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The *Brucella abortus* CcrM DNA Methyltransferase Is Essential for Viability, and Its Overexpression Attenuates Intracellular Replication in Murine Macrophages

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The CcrM DNA methyltransferase of the α-proteobacteria catalyzes the methylation of the adenine in the sequence GAnTC. Like Dam in the enterobacteria, CcrM plays a regulatory role in *Caulobacter crescentus* and *Rhizobium meliloti*. CcrM is essential for viability in both of these organisms, and we show here that it is also essential in *Brucella abortus*. Further, increased copy number of the *ccrM* gene results in striking changes in *B. abortus* morphology, DNA replication, and growth in murine macrophages. We generated strains that carry *ccrM* either on a low-copy-number plasmid (strain GR131) or on a moderate-copy-number plasmid (strain GR132). Strain GR131 has wild-type morphology and chromosome number, as assessed by flow cytometry. In contrast, strain GR132 has abnormal branched morphology, suggesting aberrant cell division, and increased chromosome number. Although these strains exhibit different morphologies and DNA content, the replication of both strains in macrophages is attenuated. These data imply that the reduction in survival in host cells is not due solely to a cell division defect but is due to additional functions of CcrM. Because CcrM is essential in *B. abortus* and increased *ccrM* copy number attenuates survival in host cells, we propose that CcrM is an appropriate target for new antibiotics.

Changes in DNA methylation patterns signal changes in cellular physiology in both prokaryotes and eukaryotes. In bacteria, DNA methyltransferases not only participate in restriction-modification systems (5) but also play regulatory roles in the cell. For example, methylation of the origin of replication by the Dam methyltransferase governs the timing of the initiation of DNA replication in *Escherichia coli* (6, 29). Dam methylation also contributes to strand discrimination in methyl-directed DNA mismatch repair (19) and plays a role in pathogenesis (10, 12). For instance, changes in Dam methylation patterns control pyelonephritis-associated pilus (*pap*) transcription in uropathogenic *E. coli* by altering the binding of Lrp (leucine-responsive protein) to the *pap* promoter (21, 38). In addition, Dam methylation either directly or indirectly regulates the transcription of a number of genes in *Salmonella enterica* serovar Typhimurium that are induced following infection of the host (12), suggesting that DNA methylation plays a role in the virulence of this organism.

The Dam methyltransferases of *E. coli* and other γ-proteobacteria have a counterpart in α-proteobacteria, a DNA adenine methyltransferase called CcrM (for cell cycle-regulated methyltransferase). This enzyme was originally described in *Caulobacter crescentus*, a bacterium that is easily synchronized and therefore amenable to cell cycle studies (42). Both Dam and CcrM catalyze the transfer of the methyl group from *S*-adenosylmethionine to the adenine of their target DNA sequences. Both enzymes apparently lack cognate restriction enzymes and instead regulate cell cycle events. However, unlike Dam, CcrM is required for cell viability, and its activity is tightly regulated during the cell cycle (36). In *Caulobacter*, CcrM is present only in late predivisional cells, just prior to cell division (36). This pattern of CcrM expression is achieved by strict temporal regulation of *ccrM* transcription (37) and rapid turnover by Lon-mediated degradation (41). The CtrA response regulator, which controls multiple cell cycle events, activates *ccrM* transcription in late predivisional cells (23, 27). Constitutive transcription of *ccrM* throughout the cell cycle, resulting in chromosomes that remain fully methylated at all times, yields elongated cells that divide abnormally and exhibit relaxed control of DNA replication initiation (41, 42). Although CcrM-catalyzed DNA methylation appears to play a role in the initiation of DNA replication, little is known about the other essential functions of this enzyme.

CcrM homologs are widely distributed among the α-proteobacteria, including *C. crescentus*, the nitrogen-fixing soil bacterium *Rhizobium meliloti*, and the animal pathogen *Brucella abortus* (36, 40). CcrM functions and its essential nature are conserved in at least two of these bacteria, *C. crescentus* and *R. meliloti* (40). Alignment of the amino acid sequences of the *Brucella* and *Caulobacter* CcrM homologs reveals extensive conservation (78% similarity) throughout the entire proteins (40). Although the *B. abortus* homolog has an N-terminal extension of 15 amino acids, the putative catalytic and AdoMet binding sites are highly conserved in both proteins (18). Their similar structures suggest that the two enzymes share a common function.

Unlike the free-living bacterium *C. crescentus*, *B. abortus* and

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other members of the α-proteobacteria live in close association with eukaryotic cells. The brucellae are small, nonmotile bacteria that infect many mammalian species, including humans. Because the brucellae are intracellular pathogens, the ability of these organisms to survive and replicate in macrophages is critical to their ability to cause disease in the host (7). The cellular mechanisms used by the brucellae to survive and multiply within host cells, however, are poorly understood (33).

In this report, we show that the *B. abortus ccrM* promoter contains a binding site for the CtrA response regulator, suggesting that the regulation of *ccrM* transcription may be similar to that observed in *C. crescentus*. Moreover, *B. abortus* CcrM is required for cell viability, and increased copy number of the *ccrM* gene results in changes in cell morphology and chromosome replication. Aberrant *ccrM* expression also alters the ability of *B. abortus* to replicate in murine macrophages. Our results indicate that CcrM functions play a role in allowing the brucellae to appropriately adapt to their intracellular lifestyle. This role may be directly related to the role of CcrM in maintaining normal cellular physiology; alternatively, it may be related to other cellular functions of this enzyme, such as controlling the expression of as-yet-undescribed virulence factors.

