recent advances in employing chaperone systems in metabolic engineering to improve the engineered strain tolerance against external stressors and the role in heterologous protein expression. In particular, the utilization of bacterial GroE in either engineered prokaryotic or eukaryotic microorganisms is primarily discussed.

Mechanisms of the Chaperone Systems

Chaperones are essential for microbial cell survival in changing environments [28,36]. As also identified as heat shock proteins (HSPs), chaperones are categorized as HSP60, HSP70, HSP90, and HSP100, according to their molecular weights in which the numbers indicate the weights in kilodaltons. The co-chaperones or other chaperones, including HSP10 and HSP33, have also been identified. HSP70, such as the *E. coli* DnaK as well as *S. cerevisiae* Ssa and Ssb, works when the nascent proteins are first synthesized, and the functions of HSP70 consists of protein folding, translocation across organelle membranes, and disaggregation of protein aggregates [37]. HSP90, like the *E. coli* HtpG, has diverse bio-functions, but its working machinery is still less well-understood [38]. Additionally, the HSP100, including the bacterial ClpA, ClpB, ClpX and HslU as well as the eukaryotic p97 and RPT1-RPT6, are unfoldases and disaggregases, which transfer the target proteins to compartmentalized proteases or disassemble aggregates of misfolded proteins (Figure 1) [28].

Among these various chaperone systems, the HSP60 chaperones, especially the bacterial HSP60 chaperones (GroEL), which are also named as chaperonins, were the first discovered and most investigated systems [39]. The GroEL chaperone consists of two heptameric rings and forms two back-to-back cavities with the hydrophobic parts in the apical domains and hydrophilic sites inside the cavities [40]. For the *cis* mechanism, the hydrophobic sites of the GroEL interact with the hydrophobic residues of nascent proteins, and trap the proteins into the cavity [40,41]. When ATP binds to GroEL, the co-chaperonin GroES caps one of the cavities and provides an undisrupted environment for proteins to fold. The well-folded protein in its designed conformation will be released when ATP is hydrolyzed [42,43]. This mechanism works for proteins smaller than 70 kDa because of the volume of the GroE cavity. Recently, the *trans* mechanisms have also been identified, and this mechanism works on proteins that are too large to be completely trapped in the cavity [44].

Usually, the bacterial GroE was regarded as a Group I chaperonin, also including chaperonins from mitochondria and chloroplast. The Group I chaperonins share similar structures as well as working machinery, and the hydrophobic interaction based functional mechanism enables Group I chaperonins to have a broad range of substrates [28]. The Group II chaperonins include the cytosolic chaperonins in eukaryotic cells, such as the eukaryotic CCT (chaperonin containing TCP1). They have distinct mechanisms as well as specified substrates. The advantages of the bacterial GroE result in a promising tool for strain engineering with better performance, and the differences between Group I and II chaperonins may shed light on the trans-domain engineering of potentially industrially relevant microorganisms (Figure 1).

