Impaired chromosome partitioning and synchronization of DNA replication initiation in an insertional mutant in the *Vibrio harveyi* *cgtA* gene coding for a common GTP-binding protein

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The *Vibrio harveyi* *cgtA* gene product belongs to a subfamily of small GTP-binding proteins, called Obg-like proteins. Members of this subfamily are present in diverse organisms ranging from bacteria to humans. On the other hand, the functions of these proteins in the regulation of cellular processes are largely unknown. Genes coding for these proteins are essential in almost all bacteria investigated thus far. However, a viable *V. harveyi* insertional mutant in the *cgtA* gene was described recently. Therefore, this mutant gives a unique opportunity to study functions of a member of the subfamily of Obg-like proteins. Here we demonstrate that the mutant cells often form long filaments with expanded, non-partitioned or rarely partitioned chromosomes. Such a phenotype suggests impairment of the mechanism of chromosome partition. Flow cytometric studies

revealed that synchronization of chromosome replication initiation is also significantly disturbed in the *cgtA* mutant. Moreover, in contrast to wild-type *V. harveyi*, inhibition of chromosome replication and/or of cell division in the mutant bacteria caused significant increase in the number of large cells, suggesting that the *cgtA* gene product may be involved in the coupling of cell growth to chromosome replication and cell division. These results indicate that CgtA, an Obg-like GTP-binding protein, plays an important role in the regulation of chromosomal functions.

Key words: CgtA protein, chromosome replication, Obg-like protein, ODN family.

INTRODUCTION

Small monomeric GTP-binding proteins have been found in every organism examined thus far. However, relatively little information is currently available about the roles of such proteins in prokaryotes. A subfamily of small GTP-binding proteins, whose members are called Obg (or Spo0B-associated GTP-binding protein) or CgtA (or common GTP-binding protein), was discovered recently in bacteria. These proteins have homologues in diverse organisms ranging from bacteria to humans [1,2]. Until recently, Obg (CgtA) proteins were investigated mainly in bacteria that sporulate or differentiate, like *Bacillus subtilis*, *Streptomyces griseus*, *S. coelicolor* and *Caulobacter crescentus* [1,3–9]. Nevertheless, until now there has been very limited knowledge about the roles of these proteins in the regulation of cellular functions in these organisms. Even less well investigated are functions of proteins homologous to Obg (CgtA) in bacteria that do not sporulate or differentiate, including the most widely used model organism *Escherichia coli*. However, a gene coding for the Obg homologue, *yhbZ*, was found to be essential in *E. coli* [10].

As in *E. coli*, genes coding for homologues of Obg (CgtA) are essential in most other bacteria [1,3,4,8–10]. However, recently a viable insertional mutant in the *cgtA* gene of *Vibrio harveyi* was isolated [11,12] and underwent preliminary characterization [2]. This mutant reveals multiple phenotypes: it is unable to grow in minimal media, its survival in physiological saline is dramatically reduced, it is more mutagenic upon treatment with

different mutagens, its luminescence is less effective and its cells often form long filaments [2]. Although the mechanisms that result in these phenotypes are not known, the viable *cgtA* mutant provides a unique opportunity to investigate the functions of a gene coding for a small GTP-binding protein and to draw conclusions about its specific roles in the regulation of cellular processes. Here we investigated the mechanisms of defects in this mutant that cause cell filamentation, as such a phenotype may suggest impairment of regulation of important processes connected to crucial chromosomal functions. Indeed, we found that chromosome partition may be impaired in the *cgtA* mutant and demonstrate for the first time that synchronization of replication initiation is defective in a mutant deficient in the function of this gene coding for a member of the Obg-like protein subfamily.

EXPERIMENTAL

Bacterial strains and growth conditions

The *V. harveyi* wild-type strain, BB7 [13], and its *cgtA::Tn5TpMCS* derivative, BB7X [2], were used in all experiments. Bacteria were cultivated at 30 °C in the BOSS medium, described previously [14].

