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Two conserved amino acids of juxtaposed domains of a ribosomal maturation protein CgtA sustain its optimal GTPase activity

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ABSTRACT

CgtA is a highly conserved ribosome binding protein involved in ribosome biogenesis and associated with stringent response. It is a 55 KDa GTPase protein consisting of GTPase, Obg and C-terminal domains. The function of the latter two domains was not clear and despite the importance, the mode of action of CgtA is still largely unknown. Knocking out of CgtA gene is lethal and mutations lead to growth, sporulation and developmental defects in bacteria. It was found that a growth defect and pinhole size colony morphology of *Bacillus subtilis* was associated with a Gly92Asp point mutation on the Obg domain of its CgtA protein, instead of its GTPase domain. CgtA is an important and essential protein of the deadly diarrhea causing bacteria *Vibrio cholerae* and in order to investigate the mode of action of the *V. cholerae* CgtA we have utilized this information. We measured the GTPase activity of *V. cholerae* CgtA ($CgtA_{vc}$) protein in the presence of purified ribosome. Our results showed 5-fold increased GTP hydrolysis activity compared to its intrinsic activity. Then we explored the GTPase activity of the mutated $CgtA_{vc}$ (Gly98Asp) located at the Obg domain, which reduced the GTP hydrolysis rate to half. The double point mutations (Gly98Asp, and Tyr194Gly) encompassing another conserved residue, Tyr194, located at the diagonally opposite position in the GTPase domain largely restored (about 82%) the reduced GTPase activity, revealing a fine-tuned inter-domain movement readily associated with the GTPase activity of CgtA and thus maintaining the proper functioning of the CgtA protein.

1. Introduction

GTPase proteins belong to the larger family of hydrolase enzymes, which can bind and hydrolyze GTP and undergo conformational changes upon hydrolysis of GTP to GDP. GTPases act as key proteins in many critical biological processes playing crucial roles in regulating cellular processes in both prokaryotes and eukaryotes [1]. GTPase proteins were divided into many classes and families [1] that included CgtA (Caulobactor GTPase A) [2], an essential and highly conserved protein belong to Obg GTPase family [3]. Studies were done in various bacterial species to understand the functions of CgtA. Eukaryotic homologs of this protein were also studied in yeast [4], mitochondria (*Homo sapiens*) [5], and chloroplast [6]. It was observed that knocking out of *cgtA* was lethal to prokaryotic organisms and mutations had affected many cellular processes in prokaryotes like, ribosome maturation [7], initiation of sporulation, DNA replication, chromosome partitioning [8–10], DNA repair [11], and stress response [12–14]. In eukaryotes, mutations in homologs of CgtA led to improper mitochondrial and chloroplast ribosome maturation [15], and disorganized nucleolar architecture [5]. However, it was still not clear that how CgtA was involved in such a wide variety of cellular processes.

Although $CgtA_{vc}$ has an intrinsic GTPase activity (this study, [16]), it is not clear whether it has any dedicated external GTPase activator. We reported previously that the GTPase activity of $CgtA_{vc}$ increased upon interaction with 50S ribosome [16], and similar phenomenon has been reported by a recent study in *Escherichia coli* [17], who also reported the localization of CgtA in the inter-subunit side of the 50S in a *E. coli* 50S·CgtA cryo-EM structure. It was known by affinity blot experiments that L13 ribosomal protein interacted with CgtA protein in *Bacillus subtilis* [18]. L13 protein is present in the exterior portion (solvent side) of the 50S ribosome. Therefore, binding of CgtA to the ribosomal inter-subunit space alone, could not explain, how it would interact with the L13 protein and thus the

Abbreviations: CgtA, Caulobactor GTPase A or common GTPase A; Obg, *spoOB* associated GTP binding protein; $CgtA_{vc}$, CgtA of *Vibrio cholerae*; ppGpp, Guanosine 5′-diphosphate 3′-diphosphate; DM, double mutant.

