Molecular Microbiology (2002) **43**(4), 945–960

Plasticity of a transcriptional regulation network among alpha-proteobacteria is supported by the identification of CtrA targets in *Brucella abortus*

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Summary

CtrA is a master response regulator found in many alpha-proteobacteria. In *Caulobacter crescentus* and *Sinorhizobium meliloti*, this regulator is essential for viability and is transcriptionally autoregulated. In *C. crescentus*, it is required for the regulation of multiple cell cycle events, such as DNA methylation, DNA replication, flagella and pili biogenesis and septation. Here, we report the characterization of the *ctrA* gene homologue in the α_2-proteobacteria *Brucella abortus*, a facultative intracellular pathogen responsible for brucellosis. We detected CtrA expression in the main *Brucella* species, and its overproduction led to a phenotype typical of cell division defect, consistent with its expected role. A purified *B. abortus* CtrA recombinant protein (His_6–CtrA) was shown to protect the *B. abortus ctrA* promoter from DNase I digestion, suggesting transcriptional autoregulation, and this protection was enhanced under CtrA phosphorylation on a conserved Asp residue. Despite the similarities shared by *B. abortus* and *C. crescentus ctrA*, the pathway downstream from CtrA may be distinct, at least partially, in both bacteria. Indeed, beside *ctrA* itself, only one (the *ccrM* gene) out of four *B. abortus* homologues of known *C. crescentus* CtrA targets is bound *in vitro* by phosphorylated *B. abortus* CtrA. Moreover, further footprinting experiments support the hypothesis that, in *B. abortus*, CtrA might directly regulate the expression of the *rpoD*, *pleC*, *minC* and *ftsE* homologues. Taken together, these results suggest that, in *B. abortus* and *C. crescentus*, similar cellular processes are regulated by CtrA through the control of distinct target genes. The plasticity of the regulation network involving CtrA in these two bacteria may be related to their distinct lifestyles.

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Introduction

The *ctrA* gene (CtrA stands for cell cycle transcriptional regulator A) was first identified in the asymmetrically dividing α-proteobacterium *Caulobacter crescentus* (Quon *et al*., 1996). It encodes an essential response regulator belonging to the OmpR family of two-component signal transduction proteins (Volz, 1993; Stock *et al*., 2000; West and Stock, 2001). *C. crescentus* CtrA is activated by phosphorylation (Domian *et al*., 1997) through a multicomponent phosphorelay signal transduction pathway involving the DivJ and PleC histidine protein kinases (HPKs) and the essential DivK response regulator (Sommer and Newton, 1991; Ohta *et al*., 1992; Wang *et al*., 1993; Hecht *et al*., 1995; Burton *et al*., 1997; Wu *et al*., 1998; Wheeler and Shapiro, 1999; reviewed by Jenal, 2000; Martin and Brun, 2000). In addition, *C. crescentus* CtrA is activated, directly or indirectly, by two other essential kinases, the HPK CckA (Jacobs *et al*., 1999) and the tyrosine kinase DivL (Wu *et al*., 1999).

In *C. crescentus*, CtrA is a global regulator that orchestrates many cell cycle events through the transcriptional control of about a quarter of the cell cycle-regulated genes (Laub *et al*., 2000). CtrA represses DNA replication initiation through regulation of the *hemE* gene, whose promoter overlaps *Cori*, the *C. crescentus* chromosomal origin of replication (Marczynski and Shapiro, 1992; Quon *et al*., 1998; Siam and Marczynski, 2000). Just before cell division, CtrA activates *ccrM* expression, a gene encoding a DNA methyltransferase that specifically recognizes and methylates GAnTC sites, converting the newly replicated hemi-methylated chromosome into the fully methylated form (Stephens *et al*., 1995; 1996; Quon *et al*., 1996; Reisenauer *et al*., 1999a,b). *C. crescentus* cell division also relies on CtrA activity through the negative control of the *ftsZ* gene encoding a tubulin-like GTPase, which polymerizes and forms the so-called septal Z-ring at the site of cell division (Bi and Lutkenhaus, 1991; Kelly *et al*., 1998). CtrA is required for the transcriptional regulation of

many genes involved in flagella and pili biogenesis (Quon *et al*., 1996; Skerker and Shapiro, 2000). *C. crescentus* CtrA controls its own transcription by a positive feedback loop (Domian *et al*., 1999). Interestingly, all these CtrA-regulated promoters in *C. crescentus* present a conserved sequence motif consisting of two TTAA half-sites spaced by seven nucleotides, the TTAA-N_7-TTAA motif (followed most of the time by a C and therefore named the 9-mer consensus) that was shown to be bound *in vitro* by *C. crescentus* CtrA (Ouimet and Marczynski, 2000). Before the cloning of *C. crescentus ctrA*, this sequence motif had been identified in several class II flagellar genes and shown to be essential for proper temporal expression of these genes (Stephens and Shapiro, 1993; Van Way *et al*., 1993; Zhuang and Shapiro, 1995; Quon *et al*., 1996).

