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Facilitated Oligomerization of Mycobacterial GroEL: Evidence for Phosphorylation-Mediated Oligomerization▽†

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The distinctive feature of the GroES-GroEL chaperonin system in mediating protein folding lies in its ability to exist in a tetradecameric state, form a central cavity, and encapsulate the substrate via the GroES lid. However, recombinant GroELs of *Mycobacterium tuberculosis* are unable to act as effective molecular chaperones when expressed in *Escherichia coli*. We demonstrate here that the inability of *M. tuberculosis* GroEL1 to act as a functional chaperone in *E. coli* can be alleviated by facilitated oligomerization. The results of directed evolution involving random DNA shuffling of the genes encoding *M. tuberculosis* GroEL homologues followed by selection for functional entities suggested that the loss of chaperoning ability of the recombinant mycobacterial GroEL1 and GroEL2 in *E. coli* might be due to their inability to form canonical tetradecamers. This was confirmed by the results of domain-swapping experiments that generated *M. tuberculosis-E. coli* chimeras bearing mutually exchanged equatorial domains, which revealed that *E. coli* GroEL loses its chaperonin activity due to alteration of its oligomerization capabilities and vice versa for *M. tuberculosis* GroEL1. Furthermore, studying the oligomerization status of native GroEL1 from cell lysates of *M. tuberculosis* revealed that it exists in multiple oligomeric forms, including single-ring and double-ring variants. Immunochemical and mass spectrometric studies of the native *M. tuberculosis* GroEL1 revealed that the tetradecameric form is phosphorylated on serine-393, while the heptameric form is not, indicating that the switch between the single- and double-ring variants is mediated by phosphorylation.

GroEL, an essential chaperonin, is known to form a ring-shaped structure for sequestering substrate proteins from the crowded cellular milieu and is responsible for the occurrence of various cellular processes, such as de novo folding, transport, and macromolecular assembly, within a biologically relevant time scale (7, 26, 48, 53). In *Escherichia coli*, GroEL, along with its cofactor GroES, assists the folding of about 10 to 30% of cytosolic proteins, among which some are known to be essential for cell viability (15, 26, 27, 31). GroEL was originally identified as the host factor responsible for phage λ and T4 capsid protein assembly and was subsequently shown to be essential for cell viability (17, 20). *E. coli groEL* is found in an operonic arrangement with *groES* (*groESL*), and its expression is regulated by multiple promoter elements.

GroEL function has been shown to be a complex interplay between its interaction with and encapsulation of substrate proteins, with concomitant conformational changes induced by ATP binding, hydrolysis, and GroES binding (24, 56, 62). *E. coli* GroEL exists as a homotetradecamer forming two isologous rings of seven identical subunits each. Crystallographic analyses have delineated the three-domain architecture of GroEL monomers and the GroES-GroEL interactions (4, 63). The central region of the GroEL polypeptide, spanning amino acid residues 191 to 376, constitutes the GroES and substrate polypeptide-binding apical domain. The equatorial ATPase domain spanning two extremities of the GroEL polypeptide, that is, residues 6 to 133 and 409 to 523, is responsible for the ATPase activity and the bulk of intersubunit interactions. The hinge-forming intermediate domain, spanning two regions on the polypeptide, namely, residues 134 to 190 and 377 to 408, connects the said two domains in the tertiary structure. The conformational changes resulting from ATP binding and hydrolysis at the equatorial domain are coupled to those occurring at the apical domain via this hinge region (4, 63).

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, on the order of 104 kDa (10, 27, 35, 46). Productive in vivo folding of the proteins larger than the usual size limit, such as the 86-kDa maltose binding protein fusion and 82-kDa mitochondrial aconitase, has also been reported (9, 29). 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.

Recent genome annotation studies of various bacteria have revealed that a few bacterial genomes possess multiple copies of *groEL* genes (2, 18, 30). The *Mycobacterium tuberculosis* genome bears two copies of *groEL* genes (*groEL*s). One of

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these, *groEL1*, is arranged in an operon, with the cognate cochaperonin *groES* being the first gene, while the second copy, *groEL2*, exists separately on the genome (13). Recombinant mycobacterial GroELs were shown to possess biochemical features that deviated significantly from the trademark properties of *E. coli* GroEL. The most striking feature of *M. tuberculosis* GroELs, however, was their oligomeric state, where contrary to expectations, in vitro they did not form the canonical tetradecameric assembly when purified from *E. coli*. The proteins instead existed as lower oligomers (dimers) irrespective of the presence or absence of cofactors, such as the cognate GroES or ATP (40, 41). Furthermore, they displayed weak ATPase activities and GroES independence in preventing aggregation of the denatured polypeptides.

Evolutionary studies of *M. tuberculosis groEL* sequences have suggested rapid evolution of the *groEL1* gene, yet without turning these into pseudogenes (21). The other hypothesis suggests that *M. tuberculosis*, being an organism that grows slowly, might require GroEL function that does not utilize ATP rapidly but, rather, with a slow turnover rate. Alternately, additional mechanisms might exist in *M. tuberculosis* which could mediate regulated oligomerization of *M. tuberculosis* chaperonins. Such regulation might help in the controlled utilization of ATP in nutrient-deprived *M. tuberculosis*, as observed for other chaperones, such as small heat shock proteins (23).

In the present study, we have exploited the unusual oligomeric status of the recombinant *M. tuberculosis* GroELs to study the significance of oligomer formation for GroEL's function as a molecular chaperone. Furthermore, we have explored the possibility of the existence of regulated oligomerization for native *M. tuberculosis* GroELs in their natural setting. We first show that *M. tuberculosis groEL* genes are not capable of complementing a conditional allele of *E. coli groEL*, namely, *groEL44*. The results of phenotypic and biochemical analyses of GroEL variants obtained by gene shuffling and domain swapping suggest that the impaired chaperoning ability of recombinant *M. tuberculosis* GroELs is a consequence of their inability to form higher-order oligomers in *E. coli* and that oligomerization is the prelude to the formation of an active GroEL chaperonin. Further, by immunochemical and mass spectrometric (MS) analysis of native mycobacterial GroELs, we show that *M. tuberculosis* GroEL1 exists in multiple oligomeric forms, viz., monomeric, dimeric, heptameric (single ring), and tetradecameric (double ring) forms, and that the switch between single-ring and double-ring variants is operated by phosphorylation on a serine residue. These observations suggest that the determinants of oligomerization for *M. tuberculosis* GroEL1 are distinct from those of its *E. coli* counterpart and that it does oligomerize in *M. tuberculosis* (its native environment), whereas it loses its oligomerization capability when expressed in *E. coli*. It could thus be possible that *M. tuberculosis* GroEL1 requires a certain native *M. tuberculosis* protein, probably a eukaryotic-like Ser-Thr protein kinase, to oligomerize properly, though the precise reason cannot be discerned by these observations.

MATERIALS AND METHODS

Materials, bacterial strains, and growth conditions. The molecular biology procedures employed in this study were performed according to the standard protocols (47). All chemicals, enzymes, and antibiotics were purchased from Sigma, Inc. Antibodies IT3 and IT56 were procured via an NIH-NIAID TB Vaccine Testing and Research Materials contract awarded to Colorado State University, CO; anti-Cpn60.1$_{Mtb}$ (Cpn60.1 of *M. tuberculosis*) was a kind gift from A. R. M. Coates; and the phosphoaminoacyl-specific antibodies antiphosphoserine polyclonal antibody and phosphothreonine monoclonal antibody were purchased from Assay Designs and Rockland Immunochemicals, respectively. *E. coli* was cultured in standard LB broth supplemented as appropriate. *E. coli* SV2 is a derivative of the *E. coli* K-12 strain B178 (*galE groESL*$^+$) which bears a temperature-sensitive allele of *groEL*, namely, *groEL44*. *E. coli* LG6 and *E. coli* MGM100 are derivatives of MG1655 wherein the chromosomal *groESL* operon is placed downstream from the lactose- or isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{lac} and L-arabinose-inducible P_{BAD} promoters, respectively (26, 36, 54). These strains were kind gifts from Alan Fersht, Arthur Horwich, and Millicent Masters, respectively. Coliphages λcIB2 and T4GT7 were sourced from laboratory stocks. *M. tuberculosis* H_{37}Rv was propagated on Middlebrook 7H11 medium and cultured in Middlebrook 7H9 liquid medium supplemented with oleic acid-albumin-dextrose-catalase and Tween 80, as appropriate (BD Biosciences). For the studies of the effects of temperature shift, a fraction of the *M. tuberculosis* culture at mid-log phase was subjected to heat shock for 3 to 6 h at 42°C. Lysates from the resulting cultures were prepared according to the standard protocols (44). The plasmids and oligonucleotide primers used in this study are listed in Tables S1 and S2, respectively, in the supplemental material. The open reading frames (ORFs) encoding *M. tuberculosis* GroES, GroEL1, and GroEL2 and *E. coli* GroEL/S were cloned under the arabinose-inducible P_{BAD} promoter (22). *M. tuberculosis groEL1* was amplified from an *M. tuberculosis* genomic DNA library, a kind gift from Stewart Cole (13), using primers SCM7F and SCM5R and cloned into the NdeI and SmaI sites of pBAD24N to obtain plasmid pSCM1604. *M. tuberculosis groEL2* was sourced from the plasmid pSCM1000 upon digestion with restriction endonucleases XbaI and HindIII, and the resulting fragment was cloned into pBAD18 that was digested with the same enzymes to generate plasmid pSCM1605 in which the *groEL2* ORF is preceded by an optimally placed vector-borne ribosome binding site. *E. coli groEL* was amplified from the genomic DNA of strain MG1655 using primers SCM4F and SCM3R and cloned into the NcoI and HindIII sites of pBAD24 to generate plasmid pSCM1608. Furthermore, two additional plasmids, pSCM1602 and pSCM1603, were constructed in which *groEL1* and *groEL2*, respectively, were placed into pBAD24 downstream from and in an operonic arrangement with *M. tuberculosis groES*.

