Transcriptional regulation of the *Rhodobacter capsulatus* response regulator CtrA

Molly M. Leung[†], Cedric A. Brimacombe, and J. Thomas Beatty

Department of Microbiology and Immunology, The University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada

Abstract

The *Rhodobacter capsulatus* response regulator CtrA controls the expression of 227 genes, some of which are upregulated by both the phosphorylated and unphosphorylated forms of CtrA. Therefore, CtrA concentration alone, regardless of phosphorylation state, may determine expression of downstream genes, yet little is known about the regulation of *ctrA* in *R. capsulatus*. In this study we used a *ctrA* : *lacZ* fusion plasmid to study the effects of medium composition, growth conditions and growth phase on *R. capsulatus ctrA* gene expression. These experiments indicate that *ctrA* expression is higher when cultures are grown in phototrophic (anaerobic) conditions compared with chemotrophic (aerobic) conditions, and is higher when grown in a minimal medium compared with a rich medium. We used several mutants to investigate possible regulatory pathways, and found that in *R. capsulatus ctrA* is not autoregulated but is regulated by a quorum-sensing system. The expression of *ctrA* increased as cell cultures moved through exponential phase and into stationary phase, with high levels of expression persisting long after culture turbidity plateaued. Although this growth phase-dependent pattern of expression was also observed in a quorum-sensing mutant, the magnitude of *ctrA* expression was about 50 % of the wild-type strain at all phases. Furthermore, reduction of phosphate concentration in the growth medium decreased *ctrA* expression in a culture density-independent manner, whereas reduction of malic acid (carbon source) or ammonium (nitrogen source) concentration had no effect. The regulation of *ctrA* expression in *R. capsulatus* appears to require the coordination of multiple pathways involved in detecting a variety of environmental conditions.

INTRODUCTION

The ability to detect changing conditions and regulate gene expression accordingly is essential for the survival of all organisms. Bacteria use response regulator proteins in two-component and phosphorelay signalling systems for this purpose, and changes in the concentrations of response regulators affect the processes they control. The response regulator protein CtrA is widespread in the alphaproteo-bacteria, and has been found to be essential for cell viability in several species, best-documented in *Caulobacter crescentus* (Barnett *et al.*, 2001; Hallez *et al.*, 2004; Laub *et al.*, 2000, 2002; Quon *et al.*, 1996). In *C.*

Correspondence: J. Thomas Beatty, j.beatty@ubc.ca.

[†]Present address: Department of Chemical and Biomolecular Engineering, UCLA, Los Angeles, CA 90025, USA.

Three supplementary figures are available with the online version of this paper.

crenscentus, the CtrA protein is a global regulator, and transcription of the *ctrA* gene is auto-regulated (Bowers *et al.*, 2008).

In contrast with the well-studied *C. crescentus* and some other species of alphaproteobacteria, the CtrA protein of the nonsulfur purple photosynthetic bacterium *Rhodobacter capsulatus* is not essential for cell survival (Lang & Beatty, 2000). Two functions of the *R. capsulatus* CtrA are to induce flagellum biosynthesis and the production of an unusual virus-like particle called the gene transfer agent (RcGTA) (Lang & Beatty, 2000, 2002; Leung, 2010). A recent transcriptome study identified 227 genes in the *R. capsulatus* CtrA regulon by comparing gene expression of a *ctrA* mutant to the wild-type (WT) strain (Mercer *et al.*, 2010). The majority of these genes (216) were upregulated by CtrA (i.e. increased expression in the WT strain compared with the *ctrA* mutant), by between twofold and more than 20-fold. In addition to the RcGTA and flagellar genes, a suite of potential signal transduction genes was identified as part of the CtrA regulon (Mercer *et al.*, 2010).

Alignment of the *C. crescentus* and *R. capsulatus* CtrA protein sequence shows 71 % overall identity, and 100 % identity in the helix–turn–helix motif that is thought to specify recognition of consensus DNA sequences 5′-TTAA-N7-TTAAC-3′ and 5′-TTAACCAT-3′ (Lang & Beatty, 2000; Laub *et al.*, 2002). However, many of the *R. capsulatus* CtrA-regulated genes lack a recognizable CtrA consensus sequence (Mercer *et al.*, 2010), as in *C. crescentus* (Laub *et al.*, 2002).

Many response regulators are regulated at the post-translational level by processes such as phosphorylation or binding of signalling molecules. However, although in *C. crescentus* the phosphorylated form of CtrA (CtrA~P) binds DNA with high affinity (Biondi *et al.*, 2006; Domian *et al.*, 1999), non-phosphorylated CtrA also binds to DNA to regulate gene expression (Spencer *et al.*, 2009). Analogously, in *R. capsulatus* either the phosphorylated or non-phosphorylated form of CtrA induces RcGTA expression (Mercer *et al.*, 2012). This implies that although the activation of RcGTA expression is dependent on the presence of CtrA, transcription of RcGTA genes occurs regardless of the CtrA phosphorylation state. In contrast, release of RcGTA particles and cell motility are induced by CtrA~P but not CtrA in *R. capsulatus* (Mercer *et al.*, 2012).

Because of the similarities and curious differences in structural and phenotypic properties of the *R. capsulatus* and *C. crescentus* CtrA proteins, we investigated aspects of *ctrA* expression in *R. capsulatus* to improve the understanding of species-specific transcriptional regulation of this widespread alphaproteobacterial gene. Unlike *C. crescentus*, *R. capsulatus* does not undergo asymmetrical cell division, and synchronous cultures have not been obtained. Therefore we investigated *ctrA* expression in response to culture conditions and growth phase, and in several regulatory mutants.