MATERIALS AND METHODS

Bacterial strains and media. *B. abortus* 2308 is a laboratory strain which is virulent in both natural and experimental hosts. Brucellae were grown at 37°C in brucella broth or on Trypticase soy agar (Difco Laboratories) supplemented with 5% defibrinated bovine blood (BA) and antibiotics as appropriate (kanamycin, 30 μg/ml; chloramphenicol, 5 to 15 μg/ml; and ampicillin, 50 μg/ml).

Primer extension analysis. RNA was isolated using freeze-thaw extraction with acidified phenol (11). Primer extension was conducted as described elsewhere (30) using Moloney murine leukemia virus reverse transcriptase (New England Biolabs). The primer for this reaction was 5′-GCGCGGAAACGCAA TCACCTTTGAT-3′. The corresponding nucleotide sequence was determined using dideoxy chain termination (31) with the same primer.

DNase I protection experiments. DNase I footprinting experiments were performed with purified *C. crescentus* His_6-CtrA that was phosphorylated using a maltose-binding protein–EnvZ fusion protein as described previously (27). The template DNA, a 490-bp *B. abortus ccrM* promoter fragment (P*ccrM* −89 to +402), was generated by PCR with the oligonucleotides 5′-CGGGCTTTCCCT GTGATATT-3′ and 5′-AAGCCCGGATCCTGCAACT-3′ and end labeled with [γ-^{32}P]ATP by use of T4 DNA polynucleotide kinase. The 3′ end of the labeled template was removed by digestion at an introduced *Bam*HI site.

Construction of a *B. abortus ccrM* null mutant. The *B. abortus ccrM* gene has been previously cloned and characterized (40). *Brucella ccrM* was disrupted by removing a 0.4-kb *Hin*dIII-*Xmn*I fragment from the *ccrM* coding region of pRW378 and inserting the chloramphenicol resistance gene (*cat*) from pBlue-Cm2 to generate p378::Cm. The resultant *ccrM*::*cat* cassette was then removed by digestion with *Hin*cII and *Eco*RV and ligated to the *Sma*I site of the pUC-based vector pEX100T, containing the *sacB* and *bla* genes (32), to generate plasmid pGR17. pGR17 was introduced by electroporation into *B. abortus* 2308, and two chloramphenicol-resistant (Cm^r) and ampicillin-resistant (Ap^r) integrants, GR121 and GR122, were selected for further analysis. In strains GR121 and GR122, pGR17 was integrated via homologous recombination 5′ of the native *ccrM* gene (see Fig. 2). Cells were grown in brucella broth in the absence of antibiotic selection to promote recombination, and sucrose-resistant clones were isolated by plating cells on modified Luria-Bertani sucrose plates (containing, per liter: 10 g of tryptone, 5 g of yeast extract, 50 g of sucrose, and 16 g of agar).

Fixation procedures and microscopy. Bacteria were harvested from BA plates, placed in brucella broth, pelleted by centrifugation, and either fixed in buffered neutral formalin (3.7% formaldehyde, 145 mM NaCl, 30 mM KH_2PO_4, 45 mM Na_2HPO_4) for phase microscopy or harvested in 2.5% glutaraldehyde–50 mM sodium cacodylate buffer (pH 7.4) for evaluation by transmission electron microscopy as previously described (4).

For phase microscopy, formalin-fixed cells were immobilized by placing them on a microscope slide with a thin pad of 1% agarose. The cells were observed with a Nikon Eclipse E800 microscope with a Nikon Plan Apo 100×/1.40 objective. The images were acquired with a cooled charge-coupled device camera and processed with Metamorph 4.01 (Universal Imaging Corp.). To ensure that fixation procedures did not influence cell morphology, live cells were also examined with an Olympus BH2 phase-contrast microscope under Biosafety Level 3 conditions.

Flow cytometry. For flow cytometry analysis, the *B. abortus* strains GR129, GR130, GR131, and GR132 were subcultured in brucella broth at 37°C for 6 h (mid-exponential-phase growth). The cells were harvested and fixed in 70% ethanol at 4°C for 4 days. For fluorescence-activated cell sorter analysis, an aliquot of cells was centrifuged at 4,000 × *g*, washed with 1 ml of PBS (10 mM phosphate buffer [pH 7.4], 150 mM NaCl), stained in PBS containing Hoechst dye no. 33342 (10 μg/ml) for 30 min at 37°C, washed again, and resuspended in 1 ml of PBS. For each flow cytometry experiment, DNA content in a population of 10,000 cells was measured with a Becton Dickinson FACStar Plus machine with excitation at 358 nm and emission at 440 nm. The data were collected and analyzed using FlowJo software (Tree Star Inc., San Carlos, Calif.).