In vivo assay for the GroEL function of recombinant *M. tuberculosis* GroELs. Stationary-phase cultures of *E. coli* strains SV2 and LG6 containing plasmids pSCM1601, pSCM1602, pSCM1603, and pBAD24 were serially diluted. Five microliters of each serially diluted culture of *E. coli* SV2 were spotted onto LB agar plates supplemented with either 0.2% D-glucose (to repress the P_{BAD} promoter) or 0.2% L-arabinose (to induce the expression of cloned *groEL* genes). The cultures of *E. coli* LG6 were spotted onto LB agar supplemented with 0.2% D-glucose (to repress the P_{BAD} promoter), 0.2% L-arabinose (to induce the expression of cloned *groEL* genes), or 1 mM IPTG (to induce the chromosomal copy of the *E. coli groESL* operon). The plates bearing SV2 cultures were incubated at permissive (30°C) and restrictive (42°C) temperatures, and those bearing LG6 cultures were incubated at 30°C.

Generation of functional GroEL ORFs from *M. tuberculosis* GroEL genes via gene shuffling. Since an ORF encoding an active version of GroEL is expected to complement the temperature-sensitive phenotype associated with the *groEL44* allele, it allows facile genetic selection for the isolation of *M. tuberculosis* GroEL variants with increased chaperone activity. Toward this end, amplified *M. tuberculosis groEL1* and *groEL2* ORFs were used for multigene DNA shuffling, following the reported method (51, 58). Briefly, PCR products were subjected to limited DNase I digestion and fragments of 50 to 150 base pairs were recovered from agarose gels, which were further used as a template for a primerless assembly PCR. The assembled PCR product was used for the next round of PCR, with *E. coli groEL*-specific primers, according to the method of Zhao and Arnold (66). For the final round of PCR, *E. coli groEL*-specific primers were used, taking into consideration the presence of the 13-residue repeat $(GGM)_4M$ at the carboxyl terminus, alteration in the size and chemical nature of which is thought to affect GroEL's function in folding a few of the substrate polypeptides (16, 52). The product obtained after the final PCR was digested and cloned under the P_{BAD} promoter, and the library of plasmids was recovered in *E. coli* strain DH5α. A pooled plasmid preparation from this library was used to transform *E. coli* strain SV2, and direct selection for recovery of plasmids encoding active versions of *groEL* was employed by plating the transformation mixture onto LB–ampicillin–L-arabinose plates at the restrictive temperature of 42°C. Nine plasmids (pSCM1622 to pSCM1637) were chosen for further study (see Table S1 in the

supplemental material). A detailed protocol followed for gene shuffling is presented in the supplemental material.

Domain-swapping experiments. Two chimeric *M. tuberculosis-E. coli* hybrid ORFs were generated, namely, $groEL_{MEF}$ and $groEL_{MER}$, by employing overlap extension PCR (60) wherein the equatorial domains of *M. tuberculosis* GroEL1 and *E. coli* GroEL were mutually exchanged (see Fig. 4A). The resulting plasmids, bearing $groEL_{MEF}$ and $groEL_{MER}$ under the P_{BAD} promoter, were designated pSCM1609 and pSCM1611, respectively. Phenotypic analysis of the cloned genes was performed in *E. coli* strains SV2 and MGM100. Detailed methods are presented in the supplemental material.

In vivo assay for bacteriophage morphogenesis. Cultures of *E. coli* SV2 bearing plasmids pSCM1604, pSCM1605, pSCM1608, pSCM1609, pSCM1611, and pBAD24 obtained after culturing in 0.4% D-maltose with the appropriate antibiotic were overlaid in soft agar on the surface of LB plates containing either 0.2% D-glucose or 0.2% L-arabinose. The plaque-forming abilities of bacteriophages λ*c*1B2 and T4GT7 were assessed by spotting 5 μl each of 100-fold diluted suspensions of bacteriophage stocks. The plates were incubated at 30°C.

ATPase activity assays. The ATPase activity of the purified GroELs was quantified with a colorimetric assay performed as described previously (25, 41, 57). Briefly, 50 μl of the reaction buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM $MgCl_2$, and 2.5 μM of each GroEL was incubated with 1 mM ATP at 37°C for 20 min. Enzymatic reactions were terminated by the addition of 200 μl of an acidic solution of malachite green. The amount of inorganic phosphate liberated was measured at 655 nm using a Nanodrop ND-1000. Control reactions without ATP and GroEL were performed. A standard curve with monobasic potassium phosphate was generated concurrently with each experiment.

Prevention of the aggregation of citrate synthase by chaperonins. Pig heart citrate synthase aggregation was performed as reported previously (5). Briefly, 0.15 μg/ml citrate synthase was incubated at 43°C in the presence or absence of equimolar oligomer ratios of different GroEL variants in 40 mM HEPES–KOH buffer (pH 7.5). The ability of the said chaperones to prevent the aggregation of citrate synthase was monitored for 20 min on a Hitachi F-4000 spectrofluorimeter with emission and excitation wavelengths set at 465 nm and corresponding band passes set at 3.0 nm. The temperature of the sample was maintained with a Julabo circulating water bath and was monitored by using a Physitemp type T microcouple.

Chaperonin-assisted refolding of chemically denatured citrate synthase. The protocol for denaturing, refolding, and assaying the activity of pig heart mitochondrial citrate synthase was followed essentially as reported previously (41, 50). Briefly, citrate synthase at 15 μM was denatured in denaturation buffer containing 100 mM Tris-HCl (pH 8.0), 6 M guanidine-HCl, and 1 mM dithiothreitol for 30 min at 25°C. The denatured citrate synthase was diluted 100-fold into the refolding buffer containing 20 mM potassium phosphate buffer (pH 7.5), 10 mM $MgCl_2$, 2 mM ATP, 1 mM oxaloacetic acid, and 1 μM GroEL variant in the presence or absence of 2 μM *E. coli* GroES, and the activity of the recovered citrate synthase was assayed after 60 min. The enzyme activity was assayed as the consumption of acetyl-coenzyme A (CoA) revealed by the decrease in absorption at 233 nm for 90 s. Refolding reactions in the absence of the chaperonins and with the 1.5 nM native citrate synthase were set as controls.

Separation of multiple oligomeric forms of *M. tuberculosis* GroEL1 by gel filtration. Cell lysates were prepared from normally grown (at 37°C) and heat-shocked (at 42°C) *M. tuberculosis* cultures that were normalized for optical density. Two to 3 mg total protein of the cell lysates from both the cultures were resolved by gel filtration chromatography on a Superdex S200 16/60 (GE Biosciences, Inc.), and the resulting fractions were loaded on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and probed for the presence of *M. tuberculosis* GroEL1 by immunoblotting, using anti-$Cpn60.1_{Mtb}$ (rabbit polyclonal) antibody at a 1:10,000 dilution. Fractions bearing individual oligomeric forms of *M. tuberculosis* GroEL1, as determined by comparison to the molecular weight standards that were also resolved by gel filtration on Superdex S200 16/60, were pooled, and an equal proportion of each pool was resolved on 10% SDS–PAGE gels and probed by immunoblotting using anti-$Cpn60.1_{Mtb}$ for quantitative determination of the level of each oligomeric form.

Determination of phosphorylation by immunoblotting. Using antibodies specific to *M. tuberculosis* GroEL1 and GroEL2, these proteins were immunoprecipitated from *M. tuberculosis* cell lysates using standard protocols (3). Briefly, 50 to 100 μg total protein of cell lysates from the normally grown and heat-shocked cultures were mixed separately with antibodies anti-$Cpn60.1_{Mtb}$ and IT56 at equal titers and were incubated at 4°C overnight with constant mixing. Forty microliters of protein A-coupled Sepharose beads (GE Biosciences) equilibrated in buffer P (50 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40) was added to each tube and was incubated for 3 h with constant mixing. The protein A-coupled Sepharose beads, which were bound by the antibody-GroEL complex, were collected by centrifugation, and the pellet was washed three times in buffer P to remove the unbound cell proteins. Equal proportions of the protein-bound beads were resolved on 10% SDS–PAGE gels to estimate the levels of GroELs in the *M. tuberculosis* cell lysates. The immunoprecipitated samples were probed further by immunoblotting for the presence of phosphorylation using antibodies specific to phosphoseryl and phosphothreonyl residues at 1:1,000 dilutions. Once it was confirmed that the phosphorylation was on the seryl residue, equal amounts of individual oligomeric forms of GroEL1 were probed for the presence of phosphorylation using antiphosphoserine antibody as described above. To ensure equal loading, the transferred proteins were visualized by staining with Ponsue red, followed by immunoblotting with the antiphosphoseryl antibody.

Determination of the site of phosphorylation on *M. tuberculosis* GroEL1 by MS. Different oligomeric forms of recombinant *M. tuberculosis* GroEL1 were resolved on 10% SDS–PAGE gels, and the Coomassie-stained bands corresponding to GroEL1 were sliced out as polyacrylamide gel plugs. These were subjected to in-gel digestion using trypsin gold (Promega), followed by a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) study for peptide mass fingerprinting and protein sequencing using an Ultraflex MALDI-tandem TOF (TOF-TOF) instrument (Bruker Daltonics). The peptides which hosted a seryl residue were subjected to tandem MS (MS-MS) using an ion trap mass spectrometer (Agilent) to determine the presence of phosphorylation. The MALDI-TOF and MS-MS data were analyzed by using the MASCOT search engine or Spectrum Mill software. In these experiments, purified recombinant GroEL1 was included as a control for peptide identification. The MS studies were carried out in collaboration with The Centre for Genomic Applications (TCGA), New Delhi, India.