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cryo-EM localization of CgtA in 50S [17], is not sufficient to explain its role in maturation. Thus the mode of CgtA's activity has to be investigated. The structural details of the protein were obtained from two crystal structures of CgtA solved till now; one from *B. subtilis* which did not contain the C-terminal domain (CTD) [19], and another from *Thermus thermophillus* [20] with incomplete CTD. The structures showed that CgtA consisted of three domains, GTP hydrolysis domain, Obg domain and the CTD. The GTP hydrolysis domain contained the conserved P-loop, switch I and switch II regions which were involved in GTP hydrolysis. Nevertheless, the functions of the Obg domain and the CTD remained unclear. It was known for *B. subtilis* CgtA, that just a point mutation from Gly92 to Asp92, (equivalent to Gly98 of in $CgtA_{vc}$), located in the Obg domain caused a detrimental effect on the cell growth, resulting in formation of pinhole size colonies [21].

We investigated the underlying cause of the above phenomenon *in vitro*, and we report here that the introduction of a slightly larger amino acid, i.e., Asp in place of the Gly98 (Gly98Asp) of the Obg domain and proximal to the GTPase domain significantly hindered the GTPase activity of CgtA. The hindrance could be relieved by abolishing the bulky side chain of Tyr194 (Tyr194Gly) which is located opposite to Gly98 in the GTPase domain revealing a fine tuned intra-molecular dynamism of CgtA closely associated with its GTPase activity. Overall, our biochemical studies aid in understanding towards the mode of action of the CgtA protein, and confer functional insights on the roles played by two of its strategically located and phylogenetically conserved amino acids, Gly98 and Tyr194 in regulating the GTPase activity of the protein.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Vibrio cholerae N16961 strain grown overnight in LB medium at 37 °C, 150 rpm was used for isolation of genomic DNA, that was used as a template for PCR amplification of the CgtA gene with appropriate primers.

E. coli DH5α cells were used to maintain the recombinant plasmids constructed and *E. coli* BL21 cells were used to express the CgtA protein upon induction with 1 mM IPTG (Isopropyl β-D-1 thiogalactopyranoside).

2.2. PCR amplification of wild type cgtA and site directed mutagenesis

CgtA gene of *V. cholerae* was PCR amplified by primers 5′CGCGGATCCCATGAAATTCGTAGATG3′ and 5′CCGCTCGAGGTCACGAACATAGATAA3′, using genomic DNA isolated from the *V. cholerae* N16961 as a template. The PCR amplified product was then directionally cloned in pET21b (Novagen) vector. The resulting vector coded for full-length CgtA protein with six Histidine tag at the C-terminal end of the full-length protein. Site directed point mutations were introduced at Gly98 and Tyr194 by mega-primer based mutagenesis technique using two mutagenic primers and the above-mentioned primers for each mutation (details of primers are given in Table 1). The mutants were then confirmed by sequencing and then cloned into pET21b expression vector.

Table 1
List of primers used in this study.

Primer name	Primer 5–3′
$CgtA_{vc}$ F	CGCGGATCCCATGAAATTCGTAGATG
$CgtA_{vc}$ R	CCGCTCGAGGTCACGAACATAGATAA
MutG2DF1	CGCGTACCGGTAGATACTCGTGCCGTC
MutG2DR2	GACGGCACGAGTATCTACCGGTACGCG
MutY2GF7	CCTAAAGTGGCGGATGGCCCGTTTACCACG
MutY2GR8	CGTGGTAAACGGGCCATCCGCCACTTTAGG

