JOURNAL OF BACTERIOLOGY, Feb. 2006, p. 1205–1210 Vol. 188, No. 3
0021-9193/06/$08.00+0 doi:10.1128/JB.188.3.1205–1210.2006


# The *Vibrio harveyi* GTPase $CgtA_V$ Is Essential and Is Associated with the 50S Ribosomal Subunit

A. E. Sikora,[1,2] R. Zielke,[1,2] K. Datta,[1] and J. R. Maddock[1]*

*Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830 North University, Ann Arbor, Michigan 48109,[1] and Department of Molecular Biology, University of Gdansk, Kładki 24, 80-822 Gdansk, Poland[2]*

Received 14 September 2005/Accepted 28 October 2005

**It was previously reported that unlike the other *obg*/*cgtA* GTPases, the *Vibrio harveyi* $cgtA_V$ is not essential. Here we show that $cgtA_V$ was not disrupted in these studies and is, in fact, essential for viability. Depletion of $CgtA_V$ did not result in cell elongation. $CgtA_V$ is associated with the large ribosomal particle. In light of our results, we predict that the *V. harveyi* $CgtA_V$ protein plays a similar essential role to that seen for Obg/CgtA proteins in other bacteria.**

All living organisms express one or more Obg/CgtA GTPase. Elucidating the function of these GTPases has been complicated by somewhat contradictory phenotypes associated with specific mutants in different model systems. Some of the confusion stems from the dual function of these proteins (ribosome assembly and stress response) in at least some organisms, as well as from potential species-specific differences that may result in different phenotypes observed for orthologous *obg*/*cgtA* mutants. Perhaps the most surprising of these inconsistent reports is that of the nonessential nature of the *Vibrio harveyi* $cgtA_V$ gene (5) and its pleiotropic phenotypes (32, 34, 42), observations at odds with what has been reported for other *obg*/*cgtA* mutants. Here we show that, in contrast to these studies, the *V. harveyi* $cgtA_V$ gene is essential. Furthermore, we demonstrate that the trimethoprim resistance associated with a $cgtA_V$ clone was conferred by the linked *folA* gene. We also show that $CgtA_V$ is associated with the 50S ribosomal particle. From these studies, we predict that the role of $CgtA_V$ is similar to that of other bacterial Obg/CgtA proteins.

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used in the present study are listed in Table 1. *Escherichia coli* cells were grown at 37°C (unless otherwise indicated) in Luria-Bertani media (LB; 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl/liter) or LB agar (1.5% agar) containing antibiotics, as required (gentamicin, 30 μg/ml; kanamycin, 30 μg/ml; chloramphenicol, 10 μg/ml; trimethoprim, 100 μg/ml; ampicillin, 100 μg/ml). *V. harveyi* strains were derived from BB7 (2) and maintained at 30°C on BOSS medium (10 g of Bacto Peptone, 3 g of beef extract, 30 g of NaCl, and 1 ml of 100% glycerol/liter) or BOSS agar (1.5% agar) supplemented with antibiotics (gentamicin, 30 μg/ml; kanamycin, 50 μg/ml).

**The *V. harveyi* $cgtA_V$ gene is essential.** We were perplexed by the reported viability of the *V. harveyi* $cgtA_V$ transposon insertion mutant BB7X (5) for several reasons. First, in all other organisms examined, *obg*/*cgtA* is an essential gene (1, 16, 22, 25, 37, 40). Second, given the sequence similarity among the Obg/CgtA proteins, we would predict that CgtA proteins play similar roles in all bacteria, and yet the viable nature and reported phenotypes of the *V. harveyi* $cgtA_V$ insertional mutant (5, 32, 34, 42) were different from those reported for other *obg*/*cgtA* mutant strains (7, 16, 17, 22, 26, 38). Therefore, we reinvestigated the consequences of $CgtA_V$ depletion in *V. harveyi*. Plasmids used in the present study are listed in Table 1 and were introduced into *V. harveyi* by conjugation with *E. coli* S17.1. Cloning and plasmid amplification were performed in *E. coli* DH5α.