RESULTS

Mycobacterial GroELs do not complement the *groEL44* allele. Owing to their unusual behavior in vitro, we tested whether the two *M. tuberculosis groEL* ORFs could complement the loss of GroEL function in *E. coli*. This was tested in two *E. coli* strains, SV2, which harbors a temperature-sensitive *groEL44* allele, and LG6, in which the expression of the chromosomal *groESL* operon is under the control of the P_{lac} promoter. The expression of *M. tuberculosis groEL1* and *groEL2* from the P_{BAD} promoter in SV2 did not lead to rescue of its temperature-sensitive phenotype even when the *M. tuberculosis groELs* were coexpressed with their cognate *groES* (Fig. 1A). For these studies, a plasmid bearing *E. coli groESL* under the control of the P_{BAD} promoter, which complemented the temperature-sensitive phenotype of the *groEL44* allele, was also employed. Immunoblots with GroEL1- and GroEL2-specific antibodies established that the proteins were expressed and, hence, that the lack of complementation was not due to the lack of expression (Fig. 1B).

Likewise, *M. tuberculosis* GroELs could not support the growth of *E. coli* LG6 (Fig. 1C). The inability of *M. tuberculosis groEL*s to complement the loss of GroEL function in *E. coli* is consistent with the weakened in vitro chaperonin activity shown by our earlier studies of *M. tuberculosis* GroELs (41).

The apical domain of GroEL can tolerate considerable variation. In order to investigate the molecular features which lead to the differences in *E. coli* and *M. tuberculosis* GroELs, we generated a pool of *groEL* variants via DNA shuffling, starting with ORFs encoding *M. tuberculosis* GroEL1 and GroEL2 as template DNA. ORFs capable of encoding active versions of GroEL were selected in *E. coli* SV2. The mutants thus generated were chimeras of *M. tuberculosis groEL1* and *groEL2* and *E. coli groEL*. Sequence analysis of the clones and the comparison of G+C contents show that the gene-shuffled *groEL* variants are in fact derived from *M. tuberculosis groEL*s

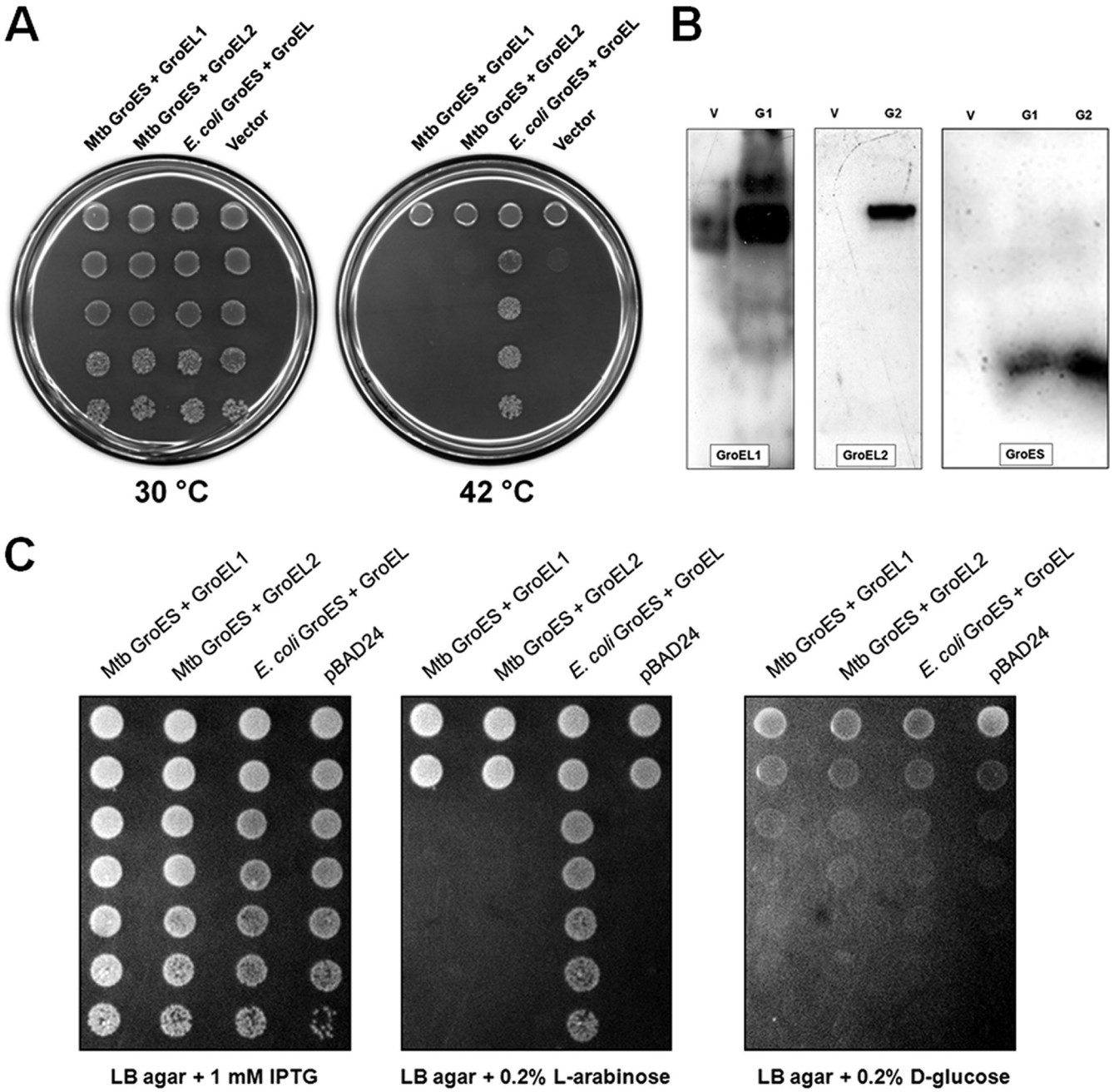

FIG. 1. Complementation of the GroEL function by *M. tuberculosis groELS* genes. (A) Serially diluted cultures of *E. coli* strain SV2 (*groEL44*) expressing the indicated *groELS* genes were spotted onto the surface of LB agar plates supplemented with 0.2% L-arabinose, and the plates were incubated at the indicated temperatures. (B) *E. coli* SV2 cultures harboring plasmids pSCM1602, pSCM1603, and pBAD24 were cultured in the presence of 0.2% L-arabinose. Cells were recovered at mid-log phase, and the cell lysates were probed with *M. tuberculosis* GroES-, GroEL1-, and GroEL2-specific antibodies. V, vector control (pBAD24); G1, GroES plus GroEL1 (pSCM1602); G2, GroES plus GroEL2 (pSCM1603). (C) Serially diluted cultures of *E. coli* strain LG6 expressing the indicated *groESL* genes were spotted onto the surface of LB agar plates supplemented as indicated, and the plates were incubated 30°C. Mtb, *M. tuberculosis*.

(Table 1). A quantitative phenotypic analysis of nine of the gene-shuffled *groEL* variants is shown in Fig. 2. Multiple sequence alignment of the polypeptides encoded by the said *groEL* alleles with the *E. coli* and *M. tuberculosis* GroEL sequences interestingly showed that their putative apical domains are subject to considerable sequence variation, with some variants bearing fairly large deletions and insertions, whereas their putative equatorial domains are conserved among the variants and are analogous to *E. coli* GroEL (Fig. 3; also see the supplemental material). This feature is most noticeable in the polypeptide encoded by the variant $groEL_{Sp24}$, which bears a deletion of about 90 amino acids in its putative apical domain but has an equatorial domain homologous to that of *E. coli* GroEL and is able to complement the temper-

TABLE 1. GC contents in the gene-shuffled *groEL* variants[a]

groEL variant	G+C content (%)
$groEL_{Sp22}$	68.1
$groEL_{Sp24}$	69.4
$groEL_{Sp25}$	67.8
$groEL_{Sp26}$	68.1
$groEL_{Sp27}$	67.5
$groEL_{Sp32}$	68
$groEL_{Sp35}$	69.3
$groEL_{Sp36}$	67.9
$groEL_{Sp37}$	67.9
E. coli groEL	52.9
M. tuberculosis groEL1	65.1
M. tuberculosis groEL2	65.4

[a] Gene-shuffled *groEL* variants are compared with *E. coli* and *M. tuberculosis groEL*s for their G+C contents.

ature-sensitive phenotype of the *groEL44* allele (see the supplemental material). These studies lend additional support to the supposition that the ability to oligomerize, due to the presence of an "*E. coli* GroEL-like" equatorial domain, correlates with biologically relevant GroEL activity and suggest that the apical domain can tolerate substantial variation. However, variants $GroEL_{Sp22}$ and $GroEL_{Sp25}$ displayed weak complementation, despite possessing homologous equatorial domains. A future comprehensive study including site-directed mutagenesis might be able to examine such behavior.