A plasmid containing the *R. capsulatus* *ctrA* promoter region and the first eight codons fused translationally in-frame with the *Escherichia coli* *lacZ* coding sequence was used to monitor *ctrA* expression (transcription and translation). The reporter plasmid yielded data in accordance with independent measurements of *ctrA* mRNA amounts (Lang & Beatty, 2000),

verifying both approaches, and indicating that regulation of transcription is the major process in the control of *ctrA* expression.

The effects of culture conditions on *ctrA* gene expression were studied, using cultures grown in a minimal or rich medium under illuminated phototrophic (anaerobic) or unilluminated chemotrophic (aerobic) conditions. The levels of *ctrA* expression as cultures progress through the exponential phase and long into the stationary phase of growth were also studied. Several mutant strains were evaluated for *ctrA* : : *lacZ* expression, revealing that the *R. capsulatus ctrA* gene is not autoregulated and that the *gtaI* gene is needed for maximal stationary phase expression of *ctrA*. The *gtaI* gene encodes an acylhomoserine lactone synthase (Schaefer *et al.*, 2002) and functions in a quorum-sensing system (Leung *et al.*, 2012). However, culture density alone does not control *ctrA* expression because cultures at a low culture density in stationary phase, due to carbon limitation, expressed *ctrA* : : *lacZ* at the same level as cultures grown to a high cell density in the replete culture medium.

A 5' end of *ctrA* mRNA was mapped, and *cis*-active sequences in the 5' regulatory region of the *ctrA* gene were found by measuring the activity of a series of deletions in a *ctrA* : : *lacZ* fusion plasmid. Promoter -10 and -35 regions were identified, and two inverted repeat sequences were found to be needed for maximal expression of *ctrA*.

Our results show greater variability from the *C. crescentus* model for the functions of *ctrA* in the alphaproteobacteria than previously thought, and that in *R. capsulatus* there are unusual connections between *ctrA* expression, quorum-sensing, culture growth phase and nutrient availability.

METHODS

Bacterial strains and growth conditions

E. coli strain DH10B was used for cloning, and HB101(pRK2013) (Ditta *et al.*, 1985) was used for conjugation of plasmids into *R. capsulatus*. The *E. coli* strains were grown at 37 °C in Luria broth supplemented with appropriate antibiotics for plasmid selection and maintenance at the following concentrations: kanamycin sulphate, 50 µg ml⁻¹; tetracycline/HCl, 10 µg ml⁻¹.

The *R. capsulatus* strains B10 (WT) (Marrs, 1974), BCKF (*ctrA* mutant), BKKR (*cckA* mutant) (Lang & Beatty, 2002), BLKI (*gtaI* mutant), BLKR (*gtaR* mutant) and BLKO (*gtaR/gtaI* double mutant) (Leung *et al.*, 2012) have been described. Except when noted otherwise, *R. capsulatus* strains were grown in either rich YPS (Wall *et al.*, 1975) or minimal RCV (Beatty & Gest, 1981) media under phototrophic (anaerobic) or chemotrophic (aerobic) conditions at ~30 °C. YPS medium contains 0.3 % yeast extract and 0.3 % peptone, whereas RCV medium contains malate as the sole carbon source, and ammonium as the sole nitrogen source (see below). Media were supplemented with tetracycline/HCl (0.5 µg ml⁻¹) and kanamycin sulphate (10 µg ml⁻¹) as required for plasmid maintenance and strain selection. The media used for nutrient-limited experiments were identical to RCV medium except for the following changes: the medium with decreased nitrogen concentration contained 3.8 mM (NH₄)₂SO₄ instead of 7.6 mM [under these conditions cultures produced hydrogen gas,

indicating nitrogen starvation (Hillmer & Gest, 1977)]; the carbon-limited medium contained 10 mM D,L-malic acid instead of 30 mM; the phosphate-limited media contained 1.9 mM, 15 μ M or no phosphate buffer instead of 9.6 mM, and were supplemented with 9.6 mM 3-morpholinopropanesulfonic acid (MOPS).

Culture turbidity was used as a measure of the number of cells ml^{-1} and monitored by measuring light-scattering with a Klett–Summerson photometer (filter no. 66; red); 100 Klett units represents approximately 4×10^8 c.f.u. ml^{-1} . We defined the early stationary phase time point as 4 h, after which culture turbidity changed less than 10 Klett units in a 4 h interval.

Recombinant plasmids

Standard DNA purification, restriction enzyme digestion and other modification techniques were used (Sambrook *et al.*, 1989). Plasmids are listed in Table 1, and descriptions of primers used in the construction of *ctrA* : *lacZ* gene fusion plasmids are given in Table 2. The plasmid pXCA601 contains a promoterless *lacZ* allele with a *Bam*HI site in the eighth codon, with transcription from the plasmid 5' region blocked by a transcription terminator (Adams *et al.*, 1989). Full-length and deleted segments of the *ctrA* 5' region were generated by PCR amplification using WT *R. capsulatus* genomic DNA as the template, and the downstream primer CEPS2 that annealed within the *ctrA* gene and introduced a *Bam*HI site for cloning purposes. The upstream primers annealed from 150 bp to 1.5 kb 5' of the CEPS2 primer and introduced a *Pst*I site. Each amplicon was digested with *Bam*HI and *Pst*I, and ligated with *Bam*HI/*Pst*I-digested pXCA601, yielding in-frame fusions between the eighth codon of the *ctrA* and the *lacZ* genes.