Isolation and infection of resident peritoneal macrophages. Macrophages were harvested from the peritoneal cavities of euthanatized 9-week-old BALB/c mice by lavage with Dulbecco's modified Eagle's medium (DMEM)–5% fetal calf serum (FCS) supplemented with 5 U of heparin per ml. Pooled macrophages in 200 μl of DMEM–5% FCS were cultivated in 96-well plates at a concentration of 1.6×10^5 per well at 37°C with 5% CO_2. Cell cultures were enriched for macrophages by washing away nonadherent cells after overnight incubation. *B. abortus* cells were opsonized for 30 min with a subagglutinating dilution (1:2,000) of hyperimmune BALB/c mouse serum in DMEM–5% FCS. Opsonized cells were added to macrophages at a ratio of approximately 40 bacteria per macrophage, and the mixture was incubated at 37°C for 1.5 h to allow time for phagocytosis. At this point, the culture medium was replaced with 200 μl of DMEM–5% FCS containing 50 μg of gentamicin per ml for 1 h to kill extracellular *B. abortus*. Macrophages were then washed three times in warm PBS–0.5% FCS and maintained in DMEM–5% FCS with 12.5 μg of gentamicin per ml. At various times after the addition of 12.5 μg of gentamicin per ml, individual cultures were lysed with 0.1% deoxycholate. The CFU were determined by serial dilution in PBS and duplicate plating on BA and on BA with 15 μg of chloramphenicol or 30 μg of kanamycin per ml. Statistical comparisons were made using Student's *t* test (34). *P* values of less than 0.05 were considered significant.

Biological containment and animal use. All procedures involving live *Brucella* were performed in a Biosafety Level 3 containment facility following Centers for Disease Control and Prevention-National Institutes of Health guidelines (7a). In conducting experiments with animals, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* (7b).

RESULTS

The CtrA response regulator binds the *B. abortus ccrM* promoter. The transcriptional start site of the *B. abortus ccrM* gene was determined by primer extension assays using RNA isolated from wild-type cells. A single transcript initiating 34 bp upstream of the *ccrM* translational start site was detected (Fig. 1B). A comparison of the 5′ untranslated sequences upstream of *ccrM* in *B. abortus* and *C. crescentus* revealed extensive sequence homology and a putative CtrA binding site overlapping the −35 region of the *B. abortus ccrM* promoter (Fig. 1A). In *C. crescentus*, *ccrM* transcription is activated by the phosphorylated CtrA response regulator (CtrA-P) which binds to this region of the promoter (23, 27). Because a CtrA homolog has been identified in *B. abortus* that is 80% identical to the *Caulobacter* protein (J. J. Letesson, personal communication), we investigated the possibility that CtrA binds to the promoter of the *B. abortus ccrM* gene. Based on the extensive amino acid identity shared by the *Brucella* and *Caulobacter* CtrA proteins and because a purified version of the *Brucella* CtrA was not available, we used purified *Caulobacter* CtrA for these studies.

We assessed the binding of the *Caulobacter* His_6–CtrA-P fusion protein to the *Brucella ccrM* promoter by DNase I footprinting analysis (Fig. 1C). Template DNA was generated by PCR and labeled at the 5′ end as described in Materials and Methods. Purified His_6-CtrA was phosphorylated in vitro by use of the *E. coli* EnvZ histidine kinase (27) and was used in the footprinting reactions. As shown in Fig. 1, CtrA-P specifically protected a single 25-bp region from −18 to −42 relative to the transcriptional start site in the *Brucella ccrM* promoter. In both the *Brucella* and the *Caulobacter ccrM* promoters, the protected sequence overlaps the −35 region. These in vitro data suggest that CtrA-P regulates *ccrM* transcription in *Brucella*. The *Caulobacter ccrM* promoter contains two inverted repeat structures that are not found in the *Brucella ccrM* promoter; one encompasses the CtrA binding site, and the other is immediately downstream of the transcriptional start site and includes a pair of CcrM methylation sites (37). These differences in promoter structure suggest that although *ccrM* tran-