***M. tuberculosis* GroEL1 regains chaperonin function by equatorial domain substitution.** Having obtained evidence that the selection pressure on the equatorial domain is relatively more stringent than that on the apical domain but that, nonetheless, the equatorial domains of *M. tuberculosis* GroEL orthologs have diverged to a large extent from that of *E. coli* GroEL, we studied the effect of mutual exchange of equatorial domains between *M. tuberculosis* GroEL1 and *E. coli* GroEL. For these studies, two ORFs, $groEL_{MEF}$ and $groEL_{MER}$, were generated and placed under the expression control of the P_{BAD} promoter (see Materials and Methods and Fig. 4A). Complementation studies with $groEL_{MEF}$ and $groEL_{MER}$ showed that the exchange of the equatorial domain in *M. tuberculosis* GroEL1 with that of *E. coli* GroEL, specified by the $groEL_{MEF}$ ORF, turns it into a functional GroEL in vivo. $groEL_{MEF}$ was able to complement the *groEL44* allele at the restrictive temperature of 42°C to an extent similar to that exhibited by *E. coli groEL*, whereas at 45°C, $groEL_{MEF}$ displayed weak complementation. In contrast, $groEL_{MER}$, in which the DNA encoding the equatorial domain of *E. coli* GroEL is replaced with that encoding the corresponding domain from *M. tuberculosis* GroEL1, was able to exhibit only very weak complementation at 42°C (Fig. 4B). Curing the plasmids from these transformants led to the loss of complementation, confirming that the phenotype was indeed vector borne (data not shown).

In *E. coli*, the development of bacteriophages like lambda (λ) and T4 requires a functional GroEL/S system (20). To gauge the extent of restoration of GroEL function present in $GroEL_{MEF}$, its ability to support phage morphogenesis was studied. The expression of $groEL_{MEF}$, like that of *E. coli groEL*, was able to support the growth of both phage λ and T4 in the *groEL44* mutant strain SV2, whereas the expression of *M. tuberculosis groEL1*, *groEL2*, and $groEL_{MER}$ did not promote phage development (Fig. 4C). These studies thus lend credence to the notion that the reason for the observed functional difference between the *E. coli* and *M. tuberculosis* GroELs must be a consequence of differences between the equatorial domains of the two molecules.

To avoid potential interference of the GroEL polypeptide encoded by the resident *groEL44* allele in the complementation exhibited by $groEL_{MEF}$, we studied the effect of $groEL_{MEF}$ expression under conditions of depletion of endogenous GroEL. Toward this end, plasmids bearing *tac* promoter (P_{tac})-driven expression of $groEL_{MEF}$ and one in which $groEL_{MEF}$ was cloned in an operonic arrangement with *E. coli groES* were generated. Similar plasmids capable of expressing *E. coli groES* and *E. coli groEL* individually and the *E. coli groESL* operon were included as controls. The said plasmids were transformed into strain MGM100, which does not form colonies in the absence of arabinose unless a functional copy of *groES-groEL* is expressed (See Materials and Methods). Then, the ability of $groEL_{MEF}$ to support the growth of MGM100 in the absence of exogenous L-arabinose supplementation was assessed. Of the various plasmids tested, only plasmids coexpressing *E. coli groES* with either $groEL_{MEF}$ or *E. coli groEL* allowed colony formation in the absence of L-arabinose (Fig. 5). These results

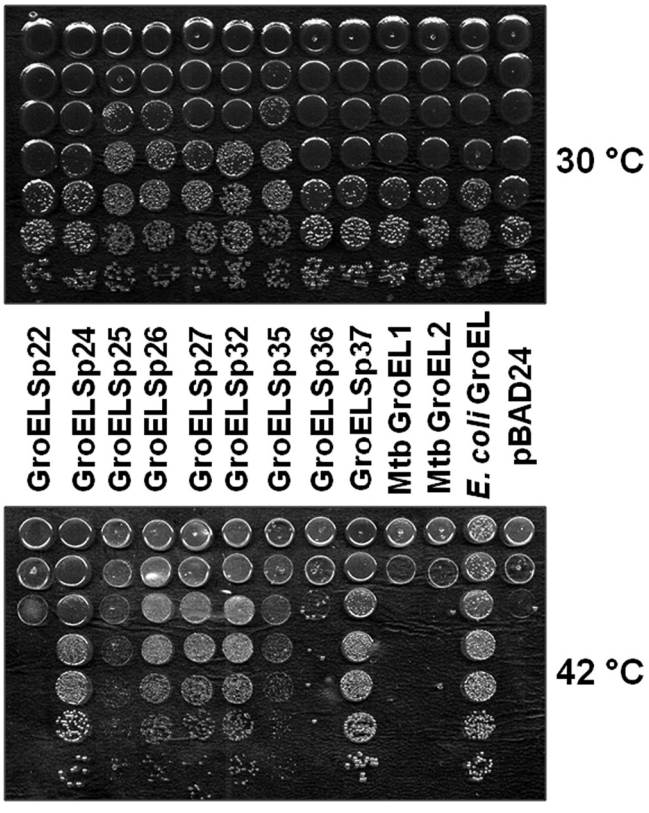

FIG. 2. Phenotypes of the gene-shuffled *groEL* mutants. Rescue of the temperature-sensitive phenotype associated with the *groEL44* allele by various *groEL* ORFs obtained by gene shuffling. Cultures of SV2 expressing the indicated *groEL* genes were serially 10-fold diluted, spotted onto the surface of LB plates supplemented with 0.2% L-arabinose, and incubated at the indicated temperatures. pBAD24 was the vector control. Mtb, *M. tuberculosis*.

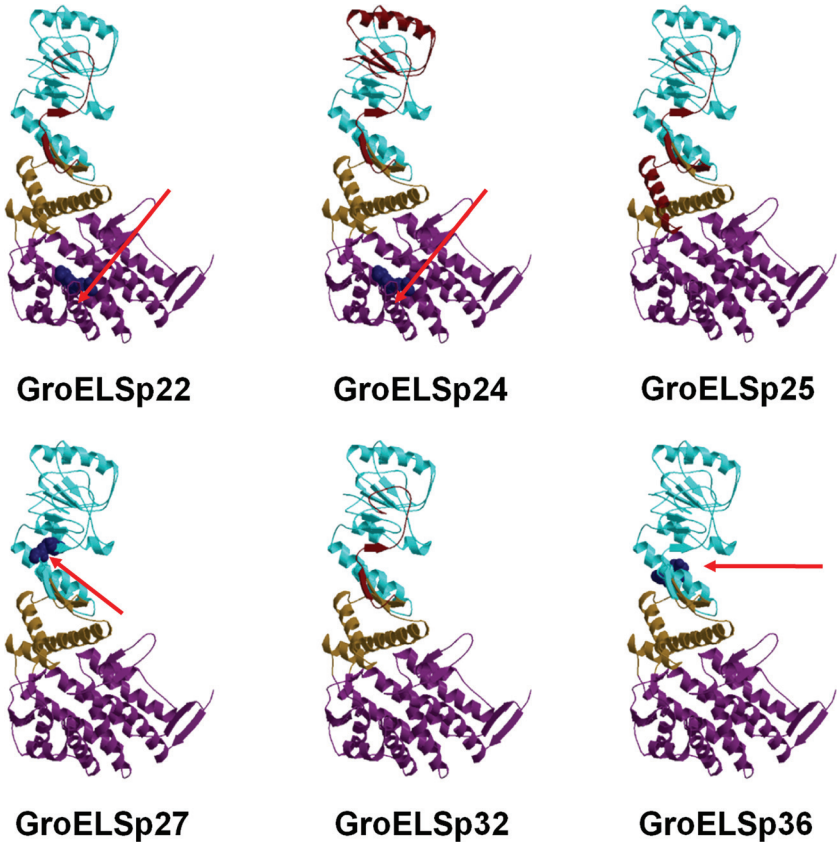

FIG. 3. Locations of variations observed in the gene-shuffled GroEL variants. GroEL monomer ribbon diagrams illustrate the sites of insertions and deletions observed in the indicated gene-shuffled GroEL variants. Variations observed in the GroEL variants are color coded. Regions marked in blue represent insertions, and those in maroon represent deletions. Insertions are also indicated with an arrow for easy distinction. GroEL domains are also shown in three different colors to highlight the occurrence of the mutations in different domains. Cyan and violet, respectively, represent the apical and equatorial domains, while brown represents the intermediate domain.

clearly show that $GroEL_{MEF}$ can substitute for *E. coli* GroEL in vivo and requires *E. coli* GroES for its activity, which is not influenced by the resident GroEL of strain SV2.

The equatorial domain of $GroEL_{MEF}$ is responsible for it attaining a higher oligomeric state. Having shown that $GroEL_{MEF}$ is functional in vivo, we wished to test whether the ability of $GroEL_{MEF}$ to complement the defect in the *groEL44* allele is a consequence of it existing in higher-order oligomeric form and thus being able to form a cavity for encapsulation, features that are lacking in the parental *M. tuberculosis* GroEL1 (40). Since the polypeptides encoded by the gene-shuffled variants of *groEL*, $groEL_{Sp24}$, and $groEL_{Sp32}$ bear considerable variation (Fig. 3; also see Fig. S1 in the supplemental material), similar attributes of the encoded polypeptides were studied. Gel filtration studies with Sephacryl S300 showed that $GroEL_{MEF}$ was capable of existing in a higher oligomeric state, similar to that seen for *E. coli* GroEL, whereas $GroEL_{MER}$ displayed a lower oligomeric character (Fig. 6), a property reminiscent of that seen for *M. tuberculosis* GroEL1 (59). One noticeable aspect of the oligomeric properties of $GroEL_{MEF}$ is that it existed in equilibrium between higher and lower oligomeric states, with the higher oligomeric state being the predominant species, which presumably explains its somewhat weakened ability to substitute for *E. coli* GroEL in vivo at 45°C (Fig. 4B). Since the higher oligomeric state of $GroEL_{MEF}$ displays gel filtration characteristics similar to those of *E. coli* GroEL, it is reasonable to presume that the said state corresponds to a tetradecameric assembly. We have also examined the oligomeric states of $GroEL_{Sp24}$ and $GroEL_{Sp32}$, variants that bear deletions in the putative intermediate apical domain boundary. Both the variants displayed tendencies to exist in a higher oligomeric state, and their gel filtration profiles were consistent with their predicted molecular weights.