The internal deletion of the *ctrA* promoter region in plasmid p601-17x was created as described above, using primers CEPS5x and CEPS2 to introduce an *Xba*I and *Bam*HI site, respectively, into the amplicon downstream of the deletion, which was ligated into pUC19 as an *Xba*I–*Bam*HI fragment, resulting in the plasmid pUC5x-17. The primers CEPS17 and CEPS11.7x were used to introduce a *Pst*I and an *Xba*I site, respectively, to the upstream amplicon, which was ligated into pUC5x-17 as a *Pst*I–*Xba*I fragment, resulting in the plasmid pUC17x. The *Pst*I–*Bam*HI fragment was excised from pUC17x and ligated into *Bam*HI/*Pst*I-digested pXCA601 resulting in the plasmid p601-17x.

Plasmids p601-SIR and p601-IRD, containing deletions of inverted repeats found in the *ctrA* gene regulatory region, were generated by site-directed mutagenesis. The *ctrA* upstream region was amplified using the oligonucleotide primers CEPS2 and CEPS17, and WT *R. capsulatus* genomic DNA as the template. This amplicon was ligated into pUC19 as a *Pst*I–*Bam*HI fragment, resulting in the plasmid pUC-c17. Site-directed mutagenesis of pUC-c17 using the mutagenic primers IRDR and IRDF to delete the inverted repeat IRD, or primers SIRR and SIRF to delete the inverted repeat SIR, resulting in the plasmids pUC-IRD and pUC-SIR, respectively. These plasmids were digested with *Pst*I and *Bam*HI, and the resulting fragments containing the deletions were ligated with *Bam*HI/*Pst*I-digested pXCA601, resulting in p601-IRD and p601-SIR, respectively.

Promoter sequence analysis, RNA 5' end-mapping and β -galactosidase assays

Analysis of sequences for putative promoter –10 and –35 sites was done using the Softberry bacterial promoter prediction computer program BPROM (<http://www.softberry.com/all.htm>).

Total cellular RNA was isolated from WT *R. capsulatus* cultures grown under phototrophic (anaerobic) conditions in RCV medium and harvested at the early stationary phase using the RNeasy kit (Qiagen). Primer extension assays to map the 5' end of the *ctrA* transcript were performed using the 5' RACE kit (Invitrogen) and the oligonucleotide primers described in Table 2. Abridged anchor primer (AAP) and abridged universal amplification primer (AUAP) were provided in the 5' RACE kit, and the oligonucleotide primers Fustin, Fustout and Fustnest were designed according to the 5' RACE kit manufacturer's specifications. The resultant amplicons were cloned into the pCR4-TOPO vector (Invitrogen) following the suggested protocol, and sequenced using the primers –21M13F and –21M13R.

β -Galactosidase specific activities of cells containing *ctrA* : : *lacZ* fusions were assayed as described previously (Leung, 2010) using sonication to break cells and the Lowry method (Peterson, 1983) with BSA as the standard to measure total protein, which was expressed as Miller units (mg total protein)⁻¹ (MU mg⁻¹).

RESULTS

Culture conditions and *ctrA* expression in WT cells

To begin to study the effects of culture medium nutrients and phototrophic versus chemotrophic energy generation on *ctrA* expression, WT cells containing the *ctrA* : : *lacZ* fusion plasmid p601-17 were collected at early stationary phase (see Methods) and assayed for β -galactosidase specific activity. The maximal growth rates (and cell numbers in the early stationary phase, given as Klett units in parentheses) of these cultures were: phototrophic YPS (513 Klett units) >phototrophic RCV (460 Klett units) >chemotrophic YPS (214 Klett units) >chemotrophic RCV (130 Klett units) (Fig. S1a, available with the online version of this paper). These experiments revealed that WT cells grown in RCV minimal medium under phototrophic (anaerobic) conditions had ~2.5 times more *ctrA* : : *lacZ* expression than cells grown in either YPS-rich medium under phototrophic conditions or cells grown in RCV medium under chemotrophic (aerobic) conditions, and almost five times more *ctrA* : : *lacZ* expression than cells grown in YPS medium under chemotrophic conditions (Fig. 1). These data show that the transcription and/or translation of *ctrA* is regulated in response to growth conditions, and that the mode of ATP synthesis (chemotrophy vs phototrophy) and the composition of the culture medium have additive effects on this regulation.

Expression of *ctrA* and nutrient limitation

To further investigate the effects of growth conditions on the expression of *ctrA*, WT cells containing the plasmid p601-17 (*ctrA* : : *lacZ* fusion) were grown under phototrophic (anaerobic) conditions in minimal, defined, RCV-derived media that contained decreased concentrations of phosphate, nitrogen or carbon source (malic acid). These cultures were

collected at early stationary phase and assayed for β -galactosidase specific activity. While decreased amounts of phosphate diminished *ctrA* : : *lacZ* expression to about 20–50 % compared with replete RCV medium, a decreased amount of nitrogen or carbon source had little effect on *ctrA* : : *lacZ* expression (Fig. 2a). Cultures grown in RCV supplemented with MOPS had increased *ctrA* : : *lacZ* expression, possibly due to increased buffering capability and greater cell concentrations. This indicates that the decreased expression seen in the phosphate-limited media (buffered with MOPS) is a phosphate effect and not a MOPS effect. One effect of phosphate-limited conditions is that cultures enter early stationary phase prior to the other conditions tested. Cultures grown in <1/5 phosphate-limited and carbon-limited media reached similar cell densities, about half that of cultures grown in replete RCV medium (Fig. 2b). Although these cultures plateaued at about the same culture density, cultures grown under carbon-limited conditions had *ctrA* : : *lacZ* expression levels similar to cultures grown in RCV medium, whereas cultures grown under all phosphate-limited conditions had significantly lower levels of expression (Fig. 2a). Thus, this nutrient-dependent control of *ctrA* : : *lacZ* expression is culture-density-independent, and instead relates to the type (phosphate vs carbon) of limitation.