2.3. Protein expression and purification

$CgtA_{vc}$ protein was expressed in *E. coli* BL21 cells after induction with 1 mM IPTG at 37 °C 150 rpm for 4 h and mutated $CgtA_{vc}$ (Gly98Asp) and Double mutant $CgtA_{vc}$ (Gly98Asp, and Tyr194Gly) were expressed in *E. coli* BL21 cells after induction with 1 mM IPTG at 18 °C 150 rpm for 12 h. The over-expressed cells were pelleted down and kept at −80 °C. The cells were thawed and re-suspended in lysis buffer (50 mM Tris–HCl pH-8, 1 mM EDTA pH-8, 200 mM NaCl, 2 mM PMSF, 2 mM DTT and 15 mM Imidazole for wild type CgtA and 50 mM Tris–HCl pH-8, 1 mM EDTA pH-8, 200 mM NaCl, 2 mM PMSF, 2 mM DTT, 0.1% Triton-X100 and 15 mM Imidazole for mutated CgtA), then sonicated to lyse the cells. The cell debris was pelleted by centrifugation at 14,000 × g for 30 min. The supernatant was loaded in 1 ml of Ni-NTA agarose beads column equilibrated in lysis buffer. The flow throw was collected and the beads were then washed twice with 5 ml wash buffer (50 mM Tris–HCl pH-8, 1 mM EDTA pH-8, 200 mM NaCl, 2 mM PMSF, 2 mM DTT) with 30 mM imidazole and 50 mM imidazole respectively. The protein was eluted with elution buffer (50 mM Tris–HCl pH-8, 150 mM KCl, 5 mM $MgCl_2$, 2 mM DTT) with 300 mM and 400 mM imidazole respectively. The elutes were dialyzed against storage buffer (50 mM Tris–HCl pH-8, 150 mM KCl, 5 mM $MgCl_2$, 2 mM DTT and 10% glycerol). The protein was then stored at −80 °C in storage buffer.

2.4. GTPase activity of isolated proteins in the presence of 50S and 70S ribosome

The GTPase activity of purified proteins were measured in the presence of 50S subunit and 70S monosome. For GTPase assay, 1 μM of purified proteins were used in the presence of excess of GTP (2 mM) in reaction buffer (50 mM Tris-HClpH-8, 150 mM KCl, 2 mM DTT, 5 mM $MgCl_2$). To study the effect of 50S and 70S in the GTPase activity of the purified protein 100 nM of ribosomal subunits or 70S monosome was used. The reactions were carried out at 30 °C and after every 30 min a 20 μl aliquot of reaction mixture were withdrawn and transferred to 96 micro-titer well plate containing 20 μl of 0.5 M EDTA solution to stop the reaction. Then 150 μl of Malachite green stock solution was added to each well and incubated for 10 min to allow the color development and then added 15 μl of 35% Citric acid to stop the color development. Then the absorbance was measured at 630 nm to identify the free Pi released [22]. The GTPase activity of CgtA was also studied in the presence of 10 μM thiostrepton, 100 nM of 16S rRNA and 23S rRNA purified from isolated ribosomal subunit by phenol-chloroform treatment such that the rRNA retained its secondary structures.

2.5. Sequence comparisons and homology modeling of $CgtA_{vc}$

Multiple sequence alignment of CgtA from different prokaryotic organisms were done to compare the sequence similarity among the prokaryotic sequences using the Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) [23]. The comparison showed high sequence similarities of *V. cholerae* $CgtA_{vc}$ proteins with others, including the *Thermus thermophilus* CgtA whose structure was solved previously and also identified few conserved residues. The homology model for *V. cholerae* CgtA was constructed using the X-ray crystallographic structure of *T. thermophilus* (PDB ID 1UDX) as template in Swiss Modeler server (http://swissmodel.expasy.org/

workspace/index.php?func=modelling_simple1) [24–26]. The homology models were then validated by PROCHECK [27] using the "Structure Assessment" tool in SWISS-MODEL server (http://swissmodel.expasy.org/workspace/index.php?func=tools_structureassessment1&userid=USERID&token=TOKEN) and from ProSA web tool (https://prosa.services.came.sbg.ac.at/prosa.php) [28]. The nucleotide (GTP) bound structures were prepared by docking the nucleotide molecules in the homology-modeled structure based on the reference *B. subtilis* CgtA structure in the UCSF Chimera software.