Initially, we constructed a chromosomal integrant in which expression of $cgtA_V$ was controlled by the $P_{BAD}$ promoter. This strain grew in the presence but not the absence of arabinose (data not shown), indicating that either $cgtA_V$ or a downstream gene was essential for viability. To rule out possible contributions from downstream genes, we disrupted the chromosomal $cgtA_V$ gene by integration of an internal $cgtA_V$ fragment and complemented with a plasmid harboring $cgtA_V$ (PCR amplified with the primers 5′-TAATCCACGCTAGCAGTAGTCGGAG and 5′-GGCTTAATGACTGCAGTAGCGATTA) controlled by the $P_{BAD}$ promoter ($P_{BAD}$-$cgtA_V$; BB7AS31) (Table 1). A control strain, BB7AS26, which expressed $P_{BAD}$-$cgtA_V$ in wild-type BB7 cells, was characterized in parallel. BB7AS31 cells grew in both liquid culture (Fig. 1A) and on plates (Fig. 1C) supplemented with 0.01% arabinose. Under these conditions, the cellular level of $CgtA_V$ in BB7AS31 and BB7AS26 was similar and was two- to fivefold more than that of wild-type *V. harveyi* (Fig. 1B). In the absence of arabinose, however, BB7AS31 cells showed a reduction in cell growth in liquid medium (Fig. 1A) and a reduction in cell viability (Fig. 1C). Furthermore, in the absence of $CgtA_V$ expression, no colonies formed on plates (Fig. 1C). We conclude that, in contrast to prior reports (5, 32, 34, 42), $cgtA_V$ is an essential gene.

In *E. coli*, overexpression of $CgtA_E$ results in elongated cells with aberrant chromosomal segregation (9, 16). Furthermore, overexpression of Obg in *S. griseus* impairs differentiation (25). To determine whether overexpression of $CgtA_V$ resulted in a growth phenotype, we induced expression of the episomal copy of $cgtA_V$ in wild-type cells (strain BB7AS26) with various amounts of arabinose. In the absence of arabinose, growth of

* Corresponding author. Mailing address: Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830 North University, Ann Arbor, MI 48109-1048. Phone: (734) 936-8068. Fax: (734) 647-0884. E-mail: maddock@umich.edu.

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Genotype[a] | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | $\lambda^-$ ϕ80d/*lac*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*($r_K^-$ $m_K^+$) *supE44 thi-1 gyrA relA1* | 13 |
| S17.1 | $F^-$ *recA pro hsdR* RP4-2 $Tc^r$::Mu $Tn^r$::Tn*7* | 33 |
| *V. harveyi* | | |
| BB7 | Wild-type *V. harveyi* | 2 |
| BB7X | BB7 with a chromosomal insertion of Tn*5*$Tp^r$MCS | 5 |
| BB7AS26 | BB7 harboring plasmid pAES26 | This study |
| BB7AS31 | BB7 with $cgtA_V$ disrupted by integration of pAES31 and harboring plasmid pAES26 | This study |
| Plasmids | | |
| PCR 2.1-TOPO | Cloning vector; $Kan^r$ $Amp^r$ | Invitrogen |
| pUC18 | Cloning vector, *ori* pMB1, *lacZ*α; $Amp^r$ | 24 |
| pGD103 | Cloning vector, *ori* pSC101, *lacZ*α; $Kan^r$ | 8 |
| pJN105 | Cloning vector *ori* pBBR1, *araC-*$p_{BAD}$, *mob*; $Gm^r$ | 23 |
| pK18mobsacB | *ori*-pMB1, *ori*T (RP4), *sacB, lacZ*α; $Kan^r$ | 30 |
| pAC1 | pUC19 containing 5,013-bp EcoRI $Tp^r$ fragment from *V. harveyi* BB7X; $Amp^r$ $Tp^r$ | 5 |
| pAES7 | 1,547-bp PCR product containing full-length $cgtA_V$ and $cgtA_V$ promoter in PCR 2.1-TOPO | This study |
| pAES10 | 541-bp $folA_V$ PCR product from BB7 in PCR 2.1-TOPO | This study |
| pAES11 | 541-bp $folA_V$ PCR product from BB7X in PCR 2.1-TOPO | This study |
| pAES12 | 517-bp BamHI-PstI $folA_V$ from pAES10 in pUC18 | This study |
| pAES13 | 517-bp BamHI-PstI $folA_V$ from pAES11 in pUC18 | This study |
| pAES14 | 517-bp BamHI-PstI $folA_V$ from pAES10 in pGD103 | This study |
| pAES15 | 517-bp BamHI-PstI $folA_V$ from pAES11 in pGD103 | This study |
| pAES25 | 1,229-bp full-length $cgtA_V$ PCR product in PCR 2.1-TOPO | This study |
| pAES26 | Plasmid with $P_{BAD}$-$cgtA_V$; 1,267-bp NheI-SacI $cgtA_V$ fragment from pAES25 in pJN105 | This study |
| pAES31 | EcoRV $cgtA_V$ from pAES25 cloned into SmaI of pK18*mobsacB* | This study |