Expression of *ctrA* in regulatory mutant strains

In *C. crescentus*, *ctrA* transcription is autoregulated by the CtrA protein and a cognate sensor kinase CckA. To determine whether *R. capsulatus* *ctrA* gene expression is regulated in a similar manner, the expression of the *ctrA* : : *lacZ* fusion in plasmid p601-17 was compared in *ctrA* mutant, *cckA* mutant and WT strains. After growth to the early stationary phase in minimal RCV medium, these three strains had essentially the same *ctrA* : : *lacZ* expression (Fig. 3). Other experiments comparing *ctrA* : : *lacZ* expression from p601-17 in the WT, *ctrA* and *cckA* knockout cultures grown under phototrophic or chemotrophic conditions in minimal RCV or rich YPS medium indicated that neither CtrA nor CckA affect *ctrA* expression under these conditions (Fig. 1). Furthermore, the growth rate and maximal growth density of the WT, *ctrA* and *cckA* mutant strains were similar when grown under the four growth conditions (Fig. S1).

Because mutation of either *ctrA* or *gtaI* decreased RcGTA expression (Lang & Beatty, 2000; Schaefer *et al.*, 2002), we were interested in determining whether *ctrA* expression is affected by mutation of the *gtaI* gene. GtaI is an acylhomoserine lactone synthase that is needed for quorum-sensing in *R. capsulatus* (Schaefer *et al.*, 2002), which appears to involve the transcription regulator GtaR (Leung *et al.*, 2012). As shown in Fig. 3, *ctrA* : : *lacZ* expression from the p601-17 plasmid in the *gtaI* mutant was less than 50 % of the WT level, indicating that the *R. capsulatus* GtaI protein is needed for maximal induction of *ctrA* transcription. Addition of exogenous C16-HSL to the *gtaI* mutant recovered *ctrA* expression to 77 % of the WT (data not shown). Further investigation into the regulatory role of the GtaI/GtaR quorum-sensing system showed that *ctrA* : : *lacZ* expression in *gtaR* and *gtaRI* mutant strains was similar to WT levels (Fig. 3). The expression profile of *ctrA* in these quorum-sensing mutants is similar to that of RcGTA (Leung *et al.*, 2012), and indicates that GtaR functions as a negative regulator of *ctrA* in the absence of the acylhomoserine lactone synthesized by GtaI. Because CtrA is needed to induce transcription of RcGTA genes (Lang & Beatty, 2000) and our data show that GtaI is needed for maximal expression of the *ctrA*

gene, it is possible that the reduced RcGTA expression in *gtaI* mutants is due to an indirect effect stemming from a decrease in *ctrA* expression.

Growth phase and *ctrA* expression

Previous RNA (Northern) blot and microarray experiments yielded a rough outline of *ctrA* expression over three time points in culture growth phases: very low expression in the mid-exponential phase, higher in the late-exponential, and highest in the early stationary phase of growth (Lang & Beatty, 2000, 2002; Mercer *et al.*, 2010). To validate our use of *ctrA* : : *lacZ* expression as an index of *ctrA* transcription, and improve the understanding of the regulation of *ctrA* expression, a long-term experiment was done by measuring β -galactosidase specific activity in WT cells containing p601-17 at multiple time points, for 6 days after cultures entered the stationary phase (Fig. 4).

Focusing on the data obtained from the WT strain, the amount of *ctrA* : : *lacZ* expression increased from early- to late-exponential phases of growth, and even more in the early stationary phase. These data show that the results obtained with the plasmid-borne *ctrA* : : *lacZ* fusion mirror the results obtained using the Northern blot and transcriptome methods, and confirm that *ctrA* : : *lacZ* β -galactosidase specific activities parallel the amount of *ctrA* mRNA in WT cells. Interestingly, this long-term experiment also indicated that *ctrA* levels in WT cultures continue to increase well beyond early stationary phase (the 30 h time point) (Fig. 4). Therefore, although cultures are in the stationary phase of growth (i.e. no change in cell numbers as indicated by culture turbidity) after about 24 h under our standard conditions, the activity of the *ctrA* : : *lacZ* gene product increases to higher levels in successive days. After 144 h, the β -galactosidase specific activity eventually drops off (Fig. 4), presumably due to cell death.

Similar long-term experiments were done on the *ctrA* and *gtaI* mutant strains. As shown in Fig. 4, the pattern of the *ctrA* mutant is qualitatively almost identical to that of the WT strain. However, although the *gtaI* mutant followed a growth-phase-dependent pattern of *ctrA* : : *lacZ* expression similar to the WT strain, the β -galactosidase specific activity of the *gtaI* mutant was approximately 50 % of the WT value at each time point, with the exception of 168 h. These results confirm that *ctrA* expression can change independently of culture cell density, and indicate that *ctrA* expression is regulated by pathways both independent of and dependent on the GtaR/GtaI quorum-sensing system.