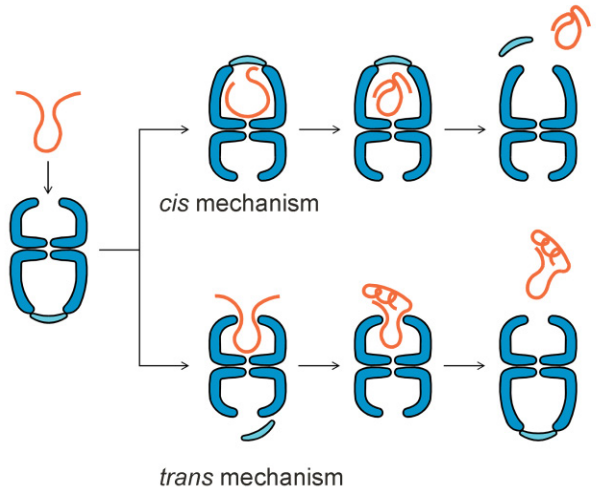

Figure 1: Mechanisms of the GroE chaperonin systems. The GroE complex consists of the HSP60 chaperonin GroEL (blue), and 14 GroEL proteins which form two back-to-back heptameric rings containing two cavities. For the *cis* mechanism (upper pathway), the hydrophobic sites in the apical domain of GroEL interact with the hydrophobic residues of nascent proteins (orange) and trap the proteins into the cavity. After ATP binds to GroEL, the co-chaperonin GroES cap (light blue), which contains seven GroES proteins, covers one of the cavity and provide an undisrupted environment for proteins to fold. The well-folded protein in its designed conformation will be released when ATP is hydrolyzed. For proteins that are too large (> 70 kDa) to be encapsulated in the GroE complex go to the *trans* mechanism (bottom pathway). The nascent protein first binds to the GroE complex, and then ATP bind to GroE while the GroES cap releases from the opposite GroEL ring to the ring that interacts with the nascent protein. After the rebinding of ATP and GroE cap in the *trans* configuration, parts of the nascent proteins are folded while some remain unfolded. The unfolded proteins re-bind to the GroE complex and undergo another round of the *trans* mechanism for proper folding.

Engineering Strain Tolerance Towards Organic Solvents

Biofuels and some value-added chemicals are natively produced organic solvents by microorganisms, and exert antimicrobial activities with eco-physiological functions in various environmental niches [45,46]. Traditionally, the inhibitory effects of organic solvents are expressed by the octanol-water partition coefficient (log P_{OW}) which indicates the hydrophobicity. A solvent with a log P_{OW} lower than 3.8 is considered toxic, such as toluene and hexanol [47,48]. However, biofuels, such as ethanol, butanol, and butanediol, exhibit unique values of log P_{OW}, and mere hydrophobicity cannot explain the inhibitory effects [49], as the log P_{OW} values of ethanol, *n*-butanol, and butanediol are -0.310, 0.839, and -0.92, respectively, implying a different interaction with water and biomolecules [46]. Some organic solvents, including ethanol, *n*-butanol, and butanediol are chaotropic solutes, and the chaotropicity of an organic solvent would disturb the hydrophobic effects needed to maintain the structure of biomolecules [46,50]. Although the functional mechanisms may differ between hydrophobicity and chaotropicity, and the concepts are still controversial, it is commonly accepted that organic solvents may disturb the structure of biomolecules, especially the conformation and structures of proteins, thus eliciting the inhibitory impacts on cells [21,51]. Therefore, it is reasonable to employ the chaperone systems, which contribute to protein folding and re-folding, to engineer host strains for higher solvent tolerance.

Transcriptome analysis in the innate ethanol producer *S. cerevisiae* reviewed that HSP33 and HSP104 were up-regulated when

challenged with sub-lethal concentrations of ethanol [52], while no significant variations have been identified in the prokaryotic producer *Zymomonas mobillis* [53]. The up-regulation of chaperones was also observed in *Lactobacillus plantarum* in the presence of ethanol [54]. Although early research failed to target the chaperones as promising machines for tolerance engineering, Tomas et al. [55] discovered that overexpression of the GroE chaperonins would increase the tolerance of *C. acetobutylicum* toward *n*-butanol. In *C. acetobutylicum*, the inhibitory effect of *n*-butanol on cell growth was reduced by 85%, the cellular metabolism was increased up to 2.5-fold, and the final concentration of *n*-butanol in the medium rose by 40% compared to the wild-type strain [55]. Recent research observed that the *C. acetobutylicum* GroE chaperonins can increase the tolerance of *E. coli* toward butanol as well [56]. Later, it has been reported that the tolerance of *E. coli* toward various organic solvents can be increased via overexpression the native GroE chaperonins [49]. The cell growth of *E. coli* increased up to 12-fold under 4% (v/v) ethanol, 2.8-fold increase under 0.75% (v/v) *n*-butanol, 3-fold under 1.25% (v/v) 2-butanol, and 4-fold under 20% (v/v) 1,2,4-butanetriol via overexpression of the GroE chaperonins. The increased solvent tolerance through the overexpression of the chaperone systems was also identified in *Lactococcus lactis*, *Lactobacillus paracasei* [57], *Lactobacillus plantarum* [58], *Pseudomonas putida* [59], and *S. cerevisiae* [60]. Moreover, a patent described that the heterologous expression of chaperones from thermophilic microorganisms can increase ethanol tolerance in prokaryotic hosts, including *C. acetobutylicum*, *E. coli*, and Z. *mobillis* against ethanol, butanol, and other short-chain alcohols [61].