[a] $Amp^r$, ampicillin resistance; $Kan^r$, kanamycin resistance; $Gm^r$, gentamicin resistance; $Tp^r$, trimethoprim resistance.

BB7AS26 cells was identical to that of the wild-type controls. Up to 10-fold induction of $CgtA_V$ protein (0.1% arabinose) had no significant effect on cell growth in liquid medium or on plates (data not shown). Thus, a modest overexpression of $CgtA_V$ is not deleterious to *V. harveyi*. In contrast, in the absence of arabinose the levels of $CgtA_V$ in BB7AS31 were significantly reduced after 1.5 h and $CgtA_V$ was not detectable after 4.5 h (Fig. 1B). In addition, we note a reduction in the level of $CgtA_V$ in the control strain, BB7AS26, as cells entered stationary phase (Fig. 1B). A reduction in the levels of CgtA protein upon entry into stationary phase has been previously been reported for *Streptomyces coelicolor* (26) and *E. coli* (16).

The Obg/CgtA proteins have been implicated in cell cycle and/or DNA replication control in a number of bacteria. In *E. coli*, $cgtA_E$ temperature-sensitive mutant cells grown to stationary phase and shifted to the nonpermissive temperature become filamentous and have defects in chromosome partitioning (16). A transposon fusion mutant of $CgtA_E$ displays defects in the coordinate timing of DNA replication initiation (12). In the developmental bacterium *Caulobacter crescentus*, $cgtA_C$(ts) mutants arrest prior to the onset to DNA replication (7). In *V. harveyi*, however, we did not observe a change in cell morphology, as judged by light microscopy, or DNA partitioning, as judged by DAPI (4′,6′-diamidino-2-phenylindole) staining, upon depletion of $CgtA_V$ (data not shown). Thus, depletion of $CgtA_V$ does not result in either a cell elongation or DNA partitioning phenotype. Therefore, it is likely that a role in cell division and/or DNA replication is not a core function for all Obg/CgtA proteins. One possible explanation is that cell division and DNA replication defects are species-specific downstream consequences of *obg*/*cgtA* depletion. In addition, discrepancies between the phenotypes of different *cgtA* mutants may be due to the nature of the lesion (temperature sensitive, depletion or protein fusion) and/or the status of the cells during analysis (stationary versus exponentially growing cells).