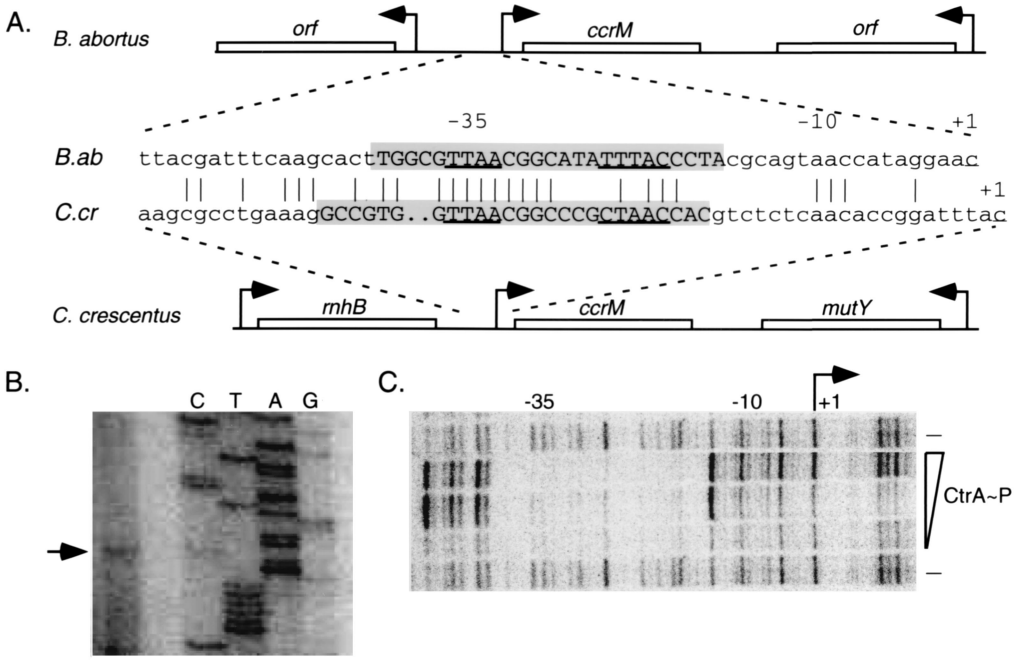


FIG. 1. *B. abortus ccrM* promoter region. (A) Comparison of the genes adjacent to *ccrM* and the *ccrM* promoter regions in *B. abortus* (*B.ab*) and *C. crescentus* (*C.cr*). The CtrA recognition sequence is underlined, and the bases protected by His_6–CtrA-P are in uppercase letters and shaded; identical bases are indicated by vertical lines. (B) Primer extension mapping of the *B. abortus ccrM* transcriptional start site. The first lane shows the primer extension product; the last four lanes show a sequencing ladder generated using the same primer. The lanes are labeled to reflect the sense strand. (C) DNase I protection of the *ccrM* promoter with purified *C. crescentus* His_6–CtrA-P. His_6–CtrA-P concentrations in the reactions were 50, 25, and 10 μg/ml. His_6–CtrA-P was omitted from the lanes marked with a minus sign. The transcriptional start site (+1) and the −10 and −35 elements are indicated.

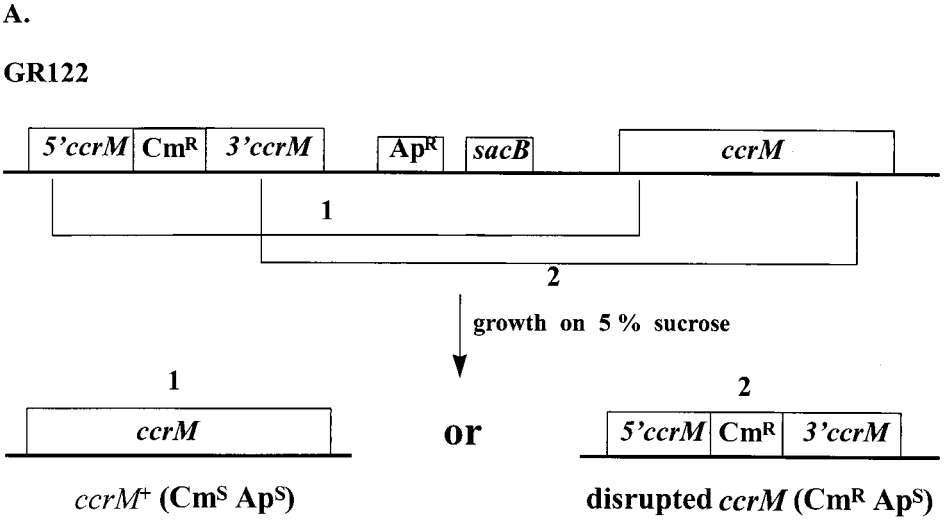
scription is likely to be regulated by CtrA-P in *Brucella*, this bacterium may have additional modes of controlling *ccrM* expression.

***B. abortus ccrM* is essential for viability.** To determine if CcrM is essential for viability in *B. abortus*, we attempted to inactivate *ccrM* using a *sacB* counterselection technique. First, a plasmid-borne *ccrM* gene was disrupted by inserting a *cat* (Cm^r) selectable marker; then, the resulting plasmid, pGR17, was integrated into the *B. abortus ccrM* chromosomal locus, generating strains GR121 and GR122 (Table 1 and Fig. 2A). This recombination event resulted in the separation of the complete wild-type chromosomal copy of *ccrM* and the disrupted *ccrM* gene by a plasmid sequence containing the *sacB* and *bla* (Ap^r) genes (Fig. 2A). Subsequent growth of these

TABLE 1. Bacterial strains and vectors used in this study

B. abortus strain or plasmid	Genotype or description	Reference or source
Strains		
2308	Wild type, virulent, smooth lipopolysaccharide	Laboratory stock
GR121	2308 *ccrM*/*ccrM*::*cat sacB bla*, integration of pGR17 occurred on the 5′ side of *cat*	This study
GR122	2308 *ccrM*/*ccrM*::*cat sacB bla*, integration of pGR17 occurred on the 5′ side of *cat*	This study
GR129	2308 containing plasmid pBBR1MCS	This study
GR130	2308 containing plasmid pGL10	This study
GR131	2308 containing plasmid pRW412	This study
GR132	2308 containing plasmid pRW414	This study
Plasmids		
pBBR1MCS	Chloramphenicol-resistant broad-host-range cloning vector (moderate copy number [10 to 12 copies per genome equivalent]) (9)	15
pGL10	Stable kanamycin-resistant RK2 derivative (low copy number [2 to 4 copies per genome equivalent]) (this study)	K. Andersen
pBlue-Cm2	*cat* amplified by PCR from pBC and cloned into the *Eco*RV site of pBluescript KS(+)	M. E. Kovach
pEX100T	Ampicillin-resistant cloning vector containing the *B. subtilis sacB* gene	32
pRW378	pBBR1MCS with a 2.0-kb *Sal*I-*Eco*RV insert containing the *B. abortus ccrM* gene and promoter region	This study
p378::Cm	pRW378 with an internal 0.4-kb *Hin*dIII-*Xmn*I fragment removed and replaced with *cat* excised from pBlue-Cm2 as a 1.0-kb *Hin*dIII-*Sma*I fragment	This study
pGR17	pEX100T with a 3.1-kb *Hin*cII-*Eco*RV fragment containing *ccrM*::*cat* from p378::Cm in the *Sma*I site	This study
pRW211	pGL10 with a 1.8-kb *Pst*I-*Hin*dIII fragment containing the *R. meliloti ccrM* gene and promoter	This study
pRW412	pGL10 with a 1.6-kb *Xba*I-*Kpn*I fragment containing the *B. abortus ccrM* gene and promoter region	This study
pRW414	pBBR1MCS with a 1.6-kb *Xba*I-*Kpn*I fragment containing the *B. abortus ccrM* gene and promoter region	This study