It was also reported that overexpression of GroE chaperones might induce the overexpression of multiple chaperone complexes, including HSP18, HSP70, and HSP90 chaperones [62], and further research found that the combination of various chaperone systems would increase multiple solvent tolerance [63]. As reported, co-expression of the GroE and GrpE would increase up to 2-fold viable cells after exposure to 5% (vol/vol) ethanol, and co-expression of GroE and ClpB resulted in 1.130%, 78% and 25% increases in viable cells under 5% ethanol, 1% *n*-butanol, and 1% *iso*-butanol, respectively. Moreover, simultaneous overexpression of GrpE, GroE, and ClpB increased 3-fold, 4.9-fold and 1.78-fold of viable cells under 7% ethanol, 1% n-butanol, or 25% 1,2,4-butanetriol, respectively [63].

These advances provide evidence that chaperone systems can be regarded as a defense mechanism against organic solvents and that strain tolerance would be improved via engineering the host strain chaperone machinery.

Improving the Temperature Tolerance

Temperature, either heat or cold shock, is a significant challenge for microorganisms in nature. For all organisms, temperatures moderately beyond the optimum growth temperature jeopardize their lives [36]. However, either innate or engineered hosts that have been employed in industrial bioprocesses may experience heat stress. Thus, besides the optimization of the bioconversion pathways, it is important to engineer the working microorganisms for being capable of adapting towards heat, cold, and temperature shocks to achieve further improvements in target chemical production levels and strain stability.

Microorganisms in nature can survive from the freezing point 0°C to 113°C and have evolved serial responses to cope with the varied or sudden variations of temperature [36]. Some of these responses are molecular chaperones. As the native or engineered microorganisms encounter varied temperatures in industrial bioprocesses, efforts have already been made to test whether chaperone systems, especially those found naturally in extremophilic bacteria, would help engineered hosts with these harsh industrial conditions.

Although homologous overexpression of the GroE system in native hosts exhibited higher tolerance to solvent stress [49], attempts to overexpress autologous GroE failed to improve thermo-tolerance in *E. coli* [64]. However, the GroE system from the extremophilic bacterium *Pseudomonas putida* was found to be a promising expression target which improved the thermal tolerance of *E. coli* [64]. Also, overexpression of GroE from the thermotolerant species *T. tengcongensis* into *S. cerevisiae* expanded the optimal growth temperature of *S. cerevisiae* from 28°C – 30°C to 28°C – 35°C [65]. Notably, engineered *E. coli* and *S. cerevisiae*, with the overexpression of thermophilic bacterial chaperones, also exhibited higher tolerance toward solvent stress with better fermentation performances [64,65]. These studies show the advantages of implanting chaperones from thermophilic bacteria into other microbes and the superior robustness of thermophilic bacteria under harsh conditions due to long-term evolution in their living niches.

Besides the heat stress, industrial microorganisms also encounter cold conditions, and the cold adaption of host strains could be significantly improved via introducing the chaperone systems from psychrophilic microorganisms, due to the unique physiologic properties at low temperatures [66]. When the GroE from the psychrophilic bacterium *Oleispira antarctica* is overexpressed in *E. coli*, the growth of the engineered *E. coli* strain was significantly improved near the natural low-temperature limit of *E. coli* [67], and cell growth was significantly improved via co-expression the GroE from the Antarctic psychrophilic bacterium, *Psychrobacter* sp. PAMC21119, at 10°C [66]. These achievements not only benefit the adaption of engineered hosts in various industrial conditions, but also contribute to the heterologous expression of cold-adapted enzymes, which require low temperatures for desired functions [68,69].