**The previously reported *cgtA*::Tn*5*TpMCS mutant, BB7X, encodes a wild-type $cgtA_V$ gene.** There has been a series of studies describing the phenotype of BB7X (5, 32, 34, 42), a strain reported to harbor a *cgtA*::Tn*5*$Tp^r$MCS allele. The plasmid pAC1, encoding at least part of the $cgtA_V$ gene, was obtained by digestion of BB7X DNA with EcoRI, ligation to pUC19, and selection for both trimethoprim and ampicillin resistance (5). The expectation was that trimethoprim-resistant transformants would harbor both the Tn*5* transposon (encoding *dhfrII*, the dihydrofolate reductase gene [21]) and flanking chromosomal DNA. Using a primer complementary to the IS*50* element of Tn*5*$Tp^r$MCS (Tn, 5′-TTCAGGACGCTACTT GTGTA-3′), the sequence of the N-terminal 99 amino acids of $cgtA_V$ was obtained, and it was concluded that BB7X contained a $cgtA_V$::Tn*5*$Tp^r$MCS insertion mutation (5).

In contrast to this conclusion, we demonstrate through several lines of evidence that BB7X encodes a wild-type $cgtA_V$ gene. First, immunoblot analysis of cell extracts from BB7 and BB7X with anti-CgtA antibodies resulted in the detection of a protein band that migrates at ca. 48 kDa, 5 kDa larger than the expected size of $CgtA_V$. A slightly slower migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been previously noted for the *C. crescentus* (19)