The biochemical properties of purified GroEL variants correlate with their in vivo activities. GroEL-assisted folding of substrate proteins typically involves (i) binding of the polypeptides by virtue of exposed hydrophobic interactions; (ii) sequestration of polypeptides into the cavity, thereby preventing irreversible aggregation; (iii) ATP hydrolysis; and (iv) GroES-dependent refolding of substrate polypeptides. Since the gel filtration results revealed that $GroEL_{MEF}$, $GroEL_{Sp24}$, and $GroEL_{Sp32}$ are oligomeric, we studied their biochemical features in hydrolyzing ATP and refolding the model substrate, citrate synthase. The ability of $GroEL_{MEF}$ to exist in a higher oligomeric state correlated with its ability, in comparison to that displayed by $GroEL_{MER}$, to refold chemically denatured substrate protein (Fig. 7A and B). Similarly, $GroEL_{Sp24}$ and

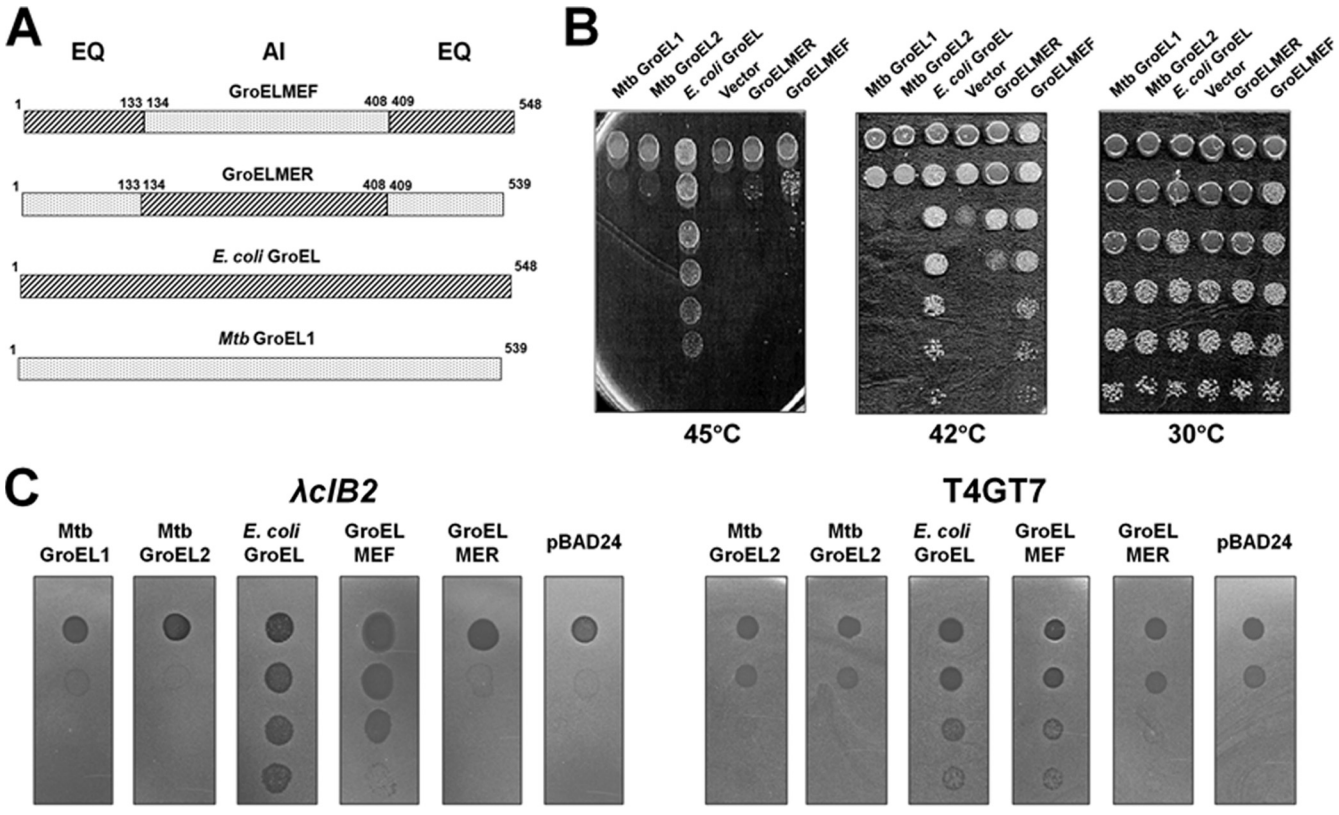

FIG. 4. Results of domain-swapping experiments. (A) Schematic representation of domain allocation in GroEL variants $GroEL_{MEF}$ and $GroEL_{MER}$. Numbering denotes amino acid residue positions of the parental *M. tuberculosis* GroEL1 and *E. coli* GroEL polypeptides. Equatorial domain regions are indicated as EQ, and those spanning apical and intermediate domains as AI. (B) Complementation of the *groEL44* allele by *M. tuberculosis groEL* genes and their derivatives. Serially 10-fold-diluted cultures of *E. coli* strain SV2 (*groEL44*) expressing the indicated *groEL* genes were spotted onto the surface of LB agar plates supplemented with 0.2% L-arabinose, and the plates were incubated at the indicated temperatures. (C) Assay for bacteriophage morphogenesis. Lawns of *E. coli* strain SV2 expressing the indicated *groEL* genes were prepared on LB agar plates supplemented with 0.2% L-arabinose. Serial 100-fold dilutions of bacteriophages λ*c*IB2 and T4GT7 were spotted onto the lawns, followed by incubation at 30°C. Mtb, *M. tuberculosis*.

$GroEL_{Sp32}$, with their ability to oligomerize, exhibited canonical chaperonin properties (Fig. 7A and B).

The well-known chaperonin properties of GroEL, as mentioned above in (i) and (ii), require two important properties, i.e., binding to exposed hydrophobic surfaces of substrate polypeptides and encapsulation in the cavity to promote folding. The former is typically studied by measuring the prevention of aggregation of substrate polypeptides, while the latter can be studied by monitoring the recovery of folded substrate proteins. Remarkably, $GroEL_{MEF}$ was poor at preventing aggregation of the substrate protein, citrate synthase, at elevated temperatures for more than 5 min. On the other hand, $GroEL_{MER}$ prevented citrate synthase aggregation for at least 20 min (Fig. 7C). Thus, $GroEL_{MEF}$, despite not being able to efficiently prevent substrate aggregation, is able to fold substrates due to its competence in encapsulation. On the other hand, $GroEL_{MER}$, despite being able to prevent aggregation, is not able to promote refolding of substrates due to its inability to encapsulate. Similarly, $GroEL_{Sp32}$ was able to prevent the aggregation of citrate synthase, whereas $GroEL_{Sp24}$ was not (Fig. 7C). The results for $GroEL_{MER}$ suggest that lower oligomeric GroELs possess substrate binding activity but are inefficient in promoting refolding.

Mycobacterial GroEL1 exists in multiple oligomeric forms. Having established that the impaired oligomerization is the basis for the inactivity of recombinant mycobacterial GroELs, we tested the nature of the oligomerization of *M. tuberculosis* GroELs in their natural settings. For these studies, we considered three aspects concerning the biology of *M. tuberculosis* and GroEL. First, multiple sequence alignment of bacterial GroEL homologues showed that several positions in the *M. tuberculosis* GroELs' equatorial domains were altered from different residues to serine/threonine, indicating potential phosphorylation sites (40, 41). Second, the *M. tuberculosis* genome encodes 11 eukaryotic-like serine/threonine kinases (ELKs), and emerging evidence that ELKs play a role in different cellular processes, including stress adaptation, across bacterial genera tempted us to speculate that there is a role for ELKs in GroEL oligomerization (38, 61, 65). Third, some heat shock proteins, including mitochondrial and chloroplast Hsp60 homologues, exhibit heat-induced or concentration-dependent oligomerization. The mitochondrial Hsp60 homologue, although predominantly found as a single ring entity, is known to display a concentration-dependent tetradecamer formation (14, 34). Hence, we attempted to explore whether *M. tuberculosis* GroEL1 exhibits regulated oligomerization and, if so, to

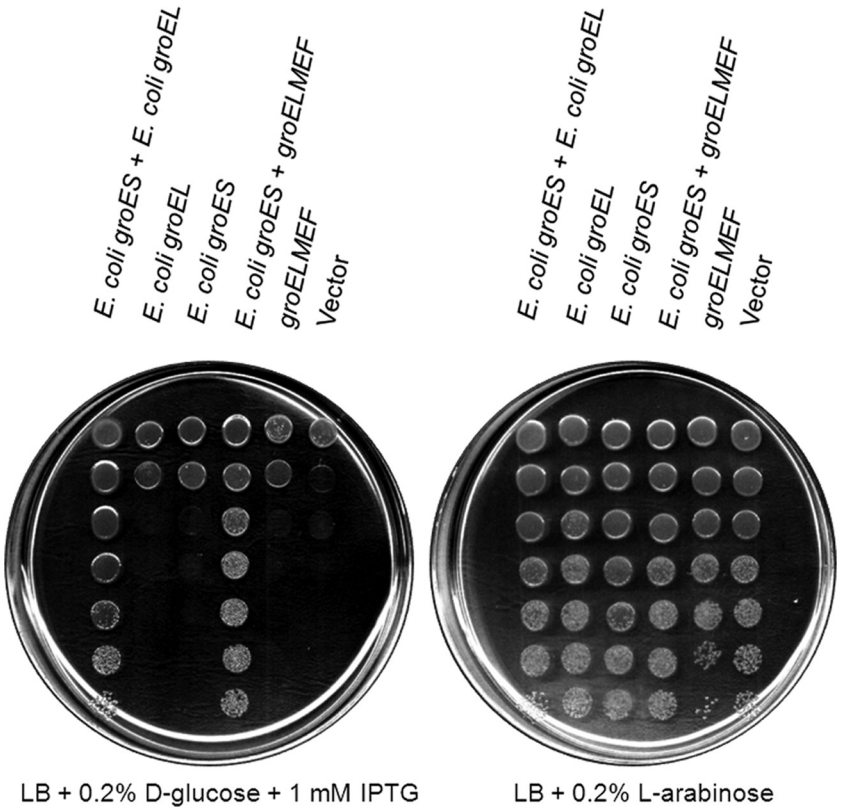

FIG. 5. In vivo activity of $GroEL_{MEF}$ in a GroEL depletion strain. Cultures of MGM100 expressing *E. coli groES*, *groEL*, $groEL_{MEF}$, and combinations as indicated were serially diluted, spotted onto the surface of LB agar plates, and incubated at 30°C under restrictive (left) and permissive (right) conditions.