A.



B.

Strain	Plasmid	Relevant genotype after pGR17 excision (% of isolates): *ccrM*$^+$ Cm^SAp^S	Relevant genotype after pGR17 excision (% of isolates): disrupted *ccrM* Cm^RAp^S
GR121	none	31	0
GR122	none	110	0
GR122	pGL10 (vector control)	49	0
GR122	pRW412 (Ba *ccrM*$^+$)	23	46 (67%)
GR122	pRW211 (Rm *ccrM*$^+$)	15	150 (91%)

FIG. 2. The *B. abortus ccrM* gene is essential for viability. (A) Strategy for disrupting the *B. abortus ccrM* gene. A schematic of the *ccrM* locus in GR122 is shown. Plasmid pGR17 was integrated into the *B. abortus ccrM* chromosomal locus, yielding strain GR122. Growth on 5% sucrose selects for a second recombination event that generates two types of isolates (labeled 1 and 2). (i) If excision occurs 5′ of *cat*, the gene encoding Cm^r, then wild-type *ccrM* is retained and sucrose-resistant colonies will be Cm^s and Ap^s. (ii) If excision occurs on the opposite side of *cat*, then disrupted *ccrM* is retained and sucrose-resistant colonies will be Cm^r and Ap^s. (B) Genotype of the *ccrM* chromosomal locus after growth on sucrose to select for excision of plasmid pGR17. The percentage of isolates that retained disrupted *ccrM* is shown in parentheses. Ba, *B. abortus*; Rm, *R. meliloti*.

strains on 5% sucrose selected for excision of the *sacB* gene and a second recombination event between the homologous regions of the two *ccrM* genes. Cells retaining the wild-type *ccrM* gene and cells with the disrupted gene were distinguished by sensitivity to chloramphenicol. As shown in Fig. 2B, it was not possible to obtain Cm^r Ap^s strains containing only the disrupted *ccrM* gene. To confirm that *ccrM* is essential, we showed that the chromosomal *ccrM* locus could be inactivated when a functional copy of *B. abortus ccrM* was provided on a plasmid. The chromosomal copy of *ccrM* could be disrupted in the presence of pRW412 but not in the presence of the pGL10 vector alone. These data demonstrate that *ccrM* is required for *B. abortus* viability under normal growth conditions. The *ccrM* gene from the closely related species *R. meliloti* also complemented the *B. abortus ccrM* null strain.

Increased *ccrM* copy number alters cell morphology and DNA replication. Constitutive expression of *ccrM* throughout the *C. crescentus* cell cycle and overexpression of *ccrM* in *R. meliloti* cause aberrant morphology and relaxation of the control of chromosome replication (40). To test the effects of increased copy number of the *ccrM* gene on *B. abortus*, the *ccrM* coding sequence and 315 bp of the 5′ region were cloned into the low- and moderate-copy-number vectors pGL10 (2 to 4 copies/cell) and pBBR1MCS (10 to 12 copies/cell) (9). The resulting plasmids, pRW412 and pRW414, were introduced into wild-type *B. abortus* 2308 to generate strains GR131 and GR132, respectively (Table 1). Strain GR132 exhibited a strikingly aberrant morphology. Electron microscopy (Fig. 3) and light microscopy (Fig. 4) showed that the majority of GR132 cells were enlarged and branched. The branched GR132 cells represented single bacteria with atypical morphology and not several attached daughter cells (Fig. 3). A similar branched phenotype has been observed for *R. meliloti* cells that overexpress *ccrM* (40). In this bacterium, the branched phenotype correlates with blocked cell division (16). In both strains GR129 (control) and GR131, the cells were short rods typical of wild-type cells (Fig. 3 and 4).