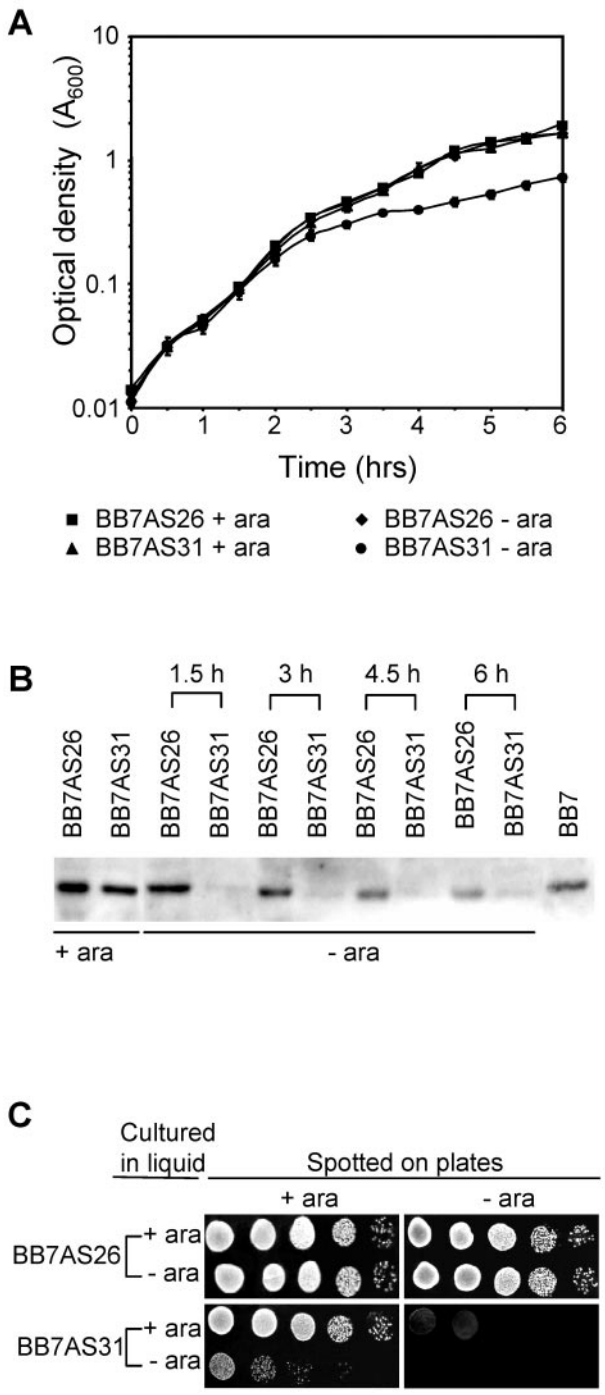


FIG. 1. Depletion of $CgtA_V$. *V. harveyi* cells expressing plasmid-borne $P_{BAD}$-*cgtA*$_V$ from wild-type cells (BB7AS26) or a strain with a chromosomal disruption of *cgtA*$_V$ (BB7AS31) were grown in the presence of 0.01% arabinose (ara) and divided, and growth was continued in the presence or absence of arabinose as indicated. (A) Cell growth as monitored by absorbance (optical density at 600 nm). (B) Immunoblot analysis showing the levels of $CgtA_V$ at representative times. BB7 is wild-type *V. harveyi*. (C) Growth of strains on plates with or without arabinose. Cells were grown in liquid medium in the presence or absence of arabinose as described above. After 6 h, serial dilutions were spotted onto BOSS agar plates with or without arabinose, as indicated. Colonies were observed only in cells expressing $CgtA_V$ (+ ara).

and *E. coli* (39) CgtA proteins. This immunoreactive band migrates at the same position as purified $CgtA_V$ protein (data not shown). Second, we designed a primer to a conserved region of *rpmA* (5′-GAGGATCCATCATCGTTCGTCAACG), the gene predicted to be upstream of *cgtA*$_V$ based on conservation of gene organization in most bacteria, including *Vibrio* species. PCR amplification of BB7 and BB7X chromosomal DNA with this primer and a primer downstream of *cgtA*$_V$ (5′-ATGGATCCGCAAATCACATCGTCT) produced a 1.6-kb PCR product regardless of the source of chromosomal DNA. Sequence analysis of the PCR products shows that the DNA is identical and encodes a full-length *cgtA*$_V$ gene (data not shown). Third, we sequenced the entire 5-kb EcoRI DNA fragment of pAC1 on both strands. This DNA insert did not contain the Tn*5*Tp$^r$MCS transposon (Fig. 2A). Finally, examination of the *cgtA*$_V$ sequence reveals that nucleotides 315 to 326 are 83% complementary to the last 12 bases of the Tn primer used to sequence the N-terminal region of pAC1 (5). We propose that the complementarity between the Tn primer and this region of *cgtA*$_V$ was sufficient to generate the DNA sequence reported previously (5). We conclude, therefore, that BB7X encodes a wild-type *cgtA*$_V$ gene and that publications referring to the characterization of the *V. harveyi* BB7X mutant (5, 32, 34, 42) do not describe a bona fide *cgtA*$_V$ mutant. The true nature of the Tn*5*Tp$^r$MCS insertion in BB7X was not explored further.

**Expression of the *V. harveyi folA* gene in *E. coli* results in trimethoprim resistance.** Because pAC1 did not contain a Tn*5*TpMCS transposon, we investigated the nature of the trimethoprim resistance conferred by the pAC1 plasmid. Introduction of pAC1, but not the high-copy vector pUC18 or the low-copy plasmid pGD103, conferred trimethoprim resistance to *E. coli* DH5α cells (Fig. 2B). Trimethoprim resistance was not due to expression of the *V. harveyi cgtA*$_V$ gene, since cells containing pAES7, a high-copy plasmid containing the *cgtA*$_V$ gene and promoter (PCR amplified with primers 5′-GAG GATCCATCATCGTTCGTCAACG and 5′-ATGGATCCG CAAATCACATCGTCT), were trimethoprim sensitive (Tp$^s$) (Fig. 2B).