Identification of the *ctrA* promoter and *cis*-active regulatory sequences

The *ctrA* gene is flanked by *ligA* and *sciP* (Fig. 5a). *In silico* analysis of sequences 5' of the *ctrA* gene identified a CtrA binding sequence, two inverted repeat sequences (SIR and IRD) and two sets of potential -10 and -35 sequences (Fig. 5b). An mRNA 5' end of the *ctrA* transcript was mapped by the 5' RACE method to the adenosine residue indicated by the asterisk in Fig. 5(b), confirming the predicted upstream -10 and -35 sequences (nt -208 to -178).

To characterize and confirm putative *cis*-active regulatory sequences in the *ctrA* promoter region, a series of progressively shorter segments of the 1.5 kb sequence 5' of *ctrA* was used to create translationally in-frame fusions of the eighth codon of *ctrA* to the eighth codon of

lacZ (as in p601-17) (Fig. 6). Each *ctrA* : : *lacZ* promoter deletion fusion plasmid (Table 1) was conjugated into the WT strain, cultures were grown in RCV medium under phototrophic (anaerobic) conditions, and cells were collected at early stationary phase and evaluated for β -galactosidase specific activity as a measure of *ctrA* expression. The longest 5' fusion, p601-17, yielded a β -galactosidase specific activity of $\sim 1400 \text{ MU mg}^{-1}$, as did the deletion in plasmid p601-11 (Fig. 6), indicating that all *cis*-active elements necessary for full *ctrA* expression in cells grown under these conditions are found in the p601-11 plasmid. Plasmid p601-11 lacks all sequences 5' of the ninth codon of the *sciP* gene, and so these results show that *ctrA* transcription is independent of possible *sciP* effects *in cis*, such as transcription read-through.

Independent deletion of either of the inverted repeats called SIR (in plasmid p601-SIR) or IRD (in p601-IRD) resulted in little decrease in *ctrA* : : *lacZ* expression (Fig. 6). This is in contrast with the drop to 35 % when both of these inverted repeats were deleted in plasmid p601-11.7. These results indicate that the SIR and IRD inverted repeat sequences have a concerted modulatory role in *ctrA* expression, because the loss of either inverted repeat did not greatly affect expression whereas the loss of both resulted in a great reduction of *ctrA* : : *lacZ* expression. Plasmid p601-9, the fusion lacking all sequences 5' of the promoter (Fig. 5b), yielded the same β -galactosidase specific activities as p601-11.7 (Fig. 6). The similarity in expression between p601-9 and p601-11.7 indicates that the 44 bp sequence difference between these two plasmids (i.e. the sequence between the IRD and -35 sequences) has no role in *ctrA* transcription.

Plasmid p601-7 lacks the upstream confirmed -10 and -35 sites and all further 5' sequences, but contains the possible CtrA binding sequence and the downstream predicted -10 and -35 sites. Plasmid p601-7 yielded β -galactosidase specific activities ~ 10 % of p601-17 in the WT strain (Fig. 6) and similar activity in the *ctrA* mutant (8.7 % of p601-17; data not shown). Plasmid p601-5 contains only 45 bp 5' of the *ctrA* start codon, and yielded little activity in both the WT and *ctrA* mutant strain. The plasmid p601-17x is identical to the plasmid p601-17 except for the absence of 226 bp that include the predicted -10 and -35 sites (Fig. 5b), and yielded almost no expression (Fig. 6). These results support the location of the -10 and -35 sites in the *ctrA* promoter region as shown in Fig. 5(b), and indicate that a positive regulatory protein binds to the SIR/IRD region.

DISCUSSION

In contrast with other alphaproteobacteria such as *C. crescentus*, CtrA is not essential for viability in *R. capsulatus*, yet CtrA is an important response regulator that affects the expression of ~ 227 *R. capsulatus* genes (Mercer *et al.*, 2010), including the RcGTA genes induced by CtrA regardless of phosphorylation state (Mercer *et al.*, 2012). Therefore the concentration of the CtrA protein, as specified by the level of *ctrA* gene expression, may determine the degree of induction of downstream genes. Here, we describe the results of experiments on the regulation of *ctrA* gene expression, with a focus on culture conditions and connections with other regulatory genes.

We found that a combination of growth in a minimal medium and phototrophic (anaerobic) conditions resulted in the highest level of *ctrA* expression. Furthermore, this effect appears to be independent of cell culture density and growth rate. This is because under phototrophic (anaerobic) conditions, although cultures grown in the YPS-rich medium had the highest cell density in the stationary phase, the *ctrA* : : *lacZ* expression was lower than in cells grown in RCV minimal medium. Cells grown in YPS medium under chemotrophic (aerobic) conditions expressed *ctrA* : : *lacZ* at a level similar to that of cells grown under phototrophic conditions (Fig. 1), despite differences in early stationary phase cell culture density. Collectively, these data show that differences in *ctrA* expression in response to growth conditions do not necessarily correlate with a difference in cell culture density or growth rate. In other words, the growth condition-associated modulation of *ctrA* expression is independent of cell culture density and growth rate differences.