determine the source of its regulation. The resolution of cell lysates on native PAGE gels, followed by probing with *M. tuberculosis* GroEL1-specific antibody, revealed the existence of *M. tuberculosis* GroEL1 in four different oligomeric forms, i.e., monomeric, dimeric, heptameric (single ring), and tetradecameric (double ring) forms (data not shown).

In order to separate these forms, *M. tuberculosis* cell lysates were resolved by molecular exclusion chromatography and an equal proportion of each fraction was probed with GroEL1-specific antibody to quantitatively determine the presence of each oligomeric form at 37°C and under heat shock conditions. It was observed that the tetradecameric form existed at both 37°C and 42°C. The presence of the tetradecameric form in cultures grown at 37°C indicates that the switch between heptameric and tetradecameric forms is not solely temperature mediated. The dimeric form was also detected under both conditions, and the monomeric form, although present, is at extremely low levels compared to the levels of the other, higher-order forms.

Tetradecameric GroEL1 is phosphorylated on serine 393. Our earlier comparative analysis with *E. coli* GroEL had indicated that at different positions, there are systematic mutations of glutamates to serines or threonines in the sequence of *M. tuberculosis* GroEL1 (41). This suggested a possibility that the serine or threonine residues are the potential sites of phosphorylation. Therefore, investigating the presence and nature of phosphorylation was attempted by immunoprecipitation of GroEL1 and GroEL2 from *M. tuberculosis* cell lysates, followed by immunoblotting with antiphosphoseryl and antiphosphothreonyl antibodies. These immunochemical analyses revealed that GroEL1 is phosphorylated on serine residues but not on any threonine residue (Fig. 8B). Having established that GroEL undergoes phosphorylation, we further investigated the phosphorylation status of the various oligomeric forms of GroEL1. Strikingly, we observed that only the tetradecameric form was phosphorylated; the heptameric and dimeric forms were not phosphorylated (Fig. 8C). The band in the lane corresponding to the heptameric form, as shown in Fig. 8C, was seen to be migrating faster than *M. tuberculosis* GroEL1 when compared with the Ponsue red-stained bands on nitrocellulose membranes. Furthermore, MS analyses of this band revealed it to be the 53.5-kDa glutamine synthetase A1 of *M. tuberculosis*. These observations lead us to believe that a phosphorylation switch might be mediating the conversion from the heptameric single-ring form to the tetradecameric double-ring form.

MS analysis of *M. tuberculosis* GroEL1 by MALDI-TOF followed by MS-MS fragmentation of the resulting peptides confirmed that serine-393 is phosphorylated in the tetradecameric form (Fig. 9A). However, we do not rule out the possibility that a few more serines could be phosphorylated, and if so, these might be located in the critical positions governing the inter-ring contacts. Our results thus reveal that there exist multiple oligomeric forms of GroEL1 molecules in *M. tuberculosis* and that the conversion from the heptameric to the

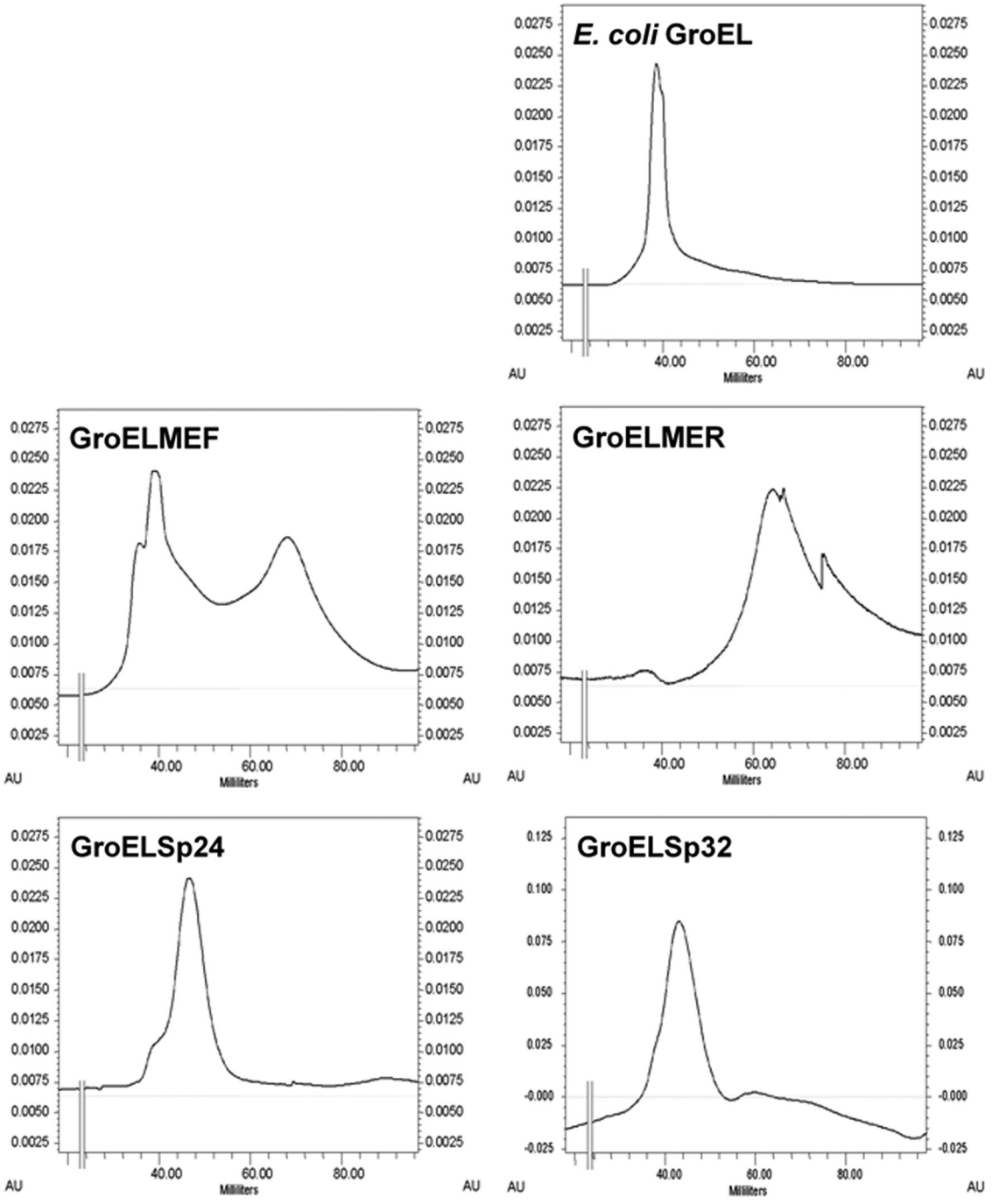

FIG. 6. Oligomeric states of GroEL variants. Gel permeation chromatograms of *E. coli* GroEL, $GroEL_{MEF}$, $GroEL_{MER}$, $GroEL_{Sp24}$, and $GroEL_{Sp32}$. The indicated proteins were separated on Sephacryl S300 16/60 (GE Biosciences) in a Biologic DuoFlow fast-performance liquid chromatography system (Bio-Rad). AU, absorbance units.

tetradecameric form is mediated by phosphorylation of Ser residues.