To determine the nature of the trimethoprim resistance, we sequenced *cgtA*$_V$ and the surrounding genomic region. Sequencing was performed at the Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland, or at the University of Michigan Sequencing Core Facility; all oligonucleotide sequences are available upon request. The entire *cgtA*$_V$ coding region was amplified by PCR (polymerase *Pfu* [Promega] or Red Taq [Sigma]) from BB7 and BB7X (4) colonies or genomic DNA (isolated by using High Pure PCR Template Preparation Kit; Roche) or from pAC1 using primers designed to conserved regions in *obg*/*cgtA* genes and by inverse PCR. Our sequence (NCBI AY623050) agrees with the partial 5′ sequence (300 bp) of the *cgtA*$_V$ gene (5) that was previously reported. The 5-kb EcoRI fragment of the pAC1 plasmid (5), as well as subclones generated in the present study, was sequenced on both strands by using appropriate oligonucleotide primers. The region upstream of *cgtA*$_V$ was amplified by using a primer complementary to a conserved region of *ispB* (a gene commonly present upstream of *obg*/*cgtA* genes) (5′-AGTTGGGCTTGAATTGTTTCATTCAC) and an internal *cgtA*$_V$ primer (5′-AACTTTTACTACCGCTTCATC

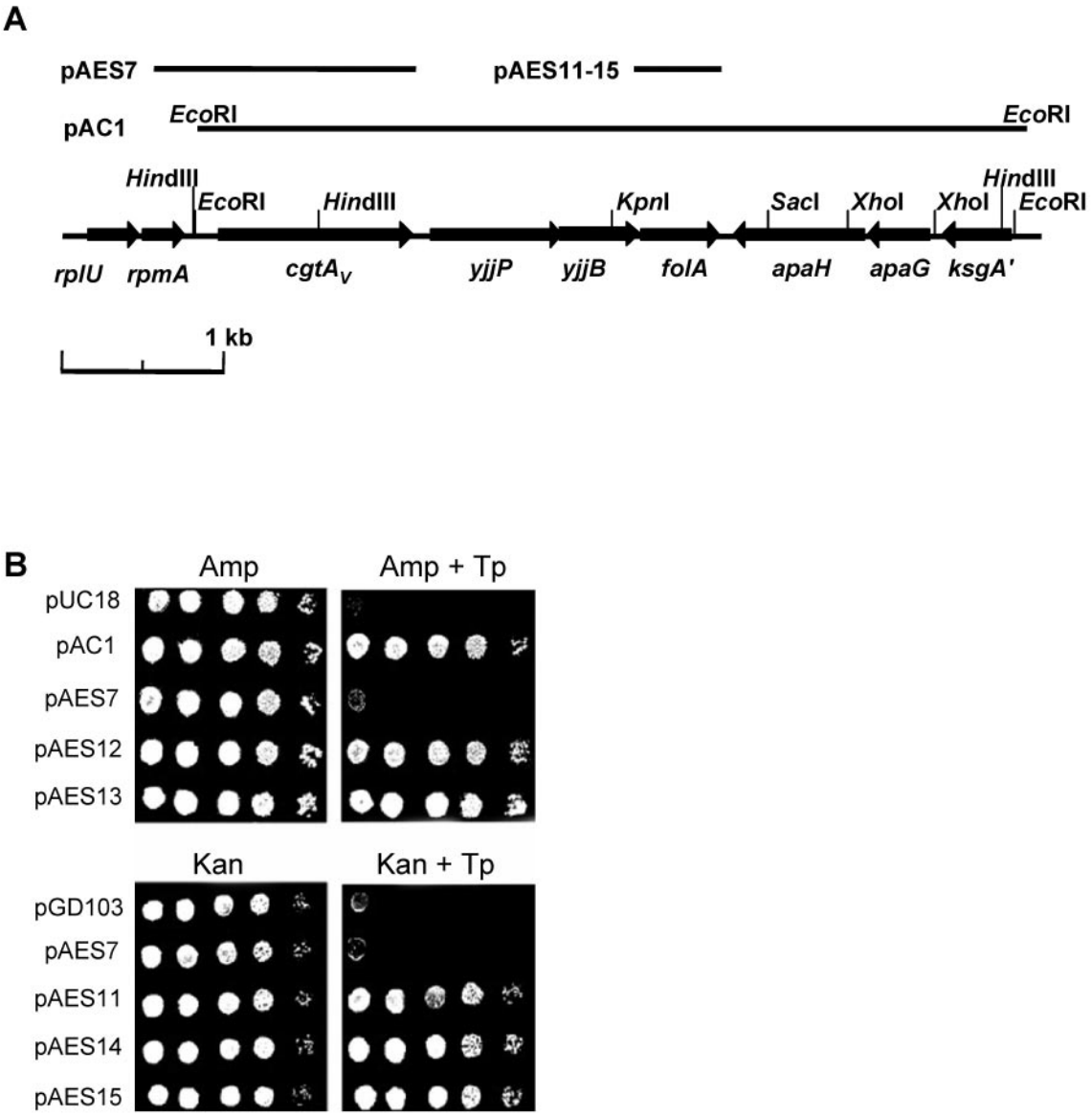


FIG. 2. Expression of the *V. harveyi folA* gene confers trimethoprim resistance to *E. coli* cells. (A) The gene organization of the $cgtA_V$ region, including gene names and selected restriction enzyme sites. The 5-kb EcoRI fragment found on the pAC1 plasmid (5) is indicated, as are the positions of the $cgtA_V$ and *folA* fragments found in the indicated plasmids. (B) *E. coli* harboring plasmids containing *V. harveyi* DNA, as indicated in Fig. 2A, were serially diluted and spotted onto LB medium supplemented with ampicillin (Amp), kanamycin (Kan), and/or trimethoprim (Tp), as indicated.