Microscopic studies

V. harveyi cells were examined using differential interference contrast under a Nikon Eclipse E800 microscope. Staining with

Abbreviations used: DAPI, 4',6-diaminidino-2-phenylindole; TCA, trichloroacetic acid.

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4',6-diaminidino-2-phenylindole (DAPI) was carried out essentially as described previously [15]. Briefly, culture samples were mixed with DAPI solution ($25 \mu\text{g} \cdot \text{ml}^{-1}$) and left in the dark for 20 min. Before examination under a microscope, the stained cells were placed on a 0.5% agar layer on a microscope slide and covered with a coverslip. Cells were photographed either under white light or using specific filters (330–380 nm).

Flow cytometry

Flow cytometric procedures were performed exactly as described previously [16], using FACStar+ equipment (Becton Dickinson). The cells were gated so as to exclude dead cells according to earlier procedures, described in detail elsewhere [16].

Measurement of DNA synthesis

Synthesis of DNA in bacterial cells was measured by labelling with [^3H]thymidine according to a procedure described previously [17]. Briefly, [^3H]thymidine was added to bacterial cultures up to $0.24 \text{ MBq} \cdot \text{ml}^{-1}$. At the indicated times, absorbance of each culture at 575 nm was measured and the number of living cells was determined by titration on BOSS plates. Samples of cultures ($50 \mu\text{l}$ each) were withdrawn, placed on to paper filters and then transferred immediately to ice-cold 10% trichloroacetic acid (TCA). Following sequential washing in 5% TCA, 1% TCA and twice in 96% ethanol, the filters were dried and radioactivity was measured in a scintillation counter.

Estimation of DNA degradation

DNA degradation of cells was estimated by pulse-chase experiments. Bacterial cultures were labelled with [^3H]thymidine ($0.24 \text{ MBq} \cdot \text{ml}^{-1}$) for 20 min. Then, an excess of unlabelled thymidine (to a final concentration of $2 \text{ mg} \cdot \text{ml}^{-1}$) was added. In some experiments, at the time of chasing, rifampicin and/or cephalixin were added up to 150 or $10 \mu\text{g} \cdot \text{ml}^{-1}$, respectively. Samples were withdrawn every 60 min (addition of unlabelled thymidine was at time 0) for 5 h. Sample preparation and measurement of radioactivity in a scintillation counter were performed as described for procedures of estimation of the efficiency of DNA synthesis.

RESULTS

It was demonstrated previously that cells of the *V. harveyi* *cgtA* mutant often form filaments, although a significant number of normal-sized bacteria are still present in a culture [2]. Such a phenotype suggested problems with regulation of chromosome functions and/or cell division. Here we investigated in more detail the distribution of nucleoids in elongated mutant cells, which might provide information about potential chromosomal defects caused by *cgtA* dysfunction.

Bacteria were stained with a DNA-specific fluorescent reagent, DAPI, and investigated microscopically. The filamentous cells contained expanded, non-partitioned or rarely partitioned chromosomes (typical cells are shown in Figure 1). They resemble phenotypes of *E. coli* *par* mutants, which are defective in the mechanisms of chromosome partition [18].

It should be noted that only long cells of the *cgtA* mutant strain are presented in Figure 1. Although no such long cells were found in cultures of wild-type *V. harveyi*, in cultures of the *cgtA* mutant there were also cells of normal size and moderately enlarged cells, as revealed by measurement of cells under a microscope (Table 1). Flow cytometry indicated less significant differences between sizes of wild-type and *cgtA* mutant cells

Figure 1 Microscopic analysis of *V. harveyi* wild-type (a, d) and *cgtA*: Tn5TpMSC (b, c, e) cells

Bacteria were stained with DAPI and photographed under a light microscope using differential interference contrast. A filter allowing detection of DAPI fluorescence (330–380 nm) was used in the photographs presented in (a–c), and no filter was employed in (d) and (e). (a) and (d), and (b) and (e) are duplicates of the same field obtained using these two methods.