Trans-Species and –Domains Expression of Heterologous Proteins

Given the progress in designing and optimizing primary metabolic pathways and the tolerance engineering as discussed above, engineered host microbes have been able to service the human society with numerous intended functions [6,16,17]. One major contribution comes from our constantly improving DNA reading and writing capabilities, which provide valuable gene resources and promising genetic tools. Nevertheless, not all target genes can be functionally expressed in non-homologous hosts, especially in trans-species and trans-domain cases [35,70]. Proteins must achieve functional conformations to work correctly. Although the structural information of proteins is usually contained in the amino acid sequences [71], protein folding needs the assistance of molecular chaperones in the complex intracellular environments [28,72].

In the prokaryotic protein expression system, such as *E. coli*, the overexpression of heterologous proteins in the cytosol might result in the aggregation of target proteins, and the undesired protein aggregates are a major issue for *E. coli* expression systems [73]. One promising solution is the co-expression of the native *E. coli* GroE chaperonins to increase the solubility of proteins [74]. However, the increased solubility of target proteins usually does not confirm higher activities [75]. A recent study observed that co-expression of the autologous chaperone systems from the gene-resource-strain show better performance. As reported, heterologous co-expression of GroE from *Aurantimonas manganoxydans* could increase the active nitrile hydratase production

at 30°C better than at lower temperatures [75]. The sarcosine oxidase from *Thermomicrobium roseum*, which was codon-optimized, was functionally expressed in *E. coli,* and the co-expression of GroE and GroE with the HSP70 complex significantly increased the solubility of expressed sarcosine oxidase [76]. Another example is that the expression of α-amylase from the hyper-thermophilic archaeum *Pyrococcus furiosus* could be improved via co-expression of the autologous chaperones [77].

The chaperone system not only prevents the aggregation of proteins but is also required for the functional expression of certain proteins. For instance, the assembly of the prokaryotic ribulose-1,5-bisphosphate decarboxylase/oxygenase (RuBisCO) from photoautotrophic *Rhodospirillum rubrum* in *E. coli* requires the GroE chaperonins [78]. Recently, a systematic study summarized the GroE-dependent proteins in *E. coli* using an engineered strain with controllable GroE expression and discovered that 57 proteins are obligate substrates of GroE chaperonins [31]. These proteins are enriched in alanine/glycine residues, and can be encapsulated in the chaperonin cavity. Interestingly, 42 of the 57 proteins are related to cellular metabolisms, including the *E. coli* xylose isomerase and arabinose isomerase, which have long been non-functionally expressed in *S. cerevisiae* [33,34]. The analog of the bacterial GroE system in yeast is located in mitochondria rather than cytosol, and the cytosolic HSP60 system in yeast is different from the bacterial GroE system regarding substrates and working mechanisms [28]. Therefore, researchers discovered that the mismatching of the chaperonin systems between prokaryotic and eukaryotic cells might be the reason some bacterial enzymes, such as *E. coli* xylose isomerase and arabinose isomerase, cannot be functionally expressed in yeast [35]. The results showed that functional expression of *E. coli* xylose isomerase and arabinose isomerase, as well as the *Agrobacterium tumefaciens D*-psicose epimerase, can be achieved in *S. cerevisiae* via co-expression the *E. coli* GroE chaperonins.

Conclusion

In this review, we summarized the recent advances in employing the chaperones, especially the bacterial chaperonin, GroE to further the development of microbial cell factories. The evidence demonstrated that co-expression of chaperones could improve the engineered strain tolerance toward organic solvents and temperature variations, and chaperones could also be a general tool for heterologous protein expression at the post-translational level.

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