TACG). This sequence includes *rplU*, *rpmA* (coding for the 50S ribosomal proteins L21 and L27, respectively), and the $cgtA_V$ promoter region, and its organization is similar to that found in most bacteria (NCBI DQ180600).

Interestingly, the gene organization downstream of $cgtA_V$ (NCBI DQ180601) is quite different from that found in the majority of bacteria, although it is conserved in other *Vibrio* species. Downstream of $cgtA_V$ are two conserved hypothetical genes, *yjjP* and *yjjB*, as well as *folA* (dihydrofolate reductase type 1). Transcribed divergently are *apaH* (diadenosine tetraphosphatase), *apaG*, and the C terminus of *ksgA* (RNA adenine dimethyltransferase) (Fig. 2A). The functions of YjjP and YjjB are unknown, although both are predicted to be transmembrane proteins. In *E. coli*, the *folA*, *apaH*, *apaG*, and *ksgA* genes are physically linked but, unlike what we observe in *V. harveyi*, this gene cluster is not located near $cgtA_E$.

We were struck by the dual observation that the third gene downstream of $cgtA_V$ is *folA* and that *V. harveyi* FolA is predicted to be 52% identical to *E. coli* FolA. Although several mechanisms of trimethoprim resistance in *E. coli* have been described (35), the most common mechanism is an increase in *folA* expression (3, 11, 28, 36). To determine whether the trimethoprim resistance conferred by pAC1 was caused by expression of *V. harveyi folA*, we PCR amplified *folA* from BB7 and BB7X cells by using the primers 5′-CTATCGCCGTGG ATCCATAGTTTAA and 5′-TTGCGGTGCTTTCTGCAG

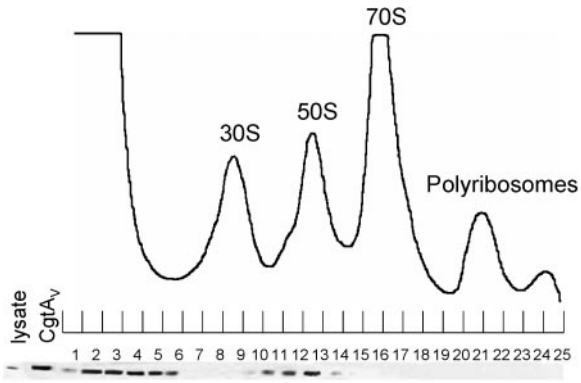


FIG. 3. CgtA$_V$ associates with the 50S ribosomal particle. Ribosomal subunits were separated by sucrose density centrifugation and monitored by UV absorbance (254 nm). The positions of the 30, 50S, and 70S ribosomes and the polysomes are indicated. The level of CgtA$_V$ in the indicated fractions was detected by immunoblot. Control lanes were loaded with *V. harveyi* BB7 cell lysate and purified CgtA$_V$ as indicated.