Table 1 Cell sizes of *V. harveyi* wild-type and *cgtA* mutant strains growing under normal conditions

The circumference of randomly chosen cells was measured under a microscope.

Cell circumference (μm)	Number of measured cells		Percentage of total cells	
	Wild-type	<i>cgtA</i>	Wild-type	<i>cgtA</i>
< 4	0	0	0	0
4–6	6	3	1.7	0.8
6–8	225	130	62.5	33.6
8–10	125	195	34.7	50.4
10–12	4	39	1.1	10.0
12–14	0	15	0	3.9
14–16	0	2	0	0.5
> 16	0	3	0	0.8
Total	360	387	100	100

Figure 2 Flow cytometric analysis of *V. harveyi* wild-type (a, c, e, g) and *cgtA*:Tn5TpMSC (b, d, f, h) cells

Panels (a–d) represent the distribution of cells according to their size before treatment with rifampicin and cephalixin (a, b), and 5 h after treatment with these antibiotics (final concentrations of 150 and 10 $\mu\text{g} \cdot \text{ml}^{-1}$, respectively; c, d). Panels (e–h) represent DNA histograms of cells before treatment with rifampicin and cephalixin (e, f), and 5 h after treatment with these antibiotics (g, h).

(Figures 2a and 2b). This ostensible discrepancy between the results obtained using two independent methods may result from the fact that in the flow cytometric experiments cells were gated so as to exclude dead cells, whereas all cells were visible under the microscope.

The process of chromosome partitioning may be impaired but not completely abolished in the *cgtA* mutant, as this strain is still viable, indicating that some chromosome partitioning must occur. Anucleate cells were rare in cultures of the *cgtA* mutant growing under normal conditions (Figures 3b and 3b'). The *cgtA*

mutant was found previously to be able to form colonies on nutrient agar plates, although its growth rate in rich liquid media was decreased [2], indicating that DNA replication occurs in the mutant at levels sufficiently high to support cellular growth. These results support the suggestion that CgtA's function may be required for chromosome segregation/partition and/or proper cell division.

To estimate the efficiency of DNA synthesis in *V. harveyi* wild-type and *cgtA* strains, bacterial cultures were labelled with [^3H]thymidine and incorporation of this precursor into TCA-precipitable material was measured. Since the *cgtA* mutant grows more slowly than the otherwise isogenic wild-type strain [2], to compare the efficiency of [^3H]thymidine incorporation per cell the radioactivity counts were divided by the number of living cells, estimated by plating. As expected, we observed a continuous increase in the amount of the incorporated precursor into DNA of wild-type bacteria (Figure 4). Surprisingly, DNA synthesis per cell was significantly greater in the *cgtA* mutant relative to that in the wild-type strain (Figure 4). These results may suggest impairment of chromosome replication regulation in the absence of *cgtA* function. On the other hand, since DNA synthesis measured *in vivo* comprises both DNA replication and repair, it was not possible to exclude the possibility that increased incorporation of [^3H]thymidine into TCA-precipitable material in the *cgtA* mutant results from enhanced DNA repair, for example due to induction of the SOS stress response. Therefore, we aimed to test regulation of chromosome replication specifically.

To investigate regulation of chromosome replication, we employed flow cytometry. Under normal growth conditions of bacterial cultures, we observed few differences in DNA distribution between cells of the wild-type strain and the *cgtA* mutants (Figures 2e and 2f). These results again support our conclusion (see above) that DNA synthesis itself is not inhibited by *cgtA* dysfunction.