DISCUSSION

The critical features of the chaperonin function reside in the ATP-driven cycles of binding, encapsulation, and controlled release of substrate polypeptides that lead to productive folding (43, 45, 48). Detailed studies have revealed mechanistic and physiological characteristics of the isologous ring form of the GroEL/S machine (11, 42, 45, 53, 54). According to the current understanding, the following properties of GroEL are significant in controlling its activity: (i) oligomerization mediated by the equatorial domain, resulting in the formation of the folding chamber and the encapsulation of substrate polypeptides (48, 59); (ii) recognition of substrate polypeptides medi-

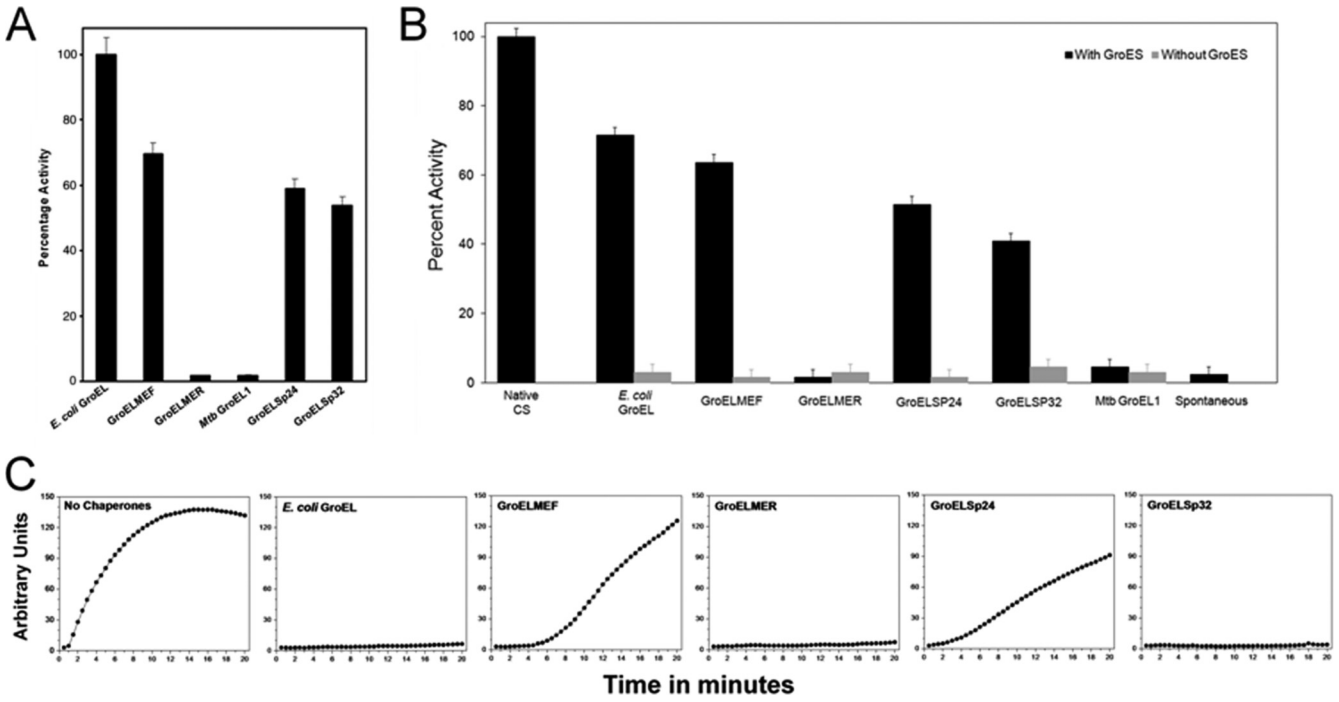

FIG. 7. Chaperonin assays of GroEL variants. (A) ATPase activities of purified GroEL variants, as indicated, were assayed by malachite green calorimetric assay. The amount of inorganic phosphate released was quantified at 655 nm. The mean for individual data sets was calculated and plotted along with the standard deviation, considering the activity of *E. coli* GroEL as 100%. (B) Effect of chaperonins in refolding of chemically denatured citrate synthase (CS). Citrate synthase was chemically denatured and then refolded with the indicated GroEL variants in the presence or absence of *E. coli* GroES. The activity of the refolded enzyme after 60 min of refolding at 25°C was measured as the decrease in absorbance at 233 nm due to the consumption of acetyl-CoA. The yield of refolded enzyme is expressed as the percentage of the activity determined for an equal quantity of nondenatured native citrate synthase. (C) Prevention of aggregation of citrate synthase by the chaperone variants as a function of time. The aggregation of citrate synthase at 43°C in the absence and presence of equimolar ratios of the indicated GroEL variants was measured as a function of light scattered at 465 nm for 20 min. Mtb, *M. tuberculosis*.

ated by the apical domain (6, 49); (iii) conformational changes between the said two domains driven by ATP and GroES binding/release (32, 63, 64); and (iv) ATP hydrolysis (19, 55). Impairment of any of these properties significantly alters the functioning of GroEL (37, 40).

Some prokaryotic species bear multiple genes encoding GroEL, one usually cotranscribed with the cognate *groES* orthologue (2, 18, 30). Since sequence conservation among GroELs from different species is an indication that the mechanism of GroEL is universally conserved, paralogous copies of GroEL in these organisms might provide redundancy of chaperonin function. However, biochemical and biophysical characterization of both of the recombinant *M. tuberculosis* GroELs showed that they failed to oligomerize in vitro despite possessing a high sequence homology with *E. coli* GroEL (40), suggesting a stringent requirement of the host environment for oligomerization. Recent studies have shown that in *Mycobacterium smegmatis*, GroEL1, a paralog of *M. tuberculosis* GroEL1, is involved in biofilm formation, whereas GroEL2 is thought to provide housekeeping chaperonin function (39). The observation that GroEL1 can physically associate with KasA, a component of the mycolic acid synthesis pathway, is thought to support the notion that GroEL1 may play the role of chaperonin in the process of biofilm formation. Furthermore, *M. tuberculosis* GroEL1 is also known to induce host inflammatory responses (28).

Since oligomerization is compromised in recombinant *M. tuberculosis* GroELs, studying the mutations accumulated in positions responsible for intersubunit interactions seems reasonable. Sequence analysis of the shuffled-gene products suggested that the apical domain is capable of absorbing considerable variation in its amino acid sequence. The large deletions observed in the shuffled-gene products occur mostly in the apical domain (Fig. 3). The tolerance of variations by the apical domain can be reconciled with the observation that GroEL can recognize a wide repertoire of substrates with almost no sequence specificity. Thus, the said domain may bear a latent plasticity. The domain-swapping experiments, by generating *E. coli* and *M. tuberculosis* GroEL hybrids, showed that the lack of in vitro activity of *M. tuberculosis* GroELs was indeed due to the loss of oligomerization (41) (Fig. 6) and that the loss of oligomerization can be exclusively attributed to the equatorial domain of *M. tuberculosis* GroEL, which when replaced with that from *E. coli* GroEL, might circumvent the requirement of its native environment for oligomerization.

Interestingly, the results of the ATPase activity assays and refolding studies with chemically denatured citrate synthase are in concurrence with those of the in vivo studies showing that $GroEL_{MEF}$ is able to exhibit characteristics of a chaperonin (Fig. 7A and B). However, $GroEL_{MEF}$ is poor at protecting the substrate from aggregation beyond 5 min, whereas $GroEL_{MER}$ acts similarly to *E. coli* GroEL in protecting the

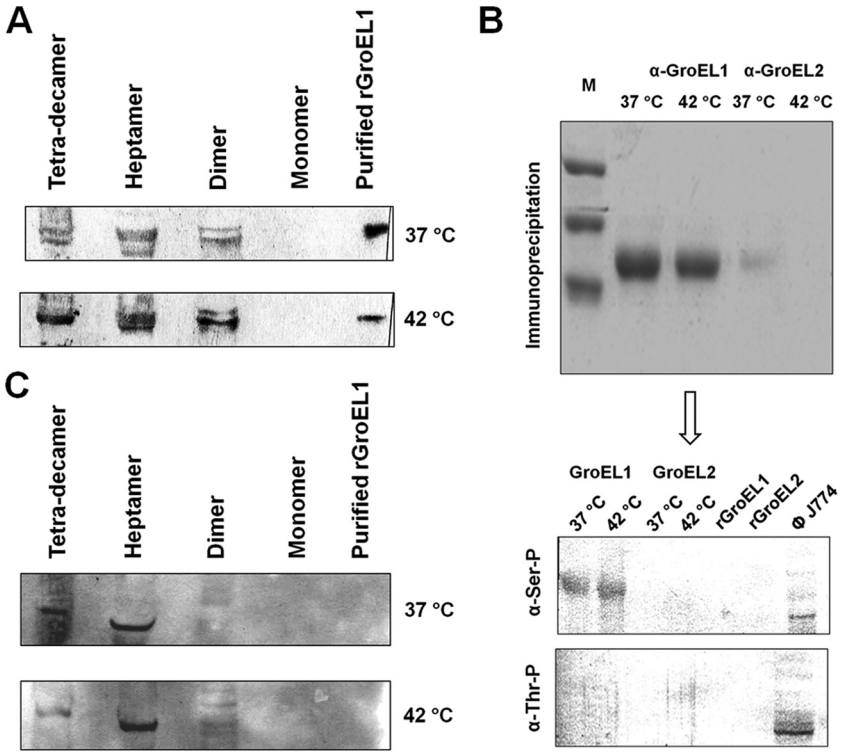

FIG. 8. Multiple oligomeric forms of *M. tuberculosis* GroEL1. (A) *M. tuberculosis* cell lysates from cells grown at 37°C and 42°C were resolved by gel filtration. Equal proportions of the indicated oligomeric forms of GroEL1 from cultures grown at the indicated temperatures were probed with anti-GroEL1-specific antibody. (B) GroEL1 and GroEL2 were immunoprecipitated from the *M. tuberculosis* cell lysates described for panel A. Equal fractions of the precipitated proteins were resolved on 10% SDS–PAGE gels, followed by probing for phosphorylation using the indicated antibodies. Cell lysates of human macrophage cell line J774 and recombinant *M. tuberculosis* GroELs (rGroEL) purified from *E. coli* were employed as positive and negative controls, respectively. α, anti; P, phospho. (C) Different oligomeric forms of *M. tuberculosis* GroEL1, as described for panel A, were resolved on 10% SDS–PAGE gels and were probed for the presence of phosphorylation using phosphoserine-specific antibody. MS analysis of the protein corresponding to the band in the heptametrical fraction, which migrated faster than GroEL1, revealed it to be glutamine synthatase A1 (GlnA1, Rv2220) of *M. tuberculosis*.

substrate from aggregation for at least 15 min (Fig. 7C). Furthermore, $GroEL_{Sp24}$ displays characteristics similar to those of *E. coli* GroEL. These observations therefore suggest that the variations in apical domain can, to some extent, be absorbed without impairing chaperonin function as long as the molecule retains its ability to encapsulate the substrate proteins. It is not surprising to find that $GroEL_{MER}$ possesses substrate binding capacity, since it bears the apical domain of *E. coli*, yet is unable to refold substrate polypeptides due to its inability to form tetradecamers. It might be possible that $GroEL_{MER}$, despite its inability to oligomerize, exhibits very weak complementation in vivo due to this property (Fig. 4B). Furthermore, the behavior of $GroEL_{Sp32}$ can be rationalized on the basis that the overt variation in $GroEL_{Sp32}$, a segmental deletion in its putative apical domain, could be neutral, leading to substantial polypeptide binding, but could contribute to its weakened chaperonin activity. These results therefore confirm that mere substrate recognition is not sufficient for the chaperonin function. On the contrary, oligomerization is an important attribute of GroEL.