TCTATTC and cloned the fragments onto the high-copy plasmids pCR2.1-TOPO and pUC18 and the low-copy plasmid pGD103. DH5α transformants harboring any of these plasmids were Tp$^r$ (Fig. 2B), indicating that expression of the *V. harveyi folA* gene was sufficient to confer trimethoprim resistance to *E. coli*, even at a relatively low copy number. We observed equivalent Tp$^r$, regardless of whether the source of *folA* was BB7 or BB7X (Fig. 2B), a finding consistent with our sequence analysis indicating that the *folA* gene was identical in each strain (data not shown). Thus, we conclude that the Tp$^r$ phenotype of *E. coli* cells harboring pAC1 was due to expression of the *V. harveyi folA* gene and not due to a transposon insertion in *cgtA$_V$*, as had previously been reported (5).

**The *V. harveyi* CgtA$_V$ protein is associated with the 50S ribosomal particle.** In all organisms, a number of GTP-binding proteins, including the Obg/CgtA proteins, are predicted to be involved in some aspect of translation (18). In the case of the bacterial Obg/CgtA proteins, direct association with the large ribosomal subunit has been demonstrated for the *E. coli* (29, 39), *C. crescentus* (20), and *B. subtilis* (31, 41) proteins. Interestingly, the eukaryotic Obg/CgtA proteins are also ribosome associated. In yeast, the mitochondrial GTPase Mtg2p is associated with the large ribosomal subunit (6), the cytosolic GTPases Rbg1p and Rbg2p are associated with translating ribosomes (P. Wout and J. R. Maddock, unpublished), and the nucleolar Nog1p is associated with the pre-60S particle (14, 15). Thus, ribosome association appears to be a conserved feature of the Obg/CgtA family. To determine whether this was also the case for the *V. harveyi* CgtA$_V$ protein, we isolated ribosomes, as previously described (10) from 50 ml of culture of *V. harveyi* BB7 that were grown at 30°C to an optical density at 600 nm of 0.5. The extract was clarified by centrifugation (10 min, 30,000 × *g*) and separated on 10 to 30% sucrose gradients (10 ml; 10 mM Tris-HCl [pH 7.5], 30 mM KCl, 5.25 mM magnesium acetate) by centrifugation in a Beckman SW41 Ti rotor at 41,000 rpm for 3 h. Fractions were collected as described previously (20) with the polysome profile monitored by UV absorbance (254 nm). Fractions were precipitated and examined by immunoblotting with purified anti-CgtA$_C$ antibodies (27). CgtA$_V$ was found in the 50S fractions and at the top of the gradient (Fig. 3). Thus, CgtA$_V$ is at least partially associated with the 50S ribosomal particle but not with the 30S particle, the 70S monosome, or with translating ribosomes.

**Concluding remarks.** In conclusion, we show that in contrast to previous publications (5, 32, 34, 42) *cgtA$_V$* is an essential gene and that depletion of *cgtA$_V$* does not lead to a cell elongation phenotype. Moreover, CgtA$_V$ is associated with the large ribosomal subunit. It is likely, therefore, that the *V. harveyi* CgtA$_V$ protein plays a cellular role similar to that of other bacterial Obg/CgtA proteins.

We are extremely grateful Susan Sullivan for careful critique of the manuscript and to Patrice Wout for technical assistance. We are also grateful to Grzegorz Węgrzyn for insightful discussions during the course of this study.

This study was supported by a collaborative FIRCA grant (TW006001) from the National Institutes of Health and by NATO funds to J.R.M. and Grzegorz Węgrzyn.

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