Our investigation into the effects of nutrient-reduced RCV-derived media revealed that growth in a medium containing a low concentration of phosphate resulted in a cell density-independent decrease in *ctrA* expression compared with the replete RCV medium, whereas growth in either the nitrogen- or carbon-reduced medium had little effect. The decrease in *ctrA* expression (to 1/2, 1/3 or 1/5 the level in replete RCV medium) paralleled decreases in the phosphate concentration (from 9.6 mM to 1.9 mM, 15 μ M or absent from the medium, respectively) and so there is not a simple linear relationship between *ctrA* expression and phosphate concentration. Similarly, growth of *R. capsulatus* in an RCV-derived medium with a decreased concentration of phosphate resulted in altered RcGTA release compared with replete RCV medium (Taylor, 2004). CtrA was shown to regulate RcGTA expression (Lang & Beatty, 2000, 2002; Mercer *et al.*, 2010) and so we suggest that either a system in common detects phosphate levels and regulates both *ctrA* expression and RcGTA release, or increased RcGTA release under low phosphate concentration stems from a decrease in *ctrA* expression. Mercer *et al.* (2012) proposed that the phosphorylated form of CtrA is needed for release of RcGTA, but we did not address the phosphorylation state of CtrA. It was reported that in *C. crescentus* guano-sine 3',5'-bispyrophosphate (ppGpp) and poly-phosphate decrease the rate of CtrA degradation in response to carbon starvation, although phosphate starvation was not studied (Boutte *et al.*, 2012).

In contrast with *C. crescentus*, the *R. capsulatus* CtrA protein does not regulate transcription of the *ctrA* gene. Rather, we found that *ctrA* expression is affected by the GtaI/GtaR quorum-sensing system in a growth phase-independent manner. The expression of *ctrA* : : *lacZ* in *gtaI* mutant cells (i.e. in the absence of acylhomoserine lactone) was decreased to and remained at approximately 50 % of the level in a WT culture throughout all phases of growth. Thus, in the *gtaI* mutant, *ctrA* expression increased as cultures progressed from log to early stationary phase (Fig. 4), albeit to a lower magnitude than in the WT strain. These observations led us to suggest that aspects of the GtaI-dependent and growth-phase-dependent *ctrA* expression are regulated additively by independent systems. GtaI-dependent and growth-phase-dependent expression patterns of RcGTA were previously observed (Lang & Beatty, 2000; Schaefer *et al.*, 2002), and our findings may indicate a regulatory mechanism based on the regulation of RcGTA transcription by CtrA (Lang & Beatty, 2000). We speculate that GtaI- and growth-phase-dependent changes in *ctrA* expression, and thus

changes in the intracellular concentration of the CtrA protein, may be the cause of increases in RcGTA expression as cultures progress from exponential to stationary phase.

Using promoter prediction software, mRNA 5' end-mapping and promoter deletion studies, we identified –10 and –35 sequences of the major promoter of the *ctrA* gene. A second possible downstream promoter may contribute up to 10 % of the *ctrA* expression. Although a possible promoter sequence is located ~20 bp 3' of a possible CtrA binding sequence, the expression from plasmids p601-5 and p601-7 relative to the full-length fusion in p601-17 was similar in the *ctrA* mutant and WT strains. This indicates that if there is a contribution to *ctrA* expression from this possible promoter, it is ~9–10 % of the total transcription initiated in the 1.5 kb region 5' of the *ctrA* start codon, which we suggest is negligible. If this possible promoter had a measurable effect on *ctrA* transcription in WT cells, there would be a slight decrease in β -galactosidase activity encoded by plasmid p601-17 in the *ctrA* mutant relative to the WT strain, which was not seen (Fig. 3).

Little is known about *R. capsulatus* promoters and no consensus sequence has been published. One study of *Rhodobacter* promoters showed much variability in –10 and –35 sequences, with the predominant aspect being an increase in A/T residues relative to the average of 33.4 % for the entire genome (Swem *et al.*, 2001). We suggest a consensus sequence (TTGAAC...N16...CAAAAT) using previously studied promoters, the *ctrA* and predicted *gtaR* and RcGTA promoters (Florizone, 2006). The *ctrA* major promoter –10 and –35 sequences share 6 of 6 bases and 4 of 6 bases, respectively, with the consensus sequence (Fig. 7). In contrast, the downstream possible promoter –35 and –10 sequences share only 3 of 6 and 2 of 6 bp, respectively, consistent with the low amount of transcription initiation associated with this region of *ctrA* 5' sequences. The *ctrA* transcriptional and translational start sites are separated by 173 bp, and it is conceivable that these sequences are involved in post-transcriptional regulation, but we did not explore this possibility.

We identified two inverted repeats (SIR and IRD) in the *ctrA* promoter region that are necessary for maximal expression of *ctrA*. To test the role of these two inverted repeats in growth-condition-modulated and quorum-sensing-regulated expression of *ctrA*, we measured the β -galactosidase activities from the fusions shown in Fig. 6 in WT cells grown under four different conditions, and in quorum-sensing mutants (*gtaI*, *gtaR* and *gtaIR* mutants). The results showed that these inverted repeats are needed for the growth-condition-modulated (Fig. S2) but not for quorum-sensing-regulated (Fig. S3) expression of *ctrA*. Specifically, two general growth-condition-modulated regulatory patterns disappeared when the inverted repeats were deleted: 1) the higher *ctrA* expression after growth under phototrophic (anaerobic) conditions than chemotrophic (aerobic) conditions; and 2) the higher *ctrA* expression after growth in RCV medium than YPS medium (Fig. S2). Because these inverted repeats are located 5' of the –10 and –35 sequences, we suggest that a transcription factor(s) binds to these sequences to induce transcription initiation. The activity from the promoter deletion plasmids in the quorum-sensing *gtaI* mutant show a similar trend as in the WT strain, but at approximately threefold lower levels. This indicates that the SIR and IRD inverted repeats are not involved in quorum-sensing-mediated regulation of *ctrA*, which is not surprising because although we show that the GtaI/GtaR quorum-sensing

system regulates CtrA, other work (Leung *et al.*, 2012) showed that this quorum-sensing system indirectly regulates *ctrA* expression.