With the establishment of the fact that oligomerization is the principal attribute of GroEL function and based on our earlier bioinformatic analysis of *M. tuberculosis groEL* genes, which showed that these genes have not accumulated mutations so as to become pseudogenes during the process of evolution, a need for GroEL's function in vivo in *M. tuberculosis* is indicated (21). We therefore attempted to explore the oligomeric status of the *M. tuberculosis* GroELs under native conditions. Surprisingly, *M. tuberculosis* GroEL1 exhibited multiple oligomeric forms, which is the first-ever observation of this for a bacterial chaperone. GroEL homologues from mammalian mitochondria and from chloroplasts have been shown to exhibit multiple oligomeric forms where the conversion from single-ring form to double-ring form is concentration and GroES dependent (61, 65). Attempts to make a single-ring version of *E. coli* GroEL by site-directed mutagenesis of four critical residues at the inter-ring surface had yielded a mutant, SR1, that was compromised in GroES release (62). However, the conversion of a temperature-sensitive GroEL mutant, GroEL44, into single-ring form, SR44, resulted in an active chaperonin (8). Studies of the assembly of bacterial chaperonin were attempted with purified *E. coli* GroEL involving cofactors and denaturing agents, such as Mg^{2+}, GroES, urea, and guanidium chloride, which showed the existence of bacterial chaperonin in multiple oligomeric forms under various denaturing conditions tested (33). In addition, several small heat shock proteins and Hsp90 were shown to exhibit temperature-regulated oligomerization. Hence, we set out to explore the possi-

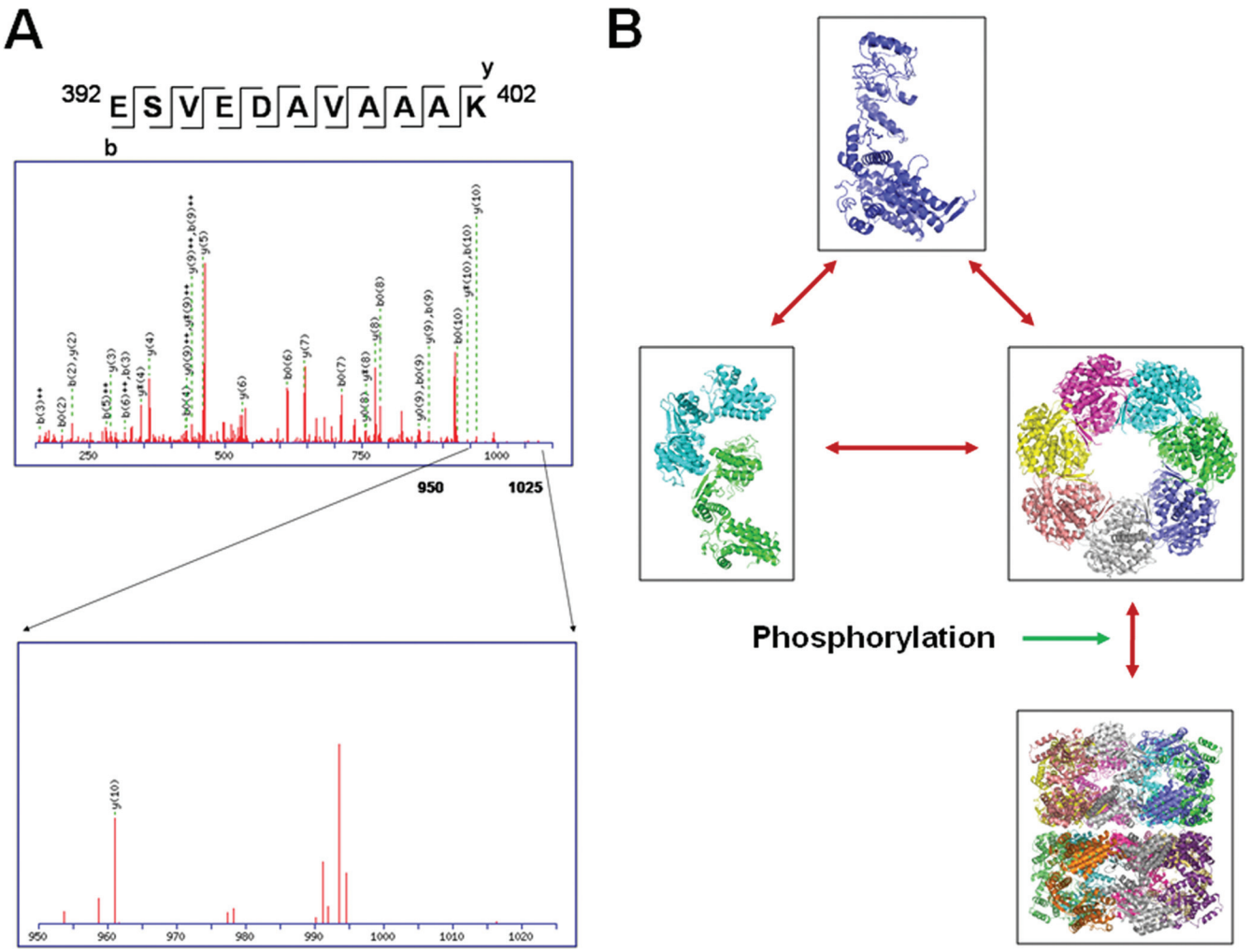

FIG. 9. Detection of phosphorylation on *M. tuberculosis* GroEL1. (A) MALDI–TOF-TOF MS-MS fragmentation analysis of the tryptic peptide ESVEDAVAAAK ($[MH]^+$ *m/z* 1088.912724) corresponding to amino acid residues 392 to 402 of *M. tuberculosis* GroEL1. Labeled peaks represent fragmented ions [y(0) to y(10) and b(0) to b(10)] corresponding to the indicated peptide. Phosphorylation on the peptide is confirmed by a mass shift of 80 Da that is due to the loss of HPO_3^-. (B) Model for the regulation of oligomerization in *M. tuberculosis* GroEL1 being mediated by phosphorylation. See the text for details.

bility of regulated oligomerization of *M. tuberculosis* GroEL1 and the source of regulation, if any. Immunochemical studies, followed by MS analysis of *M. tuberculosis* GroEL1 and GroEL2 from the *M. tuberculosis* cell lysates, confirmed that GroEL1 is phosphorylated on serine-393.

In summary, this work shows the requirement of oligomerization for the function of GroEL. Although *M. tuberculosis* GroEL1 is capable of oligomerization in its native environment, it cannot do the same in *E. coli*, but the exchange of their equatorial domains renders *M. tuberculosis* GroEL active, suggesting specific oligomerization capability of *M. tuberculosis* GroEL's equatorial domain only in the *M. tuberculosis* cellular milieu. Furthermore, *E. coli* GroEL can be rendered nonfunctional and indistinguishable from *M. tuberculosis* GroEL1 by replacing its equatorial domain with the one present in GroEL1. Since $GroEL_{MEF}$ can substitute for the lack of GroEL function in *E. coli*, it suggests that despite the heterologous apical domain of *M. tuberculosis* GroEL1 borne on $GroEL_{MEF}$, the chimeric protein (and therefore *M. tuberculosis* GroEL1) may recognize the same cellular substrates in vivo as does its *E. coli* counterpart. Despite the large functional difference between *M. tuberculosis* GroEL1 and *E. coli* GroEL, a noteworthy aspect is that GroEL1 retains the highly conserved ATP binding pocket that bears amino acid residues identical to those in *E. coli* GroEL. This tempts us to speculate that oligomerization may be a prerequisite for GroEL ATPase activity and, therefore, the ability to assist the refolding of substrate polypeptides. These studies therefore reveal that the basis of the reduced activity of recombinant *M. tuberculosis* GroEL1 in *E. coli* was its impaired oligomerization. We also show, for the first time, that mycobacterial GroEL1 exists in different oligomeric forms and that the switch between the single-ring (heptamer) and double-ring (tetradecameric) GroEL forms is mediated by phosphorylation. Thus, we propose that the naturally synthesized GroEL exists in equilibrium between a dimer and a heptamer and that heptamer-to-tetradecamer conversion is mediated by phosphorylation (Fig. 9B). Since a similar strategy of chaperonin oligomerization operates in mitochondrial

GroEL, this study, in addition to probing *M. tuberculosis* GroEL biology, might also shed light on the evolution of mitochondria and chloroplasts, which are thought to be the bacterial symbionts of the eukaryotes (1).

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Author contributions. C.M.S.K. conducted all the molecular biological, biochemical and genetic experiments. C.M.S.K. and A.A.S. designed the genetic experiments with *E. coli*, and C.M.S.K. and C.V.S. performed the gene-shuffling studies. C.M.S.K., G.K., A.K.T., and S.C.M. designed the experiments with *M. tuberculosis*, and C.M.S.K. and G.K. conducted these. Overall supervision and design was by S.C.M., A.K.T., and A.A.S.

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