To investigate synchronization of DNA replication, we added rifampicin and cephalixin to bacterial cultures. These antibiotics were found previously to be useful in studies of synchronization of bacterial chromosome replication initiation [16]. Rifampicin inhibits transcription, which causes inhibition of chromosome replication, and cephalixin inhibits cell division. Hence, after addition of these antibiotics to a bacterial culture, the cells are found to have an integral number of chromosomes, which represents the number of origins present in each cell at the time of the drug's action. When initiation of replication from all chromosomal origin regions in a cell is synchronized (as in wild-type cells), the integral number of chromosomes in the cells is 1, 2, 4 or 8. With increasing asynchrony of replication initiation, an increasing number of cells with 3, 5, 6 or 7 chromosomes appear in the culture. After treatment with rifampicin and cephalixin, we found significant asynchrony of replication initiation in the *cgtA* mutant, whereas control cells revealed a relatively high degree of synchronization (Figures 2g and 2h).

One could argue that the shape of the flow cytometric diagram obtained for the *cgtA* mutant cells treated with rifampicin and cephalixin (Figure 2h) might result from DNA degradation rather than from the appearance of a significant number of cells bearing three chromosomes. However, using pulse-and-chase experiments we found no significant DNA degradation in either wild-type or *cgtA* strains untreated or treated with rifampicin and/or cephalixin for as long as 5 h (results not shown). Therefore, we conclude that deficiency of the *cgtA* gene product disturbs synchronization of chromosome replication initiation.

Interesting results were also obtained when cell size was measured using flow cytometry and microscopy. In bacteria growing without antibiotics, considerable but not dramatic

Figure 3 Microscopic analysis of *V. harveyi* wild-type (left-hand panels) and *cgfA::Tn5TpMSC* (right-hand panels) cells

Cells were grown under normal conditions (**a**, **a'**, **b**, **b'**) and treated with rifampicin ($150 \mu\text{g} \cdot \text{ml}^{-1}$; **c**, **c'**, **d**, **d'**), cephalixin ($10 \mu\text{g} \cdot \text{ml}^{-1}$; **e**, **e'**, **f**, **f'**) or both (**g**, **g'**, **h**, **h'**) for 5 h. Bacteria were stained with DAPI and photographed under a light microscope using differential interference contrast. A filter allowing detection of DAPI fluorescence (330–380 nm) was used in the photographs presented in (**a**–**h**) and no filter was employed in (**a'**–**h'**).

differences were observed between the wild-type and mutant strains, namely the culture of the *cgfA* strain contained more cells that were bigger (Table 1, Figures 2a, 2b, 3a, 3a', 3b and 3b'). However, after treatment with rifampicin and cephalixin the size of wild-type cells was more uniform, whereas a large number of very long *cgfA* mutant cells appeared (Figures 2c, 2d, 3g, 3g', 3h and 3h'). Since cephalixin interferes with cell division,

one might speculate that the *cgfA* gene product is involved in the regulation of this process. Therefore, using both microscopy and flow cytometry, we investigated wild-type and *cgfA* cells treated with either rifampicin or cephalixin alone. No significant differences were observed in wild-type cells treated with either of these antibiotics relative to untreated bacteria (Figure 3c, 3c', 3e and 3e'). However, addition of either rifampicin or cephalixin to the

Figure 4 DNA synthesis in *V. harveyi* wild-type (○) and *cgtA*::Tn5TpMSC (■) cells

DNA synthesis was measured by incorporation of [3 H]thymidine into TCA-precipitable material. Radioactivity (c.p.m.) per cell was calculated. Means $\pm$ S.D. for three experiments are presented.

culture of *cgtA* cells and further incubation of bacteria under these conditions for 5 h resulted in formation of larger cells (Figures 3d, 3d', 3f and 3f'). For both wild-type and *cgtA* strains, results of flow cytometric studies with cells treated with either rifampicin or cephalixin were similar to corresponding results of experiments in which both antibiotics were added simultaneously (results not shown, but compare Figures 2c and 2d). These results may suggest impairment of the coupling of cell growth to DNA replication and cell division in the *cgtA* mutant.