In *R. meliloti* and *C. crescentus*, multiple copies of *ccrM*

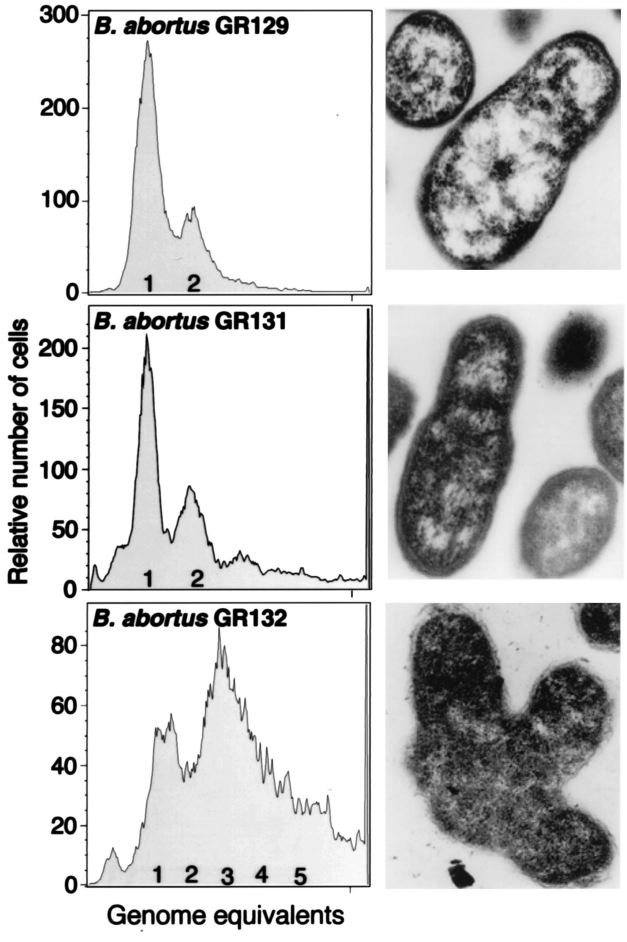


FIG. 3. Effect of overexpressing *B. abortus ccrM* on DNA replication and cell morphology. Shown are flow cytometry analysis and electron micrographs (magnification, ×27,500) of strains GR129 (*B. abortus* 2308 with vector alone), GR131 (*B. abortus* 2308 expressing *ccrM* from a low-copy-number vector), and GR132 (*B. abortus* 2308 expressing *ccrM* from a moderate-copy-number vector). For the flow cytometry analysis, the vertical axis shows the relative number of cells measured by fluorescence intensity, and the horizontal axis shows the DNA content (presented as genome equivalents).

result not only in aberrant cell division and cell morphology but also in relaxation of the control of the initiation of DNA replication (40, 41, 42). To determine if the overexpression of *ccrM* alters the control of DNA replication in *B. abortus*, we used flow cytometry to compare the DNA contents of control strain GR129 and the two strains that have multiple copies of *ccrM* (Fig. 3). In control strain GR129, a single peak representing the two chromosomes in the *B. abortus* genome (13) was observed for 70% of the cells (Fig. 3). The remaining 30% of the cells contained two genome equivalents. Similar results were obtained with control strain GR130 (*B. abortus* containing the pGL10 vector; data not shown). In strain GR131, which carries *ccrM* on a low-copy-number vector, about 40% of cells contained two or more genome equivalents (Fig. 3). Therefore, a small increase in *ccrM* copy number had little effect on DNA content or cell morphology. In contrast, over 70% of GR132 cells contained more than one genome equivalent, and many contained three to five genome equivalents. Many of the GR132 cells were also branched, suggesting a defect in the control of DNA replication or the coupling of DNA replication to cell division in this strain.

Increased *ccrM* copy number attenuates the intracellular replication of *B. abortus* in macrophages. To evaluate the intracellular replication of *B. abortus* strains carrying multiple copies of *ccrM* in cultured murine peritoneal macrophages, macrophages were infected with immunoglobulin G-opsonized *B. abortus* control strain GR129, GR131 (low *ccrM* copy number), or GR132 (moderate *ccrM* copy number). Each strain remained viable during the opsonization and internalization steps. Equivalent numbers of cells were internalized by phagocytosis, as judged by the numbers of intracellular bacteria present after gentamicin treatment (data not shown). As expected, the number of intracellular bacteria decreased dramatically during the first 24 h after infection in all groups (Fig. 4A). However, 36 to 60 h after infection the number of viable intracellular brucellae increased significantly in macrophages infected with the control strain. For both *B. abortus* strains containing multiple copies of *ccrM*, the number of intracellular bacteria increased slightly but not to control levels. These data indicate that the overexpression of *ccrM* did not affect uptake of the bacteria by macrophages but attenuated the intracellular replication of *B. abortus* in these phagocytes. Despite the absence of antibiotic selection in these experiments, no significant loss of vector-encoded antibiotic resistance was detected, indicating that the plasmids were retained throughout the study (data not shown). The difference in antibiotic resistance imparted by pRW414 (kanamycin resistance) and pRW414 (chloramphenicol resistance) also allowed us to verify the identities of GR131 and GR132 following isolation from cultured macrophages. In liquid cultures, strains GR131 and GR132 grew at the same rate as the vector-only controls (data not shown). This result suggests that gross alterations in cell growth rate are not responsible for attenuation of the replication of these strains in macrophages.

Bacteria isolated from macrophages 2, 24, and 60 h after phagocytosis were examined for morphologic abnormalities by light microscopy. As shown in Fig. 4B, cells of the *B. abortus* control strain GR129 and strain GR131 retained wild-type morphology at all times, while the majority of GR132 cells remained enlarged and branched throughout the study. Because the intracellular replication of both GR131 and GR132 was attenuated (Fig. 4A), the aberrant morphology and relaxation of the control of DNA replication observed for strain GR132 (Fig. 3) were not solely responsible for the failure of these strains to replicate within macrophages. Thus, the impaired intracellular replication of the *B. abortus* strains bearing multiple copies of *ccrM* is likely to be due to additional cellular functions of CcrM. Although the data presented in Fig. 4 are the results of a single representative experiment, equivalent results were obtained from multiple experiments.