3. Results and discussions

3.1. GTPase activity of $CgtA_{vc}$ is stimulated upon interaction with the ribosome

CgtA is a GTPase protein belonging to Obg GTPase family. It is conceivable that its GTPase activity is required for proper functioning of the protein. CgtA is associated with 50S and involved in its maturation. Therefore, we studied the activity of the $CgtA_{vc}$ in the presence of *V. cholerae* 50S subunit. Studying the GTPase activity revealed that the activity of $CgtA_{vc}$ protein (intrinsic activity) was stimulated by about 5-fold in the presence of 50S ribosomal subunit i.e.; 0.2 ± 0.1 to 0.98 ± 0.22 pmole of Pi release/pmole of protein/min (see Table S1), P-values for t-test of $CgtA_{vc}$ and $CgtA_{vc}$+50S are shown in Table S2. In some ribosome associated translation factors like EF-G [29] and in some ribosome maturation factors like YjeQ, RbgA [30,31] this kind of phenomena was seen. Thiostrepton is an antibiotic that binds to the GTPase association center of 50S ribosome [32] facing the inter-subunit side, thereby inhibiting the interaction of Elongation Factor -G (EF-G) with the ribosome. However, we found no effect of thiostrepton in the 50S ribosome associated GTPase activity of the $CgtA_{vc}$ protein, indicating a possibly different binding mechanism of $CgtA_{vc}$ with the 50S in comparison to the EF-G. Isolated rRNA alone and UV treated or inactivated ribosome was also incapable of stimulating the GTPase activity of $CgtA_{vc}$, implying that the proper structure or functional ribosome was required for proper interaction with $CgtA_{vc}$ and this interaction possibly had some direct or indirect role in the augmented GTPase activity of $CgtA_{vc}$. The GTPase activity in the presence of 70S monosomes (tight couples) in 10 mM Mg^{2+} ion concentration in the assay buffer was ineffective in stimulating the GTPase activity of the $CgtA_{vc}$ protein. However, when the Mg^{2+} ion concentration was reduced to 3.5 mM in the assay buffer (loose couple ribosomes) the $CgtA_{vc}$ GTPase activity was stimulated (Fig. 1). Thus, indicated that the $CgtA_{vc}$ protein would bind to the inter-subunit face of 30S and 50S subunit. However, may not be in a similar manner as does by the other translational factors like EF-G. Recently published work [17] also supports our observation to some extent.

3.2. Structure-function analysis of $CgtA_{vc}$ protein from V. cholerae

In *B. subtilis* CgtA it was known that just a point mutation from Gly92 to Asp92 caused deleterious effect on the cell growth, resulting in formation of pinhole size colonies [21]. The multiple amino acid sequence alignment of CgtA protein from several prokaryotic microorganisms showed many conserved residues. Among the conserved residues Gly92 (highlighted in yellow) with respect to *B. subtilis* was also present (see Fig. 2). Hence, to obtain a structural insight of the protein and to locate the Gly92 in the protein, homology model of $CgtA_{vc}$ was constructed (see Fig. 3) using SWISS-model workspace. The homology model thus obtained showed the Obg domain, and the GTPase domain and an incomplete C-terminal domain (see Fig. 3). The resultant structure was validated using the standard procedures (Fig. S1). For clarity, we removed the CTD from the homology model Fig. reported here. The proximity of Gly98 to the GTPase domain (see Fig. 3) as seen in the homology model prompted us to predict that any change in Gly98 residue would cause perturbation in the potential inter-domain movement between the Obg domain and the GTPase domain. I-mutant3 [33] and SDM [34] prediction server results supported our hypothesis that this mutation could affect the protein stability and could cause malfunction of the protein (for details table S3).

From the multiple sequence alignment, we could locate another amino acid, Tyr188 (also highlighted in yellow) of *B. subtilis* CgtA that belonged to the GTPase domain. Subsequently, we found that not only in prokaryotic CgtA protein, but also in eukaryotic homologs of CgtA those residues were conserved (chart not shown). Above two *B. subtilis* CgtA residues Gly92 and Tyr188 corresponded to Gly98 and Tyr194 respectively, of the *V. cholerae* amino acid sequence (strain N169161) and we have marked them in the $CgtA_{vc}$ homology model figure (See Fig. 3). It was known that mutational studies could aid in understanding the functionality of many proteins. Therefore, inorder to explore how the above-mentioned point mutation could possibly affected the functionality of the CgtA, which might have led to detrimental growth, we introduced Gly98Asp and other point mutations, and studied their biochemical effects on the 50S-$CgtA_{vc}$ interactions.