DISCUSSION

Although Obg-like proteins are essential for viability of almost all bacteria investigated thus far and have homologues in diverse organisms ranging from bacteria to humans, their roles in cellular processes are almost completely unknown. The most intensively investigated member of this subfamily of proteins is Obg, encoded in the *B. subtilis* genome. It was suggested that Obg may regulate initiation of sporulation [3,4], may be required for stress-dependent activation of transcription factor σ^B [6] and may interact with ribosomes [7]. However, particular functions of Obg in the regulation of sporulation, transcription and, potentially, translation are not known. It was also suggested that Obg may be involved in the control of DNA replication [5], but this proposal was based on indirect observations.

Here we show that the *cgtA* gene product of a Gram-negative non-differentiating bacterium *V. harveyi* is involved in the regulation of chromosome partition, synchronization of DNA replication initiation and the coupling of cell growth to DNA replication and cell division. Since these processes are crucial in maintenance of chromosomal functions, it is tempting to speculate that in other bacteria their disturbance is responsible for the lethal effect of mutations in genes homologous to *cgtA*. As the similarity between different bacterial Obg-like proteins is great [1,2], the probability that they have similar functions is high. However, it remains unknown why, in contrast to other bacteria, *V. harveyi* is able to tolerate *cgtA* disruption, especially

in light of the fact that neither an additional copy nor a homologue of *cgtA* has been found in the *V. harveyi* genome [2].

It is worth noting that some phenotypes of the *cgtA* insertional mutant, e.g. cell filamentation, are exhibited only by part of the strain. One might speculate that, apart from *cgtA* dysfunction, deficiency in another cellular factor is required for the filamentation phenotype to be seen. Activity of this putative factor could differ significantly from cell to cell in a bacterial culture, especially if some cellular processes which are normally strictly controlled were randomized in the *cgtA* mutant. Such randomization would lead to the creation of a population of unequal cells. Unsynchronized initiation of chromosome replication, observed in the *cgtA* mutant, may be an example of randomized processes. On the other hand, the partial filamentation phenotype, observed in the *cgtA* mutant, might arise from the fact that this is an insertion mutation, not a deletion of the gene. We cannot exclude the possibility that complete deletion of the *cgtA* gene would be lethal for *V. harveyi*. However, we failed to construct a viable *E. coli* mutant with an insertion analogous to that present in the *V. harveyi cgtA* mutant (i.e. with an insertion in the same region of the *E. coli cgtA* homologue; R. Dutkiewicz, M. Słomińska, G. Węgrzyn and A. Czyż, unpublished work). This indicates that a portion of the CgtA protein which may remain active in the *V. harveyi cgtA* mutant is not sufficient to support *E. coli*.

The *cgtA* gene product may be involved, directly or indirectly, in the processes of chromosome partition, synchronization of DNA replication initiation and the coupling of cell growth to DNA replication and cell division. Since it is a GTP-binding protein, the latter possibility seems to be more likely. In fact, it was speculated that CgtA may be involved in signal transduction processes [2], which might in turn control some chromosomal functions.

It was suggested recently that all organisms possess genes coding for homologues of bacterial Obg (CgtA) proteins, pointing to the biological significance of this family of GTP-binding proteins, named ODN [19]. Similarly to most bacteria, an Obg homologue is essential for *Saccharomyces cerevisiae* viability [19]. This protein, named NOG1, was found to be localized mainly in the nucleoli of *S. cerevisiae*, *Trypanosoma brucei* and human cells [19]. Therefore, it seems likely that eukaryotic members of the ODN family may play a role in ribosome biogenesis, which is compatible with a recent finding that *B. subtilis* Obg protein co-fractionates with ribosomes [7]. This suggests that ODN proteins may have similar functions in both eukaryotic and prokaryotic cells. Hence, it is also possible that eukaryotic ODN proteins may be involved in the regulation of certain chromosomal functions, in analogy with the CgtA protein of *V. harveyi*.

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