DISCUSSION

The phylogenetic relationship between *B. abortus* and α-proteobacteria has been well established (20). We have further investigated this relationship by examining the role of the CcrM DNA methyltransferase in the maintenance of normal physiology in *B. abortus*. As in *C. crescentus* and the plant symbiont *R. meliloti*, *ccrM* is required for the viability of *B. abortus*. This requirement is not believed to be due to the restriction of self DNA, as CcrM does not appear to have an associated restriction endonuclease (36). Therefore, CcrM methylation is required for essential cellular processes that appear to be common to at least three members of the α-proteobacteria. The physiologic basis for the essential nature of this enzymatic reaction is not known, although CcrM has been linked to normal cell cycle progression in *C. crescentus* (41, 42),

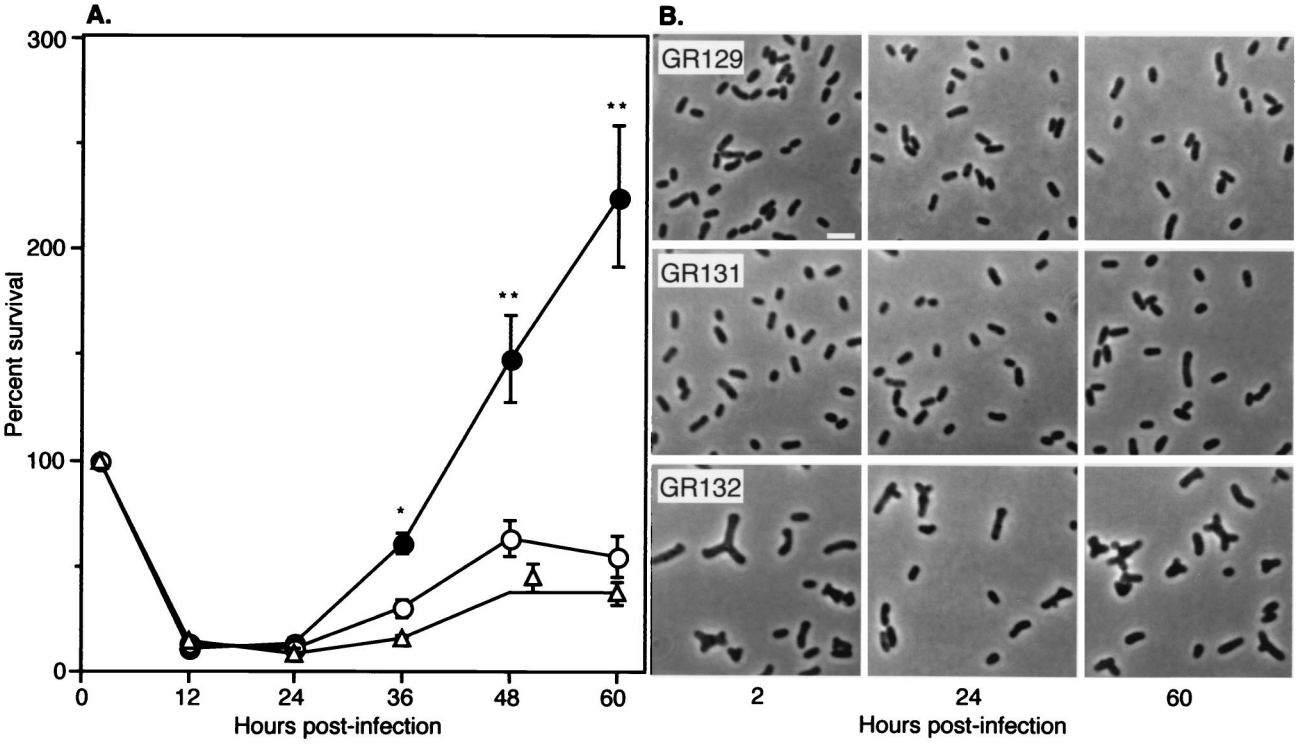


FIG. 4. Survival of *B. abortus* in cultured murine macrophages. (A) Murine peritoneal macrophages were infected with *B. abortus* GR129 (closed circles), GR131 (open circles), and GR132 (open triangles) as described in Materials and Methods. At each time point after phagocytosis, the number of surviving intracellular bacteria in four replicate wells was determined. Percent survival represents the number of intracellular bacteria present at each sampling time divided by the number present at time zero and multiplied by 100. Data are mean ± standard error. Symbols: *, survival of GR132 is significantly different from that of the GR129 control ($P < 0.05$) (Student's *t* test); **, survival of both GR131 and GR132 is significantly different from that of the GR129 control ($P < 0.05$). (B) Morphology of *B. abortus* isolated from macrophages. *B. abortus* strains GR129, GR131, and GR132 were isolated from macrophages 2, 24, and 60 h after infection, fixed in buffered neutral formalin, and examined by phase microscopy. Bar, 2 μm.

and it seems likely that deregulation of this process could be detrimental to the maintenance of normal cellular physiology. Our studies suggest a similar role for CcrM in *B. abortus*, as strains bearing multiple copies of *ccrM* exhibit aberrant cell morphology and relaxation of the control of DNA replication. These processes do not appear to be significantly altered in strains bearing fewer copies of *ccrM*, suggesting that a threshold level of the enzyme must be reached to obtain the gross morphological changes observed with strains bearing higher-copy-number plasmids. In *C. crescentus*, the Lon protease is responsible for clearing CcrM from the cells (41). Perhaps proteolysis can effectively clear CcrM from *B. abortus* cells when it is overproduced at low levels but fails to do so at higher levels.