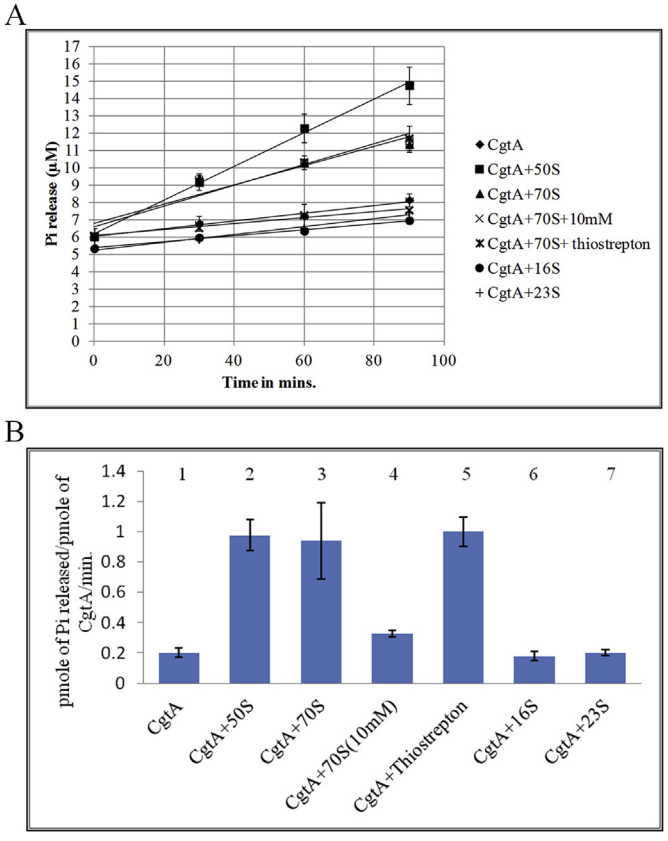

Fig. 1. GTPase activity in time Course and specific activity of the $CgtA_{vc}$ in different conditions. (A) The GTPase activity in time course with standard error. (B) The intrinsic GTPase activity of wild type $CgtA_{vc}$ protein (bar 1), the increased specific activity of $CgtA_{vc}$ in the presence of 50S ribosomal subunit (bar 2), the specific activity in the presence of 70S monosomes in 3.5 mM and 10 mM $MgCl_2$ buffer conditions (bar 3 & 4 respectively). The specific activity in the presence of thiostrepton antibiotic of $CgtA_{vc}$ in the presence of 50S (bar 5). The specific GTPase activity in the presence of isolated 16S and 23S rRNA (bar 6 and 7 respectively).