In conclusion, we studied the effects of growth conditions, growth phase and several regulatory proteins on the expression of the *ctrA* gene to better understand the role of the CtrA protein in *R. capsulatus*. As in other species, phosphorylation of CtrA is required for induction of downstream processes, which in *R. capsulatus* include RcGTA release and cell motility; however, CtrA concentration is also important because CtrA in both the phosphorylated and unphosphorylated states induces RcGTA expression (Mercer *et al.*, 2012). It is possible that, as in other species, phosphorylation of the *R. capsulatus* CtrA protein changes the affinity for DNA sequences, and it is conceivable that the RcGTA gene cluster has two CtrA binding sites, one for CtrA~P and one for CtrA, both of which induce RcGTA expression. Because the CtrA-dependent induction of RcGTA genes is not affected by the phosphorylation of CtrA, the cellular levels of CtrA should be tightly controlled. Although the *ctrA* : *lacZ* constructs reflect the frequency of *ctrA* transcription initiation, post-transcriptional regulation through 5' mRNA sequences and translation initiation, our data do not address the possibility of differential protein degradation rates on controlling CtrA concentration. Nevertheless, we discovered that expression of *ctrA* responds to multiple environmental conditions, and its regulation appears to involve multiple pathways and factors. Our work provides a foundation for future experiments to elucidate the details of these pathways and factors, and address the possibility that CtrA degradation contributes to maintenance of biologically active CtrA levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MOPS	3-morpholinopropanesulfonic acid
MU mg⁻¹	Miller units per mg
WT	wild-type

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Fig. 1.

Growth conditions affect *ctrA* expression similarly in WT and regulatory mutant strains. WT, *ctrA* and *cckA* mutant strains containing the *ctrA* : *lacZ* plasmid p601-17 were grown to early stationary phase under four different growth conditions, and β -galactosidase specific activity was determined as a measure of *ctrA* expression ($n = 3$). YPS, rich medium; RCV, minimal medium; PT, phototrophic (anaerobic, illuminated) culture conditions; CH, chemotrophic (aerobic, non-illuminated) culture conditions.

Fig. 2.

Expression of *ctrA* is affected by phosphate limitation in a culture-density-independent manner. Cultures of WT cells containing *ctrA* : *lacZ* plasmid p601-17 were grown under phototrophic (anaerobic, illuminated) conditions in RCV and nutrient-reduced RCV-derived media. The media contained: 9.6 mM phosphate (RCV); 9.6 mM MOPS in addition to phosphate (RCV+M); 1.9 mM phosphate (1/5); 15 μ M phosphate (1/640); no phosphate (trace); 10 mM malic acid (RCV-C); 3.8 mM ammonium (RCV-N). The P-reduced medium was supplemented with MOPS buffer. (a) β -Galactosidase specific activities ($n = 3$). (b) Plot of culture cell density over time; the numbers in the graph give mean culture densities (in Klett units) at time of harvest, which varied by less than 20 % ($n = 3$).

Fig. 3.

Comparison of *ctrA* expression in the WT and *ctrA*, *cckA*, *gtaI*, *gtaR* and *gtaRI* mutant strains. Cells containing the *ctrA* : : *lacZ* plasmid p601-17 were grown to the early stationary phase in RCV medium under phototrophic (anaerobic, illuminated) conditions, and β -galactosidase specific activity was determined as a measure of *ctrA* expression ($n = 3$). The specific activity of WT cells containing the empty plasmid pXCA601 as a control is indicated (p601).

Fig. 4.

Effects of culture growth phase on *ctrA* expression. WT, *ctrA* and *gtaI* mutant cells containing the *ctrA* : *lacZ* plasmid p601-17 were grown in RCV medium under phototrophic (anaerobic, illuminated) conditions. Samples were taken at the times indicated on the horizontal axis, and evaluated for β -galactosidase specific activity as a measure of *ctrA* expression ($n = 3$). Error bars show SD.

Fig. 5.

The *ctrA* gene, surrounding ORFs, and the sequence of the *ctrA* 5' regulatory region. (a) Representation of *ctrA* and flanking orfs. Gene annotations are according to GenBank accession no. CP001312. From left to right: *trmU* is annotated as a tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase; *sciP* encodes a transcription factor; *ctrA* encodes the CtrA response regulator homologue; and *ligA* is annotated as a NAD-dependent DNA ligase. (b) DNA sequence of the *ctrA* 5' region, showing key features. Bent arrows show the *ctrA* and *sciP* start codons. Solid boxes and dashed boxes indicate the -35 and -10 sites predicted by the Softberry program BPROM (<http://linux1.softberry.com/berry.phtml>) and by visual inspection, respectively. Asterisk indicates the mRNA 5' end mapped using 5' RACE. Highlighted in grey is the stop codon of *sciP*. The black horizontal bars indicate the sequences of two inverted repeats, SIR and IRD, that were deleted in plasmids p601-SIR and p601-IRD, respectively. The underlined italicized sequence resembles a CtrA binding sequence. The vertical arrow labelled '2' indicates the 3' juncture of *ctrA* : : *lacZ* fusions. The vertical arrows labelled with odd numbers indicate the 5' end of *ctrA* promoter region deletions. Each arrow is identified with a label that corresponds to the plasmid name (i.e., the arrow labelled '5' corresponds to plasmid p601-5). Numbers on the right of each line indicate the number of bases before or after the *ctrA* start codon.