In addition to the proposed role of CcrM in cell cycle progression in *C. crescentus* (26), there is growing evidence that CcrM-mediated methylation also regulates gene expression. Methylation sites are present in the promoter regions of several *Caulobacter* genes, including the *ctrA* response regulator, the *ftsZ* cell division gene, and the flagellar genes *fliL* and *fliQ* (26). Moreover, expression of the *Caulobacter ccrM* gene appears to be autoregulated by the methylation of its own promoter, as mutation of the GAnTC methylation sites in the mRNA leader region results in prolonged *ccrM* expression (37). These findings support the contention that CcrM-mediated DNA methylation controls the transcription of certain genes and may provide a means to link gene expression to cell cycle progression in this bacterium. *Brucella* and *Caulobacter* are closely related genera with at least two homologous regulatory proteins, CcrM and CtrA. The CtrA response regulator orchestrates cell cycle events in *C. crescentus*. It controls the transcription of both *ccrM* and *ftsZ* (14, 23), initiates biogenesis of the flagellum (23), and inhibits DNA replication initiation (24). The fact that the *Caulobacter* CtrA protein binds to the *Brucella ccrM* promoter strengthens the premise that the *Caulobacter* and *Brucella* CtrA proteins are functionally interchangeable. Our findings also suggest that the transcription of *ccrM* in *B. abortus* is likely to be controlled by CtrA.

In enteric bacteria, Dam methylation plays critical roles in the timing and control of basic physiologic processes, such as the initiation of DNA replication, mismatch repair, and transcription (3). Precise levels of Dam also appear to be required for the virulence of *Salmonella enterica* serovar Typhimurium (10, 12). Specifically, deletion or overproduction of Dam results in severe attenuation of this organism in experimentally infected mice (12). In uropathogenic *E. coli*, Dam methylation also regulates the expression of the *pap* operon (21, 38) and consequently the production of pili, structures known to be significant virulence determinants (8).

Brucellae are intracellular pathogens and, arguably, the true environmental niche for these organisms is within phagosomes of host macrophages (2). Within these cells, *B. abortus* exhibits major changes in gene expression (17), resulting in either the induction or the repression of at least 73 proteins (25). This alteration in gene expression cannot be mimicked in the presence of in vitro stress conditions, such as heat, acid, and nutritional and oxidative stress. These findings suggest that within macrophages, the brucellae are subject to complex global regulation of gene expression. Despite extensive studies to identify dedicated virulence factors for this organism, however,

only a select subset of genetically defined *Brucella* strains demonstrating significant and stable attenuation has been constructed. This subset includes strains with mutations resulting in the loss of normal lipopolysaccharide O-side-chain biosynthesis (1) and the inactivation of genes encoding a type IV secretion apparatus (22), the BvrR-BvrS two-component regulatory system (35), and a putative RNA binding protein (encoded by the *hfq* gene) that appears to be required for the maintenance of stationary phase (28). Intriguingly, the *Brucella hfq* also contains a putative CtrA binding sequence overlapping the −10 region of its promoter and therefore may be under the negative control of this transcriptional regulator. This finding provides indirect evidence that CtrA may regulate the expression not only of *ccrM* but also of at least one additional gene required for survival in macrophages and virulence in BALB/c mice.

The defective intracellular replication in macrophages of the *B. abortus* strains with *ccrM* on low-copy-number plasmids strongly suggests that the physiologic basis for this defect is independent of that which produces aberrant cell morphology. For example, CcrM methylation may control the expression of genes which are specifically required for successful adaptation to the intracellular environment. This activity could be dependent on some function that is directly regulated by DNA methylation status. Alternatively, this activity could be mediated by a second regulator which is itself controlled by DNA methylation. A prime candidate for the latter is the recently identified *Brucella* CtrA response regulator, which has six CcrM methylation sites within the 240 bases immediately upstream of the ATG (G. T. Robertson and R. M. Roop II, unpublished observation). CtrA likely contributes to the regulation of a wide array of cellular functions, including the regulation of *ccrM* and *hfq* transcription (28). Understanding the basis for the essential nature of CcrM in *B. abortus* and the cellular functions that are controlled by this enzyme should provide insights into the mechanisms used by brucellae to adapt to and survive within host macrophages.

It has been proposed that the Dam methyltransferase of the enteric bacteria may represent an ideal target for the design of new antimicrobial agents and the development of vaccines (12). In this same vein, our data suggest that the CcrM methyltransferase is an appropriate target for new antibiotics. CcrM homologs are widely distributed among the α-proteobacteria (40), and this group contains a number of important animal and plant pathogens, including, in addition to *B. abortus*, *Agrobacterium*, *Bartonella*, and *Ochrobactrum* (39). Given that CcrM is essential for the viability of at least three members of the α-proteobacteria, it seems likely that its essential functions may be conserved throughout this group of bacteria.

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