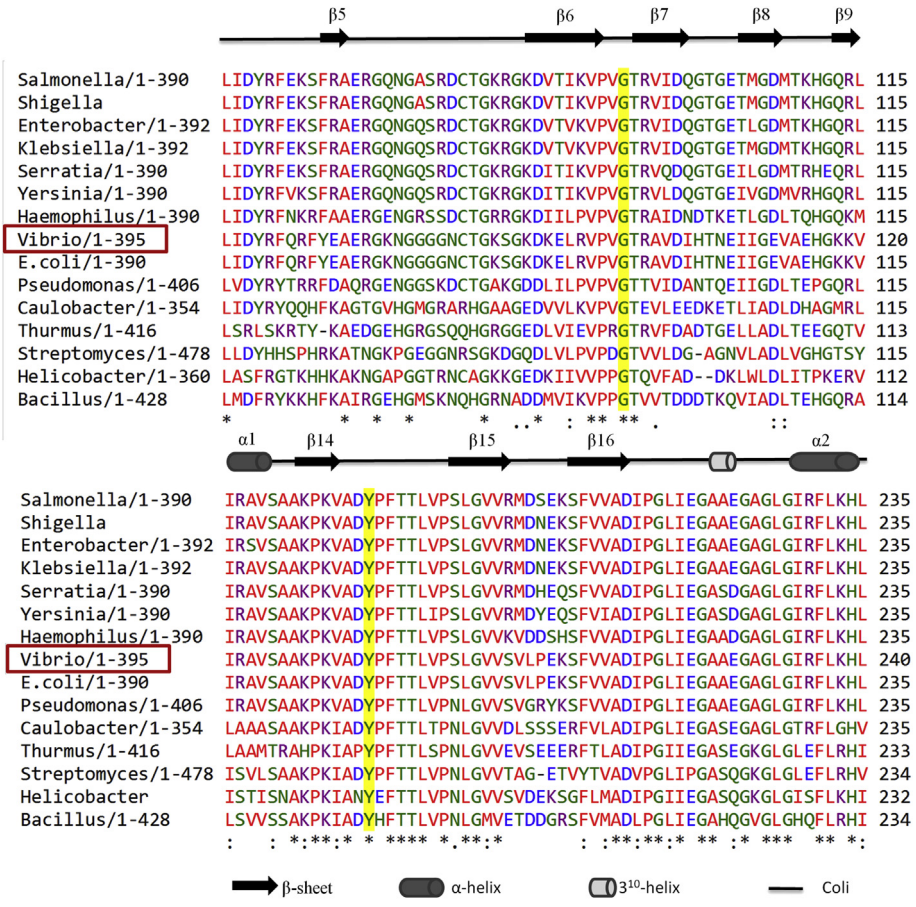

Fig. 2. Multiple sequence alignment of CgtA proteins of some prokaryotic organisms. The regions of alignment highlighted in yellow are the two conserved residues corresponding to Gly98 and Tyr194 of *V. cholerae*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A closer insight into the structure revealed that the Obg domain and the GTPase domain were inter-connected by only a single unstructured chain of amino acids (which is marked in orange in Fig. 3). That chain was connected to the conserved P-loop (marked in yellow in Fig. 3) which was mainly involved in the binding of nucleotides. Therefore, it could be predicted that any movement taking place in the Obg domain from the hinge at Leu164 would be translated though that single unstructured chain to the GTPase domain or vice versa.

3.3. *Two highly conserved amino acids Gly98 and Try194 together play important roles in maintaining optimum GTPase activity of* $CgtA_{vc}$

In order to understand the effect of the aforesaid point mutation, we replace Gly98 with Asp98 in the $CgtA_{vc}$ of *V. cholerae*. The single mutation essentially caused a decrease in the GTPase activity of the purified protein *in vitro* (Fig. 3) even though the residue was located in the Obg domain and not in the so-called GTPase domain (Fig. 3). Results shown in Fig. 3 depicted that both the intrinsic GTPase activity of the mutant protein $CgtA_{vc}$ (Gly98Asp) and the activity in the presence of 50S ribosomal subunit decreased to approximately half the activity of wild type $CgtA_{vc}$ protein (see Fig. 4). Thus, we infer that in the case of the Gly92Asp mutant CgtA of *B. subtilis* [21], similar reduced GTPase activities might be the direct or indirect cause that led to the growth defects of this bacterium.

Interestingly, after removing the bulky side chain of Tyr194 by introducing a point mutation in Tyr194Gly located diagonally opposite to the Gly98 (according to the X-ray crystallographic structure of *T. thermophilus* (PDB ID 1UDX)), the intrinsic GTPase activity of the resultant $CgtA_{vc}$ (Tyr194Gly) mutant increased heavily (Fig. 4). In the presence of 50S ribosome also the GTPase activity of the $CgtA_{vc}$ (Tyr194Gly) mutant increased excessively (Fig. 4). Therefore, it was clear that location and nature of the above two amino acids, Gly98 and Tyr194 were very important for properly maintaining the GTPase activity of $CgtA_{vc}$. Eventually, the