Fig. 6.

Expression of *ctrA* promoter region deletions. On the left are representations of *ctrA* : *lacZ* fusions. WT cells containing *ctrA* : *lacZ* promoter deletion plasmids (named p601-11, p601-SIR, p601-IRD, p601-11.7, p601-9, p601-7, p601-5 and p601-17x) were grown to the early stationary phase in RCV medium under phototrophic (anaerobic, illuminated) conditions and assayed for β -galactosidase specific activity ($n = 3$), as shown on the right.

Fig. 7.

Alignment of *R. capsulatus* promoter -10 and -35 sequences. Promoters were identified in previous publications (Du *et al.*, 1998; Dubbs *et al.*, 2000; Elsen *et al.*, 2000; Florizone, 2006; Karls *et al.*, 1999; Leung, 2010; Swem *et al.*, 2001; Vichivanives *et al.*, 2000) and this study. The frequency of bases found at each position is indicated by the size of the coloured letters on the bottom, created by the Weblogo 3.0 software (Crooks *et al.*, 2004; Schneider & Stephens, 1990).

Table 1Plasmids used in the *ctrA* : : *lacZ* fusion plasmid and 5' RACE experiments

Plasmid (use)	Description	Marker [*]	Reference or source
<i>ctrA</i> : : <i>lacZ</i> fusion studies			
pXCA601	Promoter probe vector, for construction of promoter : : <i>lacZ</i> fusions	Tc ^R	Adams <i>et al.</i> (1989)
p601-5	47 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pXCA601 as a <i>Pst</i> I to <i>Bam</i> HI fragment	Tc ^R	This study
p601-7	116 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pXCA601 as a <i>Pst</i> I to <i>Bam</i> HI fragment	Tc ^R	This study
p601-9	227 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pXCA601 as a <i>Pst</i> I to <i>Bam</i> HI fragment	Tc ^R	This study
p601-11.7	270 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pXCA601 as a <i>Pst</i> I to <i>Bam</i> HI fragment	Tc ^R	This study
p601-11	379 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pXCA601 as a <i>Pst</i> I to <i>Bam</i> HI fragment	Tc ^R	This study
p601-17	1463 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pXCA601 as a <i>Pst</i> I to <i>Bam</i> HI fragment	Tc ^R	This study
p601-17x	p601-17 with a deletion of the predicted 210 and 235 sites, and surrounding sequences	Tc ^R	This study
p601-SIR	p601-17 with a deletion of the SIR inverted repeat	Tc ^R	This study
p601-IRD	p601-17 with a deletion of the IRD inverted repeat	Tc ^R	This study
pUC19	Cloning Vector, <i>lacZ</i> a (Invitrogen)	Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
pUC19-17	1463 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pUC19 as a <i>Pst</i> I to <i>Bam</i> HI fragment	Ap ^R	This study
pUC5x-17	47 bp 5' of <i>ctrA</i> start codon and the first 9 <i>ctrA</i> codons cloned into pUC19 as a <i>Xba</i> I to <i>Bam</i> HI fragment	Ap ^R	This study
pUC17x	Subclone for the <i>Pst</i> I to <i>Bam</i> HI fragment cloned into p601-17x	Ap ^R	This study
5' RACE			
pCR 4-TOPO	Plasmid used for TA cloning of PCR products	Ap ^R	Invitrogen

^{*} Tc, Tetracycline/HCl; Ap, ampicillin.

Table 2
Oligonucleotides used in the construction of *ctrA* : *lacZ* fusion plasmids and mRNA 5' end-mapping experiments

All primers were generated for this study, except for AAP, AUAP, –21M13F and –21M13R, which were purchased from Invitrogen.

Primer (use)	Primer sequence (5'–3')
Constructing <i>ctrA</i> : <i>lacZ</i> fusion plasmids	
CEPS2	GAGAGGTCGTCGGATCCTCCTCCAC
CEPS 17	GATCTGCAGCGAGACCTCCCGTTC
CEPS 15	CCTTTTCTGCAGGGCCGCGC
CEPS 13	GCCCGACCTGCAGACGACCAC
CEPS 11	GGTGAGCCCCTGCAGCTGAAAAAAG
CEPS11.7	GCGTCGTTGGCTGCAGTCGCGCAAGG
CEPS 9	CGCATGGTCTGCAGGGACGCTC
CEPS 7	GATCGAGGGCTGCAGGCGGAGAAC
CEPS 5	CGTGTTAACCATCTGCAGACAAGGTCG
CEPS5x	CGTGTTAACCATCTAGAGACAAGGTCGAACG
CEPS11.7x	CCTTGCGCGACTCTAGACAACGACG
IRDR	CGATGATCGCCTTGCGCGACTCTAGATCGGCCCGGGTCAGAAT
IRDF	ATTCTGACCCGGGCCGATCTAGAGTCGCGCAAGGCGATCATCG
SIRF	ATCTGAAAAAAGTGATGGTCTAGACGATTCTGACCCGGGCCG
SIRR	CGGCCCGGGTCAGAATCGTCTAGACCATCCACTTTTTTCAGAT
mRNA 5' end-mapping and sequencing	
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
AUAP	GGCCACGCGTCGACTAGTAC
Fustin	GTCCTCCACCAACAAAATCCGCATCC
Fustout	GATGCTGCGAGAGGTCGTCGGGTC
Fustnest	CGCATCCTGGGTTCTCCGCATTA
–21M13F	GTTTTCCCAGTCACGACGTTGTA
–21M13R	CAGGAAACAGCTATGACC