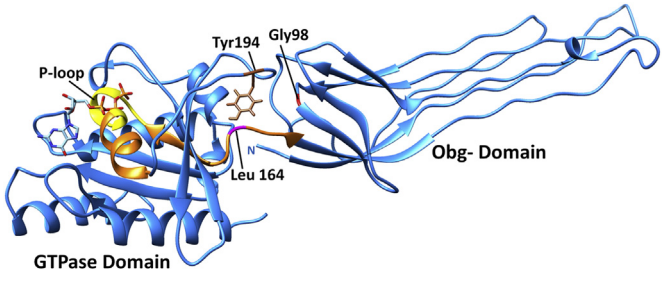

Fig. 3. Homology model of *V. cholerae* $CgtA_{vc}$. The Obg domain and the GTPase domain are connected with each other through a single chain (in orange), which is directly connected to the P-loop (marked in yellow) of the GTPase domain where binding and hydrolysis of GTP takes place. The two conserved residues Gly98 and Tyr194 are marked in red and brown respectively. Leu164 is marked in magenta. Leu164 flanked the GTPase and the Obg domains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

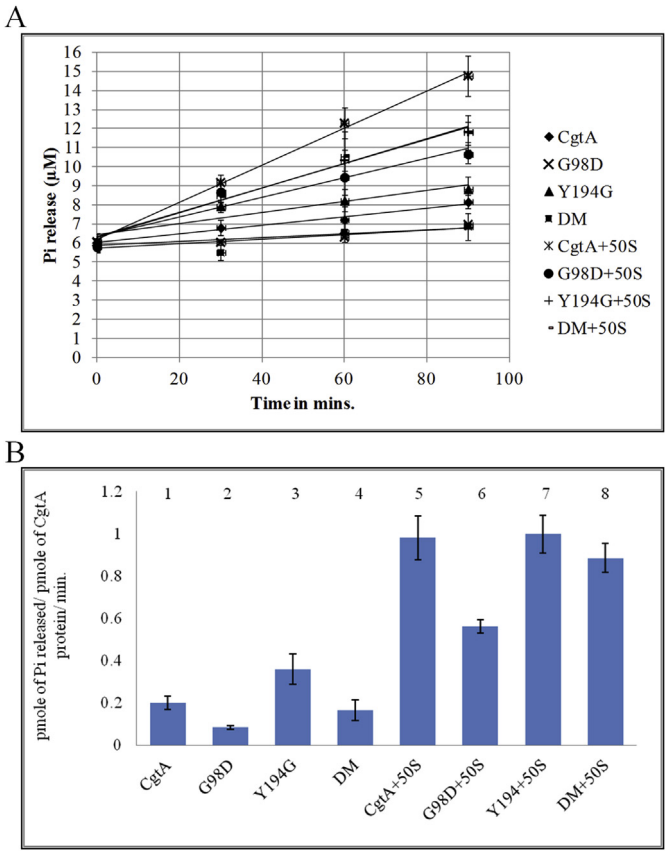

Fig. 4. Specific GTPase activity of the wild type and the mutant $CgtA_{vc}$ proteins. (A) The GTPase activity in time course with standard error. (B) The intrinsic GTPase activity of wild type $CgtA_{vc}$ and various mutants (bars 1–4). The specific activity in the presence of 50S ribosome of wild type $CgtA_{vc}$ and various mutants (bars 5–8).

double mutant i.e., $CgtA_{vc}$ (Gly98Asp, and Tyr194Gly) could complement each others' effect and restored the overall GTPase activity up to normalcy (about 82% compared to the wild type) (Fig. 4, Table S4). Table S2 shows the P-values for t-test of the above experiments. The above results depicted that those two amino acid residues were at the pinnacle of the inter-domain interactions of $CgtA_{vc}$ and probably that is the reason they are so conserved in nature. In future, the effects of those mutations on the physiology of the bacteria are to be tested and also how CgtA is involved in the ribosome maturation is to be investigated.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.079.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.079.

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