Besides *C. crescentus*, *ctrA* is conserved in at least five other α-proteobacteria: *Rhodobacter capsulatus*; *Sinorhizobium meliloti*, a nitrogen-fixing endosymbiont of some leguminous plants; *Agrobacterium tumefaciens*, the causative agent of crown gall disease in plants; *Rickettsia prowazekii*, the aetiological agent of epidemic typhus in humans; and *Brucella abortus*, the causative agent of brucellosis, the last four organisms all being involved in a host–bacteria interaction. In *R. capsulatus*, the-non-essential CtrA and CckA homologues are part of a signalling pathway required for the expression of GTA, a defective prophage that has been adopted by *R. capsulatus* for genetic exchange (Lang and Beatty, 2000). In *S. meliloti*, the *ctrA* gene was shown to be essential for viability and to contain five CtrA binding sites in its promoter, suggesting that it could be autoregulated, as already demonstrated in *C. crescentus* (Barnett *et al*., 2001). The *ctrA* gene is conserved in *A. tumefaciens*, as shown by Southern blot hybridization with a *C. crescentus ctrA* probe (Barnett *et al*., 2001); its sequence was made available recently (GenBank AAK88170). In *R. prowazekii*, there is no experimental evidence that the CzcR response regulator is a CtrA functional homologue, but its 59% identity with *C. crescentus* CtrA (higher than the 35% identity shared by all helix–turn–helix transcriptional regulators) makes CzcR a good CtrA candidate in this organism (Andersson *et al*., 1998; Barnett *et al*., 2001). In *B. abortus*, the *ctrA* homologue (GenBank AF051939) encodes a predicted protein of 232 amino acids that is 93%, 91%, 81% and 77% identical to *S. meliloti*, *A. tumefaciens*, *C. crescentus* and *R. capsulatus* CtrA, respectively, and 56% identical to *R. prowazekii* CzcR (for a multiple alignment, see Barnett *et al*., 2001).

The brucellae are a family of small, non-motile and facultative intracellular Gram-negative coccobacilli, pathogenic for mammals and occasionally for humans. They are responsible for a chronic infectious disease known as brucellosis, a worldwide zoonosis (Enright *et al*., 1990; Corbel, 1997; Boschiroli *et al*., 2001). These class III pathogens require appropriate facilities and precautions, and very few genetic tools are available for their study.

Up to now, the CtrA role in *B. abortus* has been poorly documented. The only indirect data concerning CtrA function in *B. abortus* were obtained by Robertson *et al*. (2000), who showed the *in vitro* binding of *C. crescentus* CtrA to the *B. abortus ccrM* promoter. This result suggests that, in *B. abortus*, *ccrM* transcription is also CtrA regulated and that the CtrA recognition motif might be conserved between *Caulobacter* and *Brucella*. In addition, Robertson *et al*. (2000) showed that overexpression of *B. abortus ccrM* impairs proper intracellular replication in murine macrophages and that it is essential for viability, supporting the hypothesis that CtrA might also be a central regulator in *B. abortus*.

To gain insight into the role of the CtrA central regulator in *B. abortus*, we characterized its function as a response regulator and searched for specific target genes. Our data indicate that this protein is a functional response regulator that is phosphorylated on a conserved Asp residue, binds to specific sites *in vitro* and plays a role in cell division, as demonstrated by the phenotype of an overexpression mutant. Using DNase I footprinting assays, we identified putative *B. abortus* CtrA target genes that are different from those described in *C. crescentus*, supporting the hypothesis of CtrA network plasticity in these two bacteria. The predicted function of the product of these genes suggests that, in *B. abortus*, CtrA could control cellular events similar to those regulated by CtrA in *C. crescentus*, particularly cell division.

Results

Brucella abortus *CtrA is the functional homologue of* C. crescentus *CtrA*

Previous work from our laboratory has shown that the *B. abortus ctrA* gene (GenBank AF051939, unpublished) encodes a predicted 26 kDa protein, containing each of the conserved residues that define members of the response regulator superfamily, including the putative phosphorylation site at position 51 (Volz, 1993; 1995). *B. abortus* CtrA is 93%, 91%, 81% and 77% identical to *S. meliloti*, *A. tumefaciens*, *C. crescentus* and *R. capsulatus* CtrA respectively (Quon *et al*., 1996; Lang and Beatty, 2000; Barnett *et al*., 2001).

Using a rabbit polyclonal antibody raised against the *B. abortus* His_6–CtrA recombinant protein, we observed by Western blotting that *ctrA* is expressed in six *Brucella* reference strains (*B. melitensis* 16M, *B. suis* 1330, *B. abortus* 544, *B. canis* RM 6/66, *B. ovis* 63/290 and *B. neotomae* 5K33) and that these antibodies recognize *C. crescentus* CtrA (data not shown).

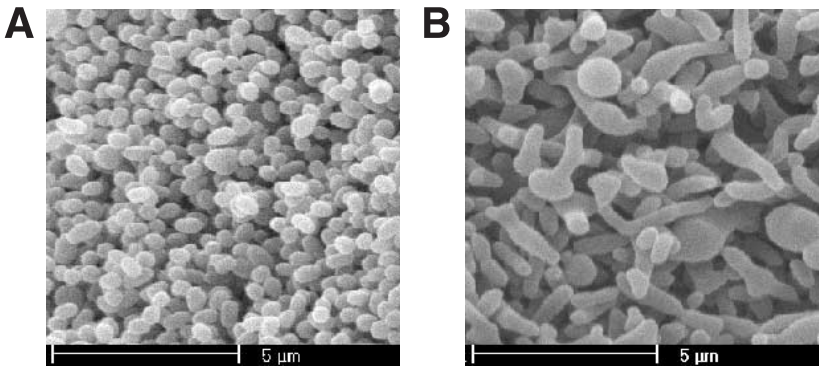

Fig. 1. Morphology of *B. abortus* wild-type strain (A) compared with the *ctrA* overexpressing mutant strain (B). The overproduction of CtrA leads to larger and longer cells, sometimes presenting a branched form. These data strongly suggest that CtrA controls cell division, as already demonstrated in *C. crescentus*. Scanning electron microscopy was performed at the Unité Interfacultaire de Microscopie Electronique (University of Namur, Belgium).

Deletion of the *ctrA* gene was tried using allelic replacement, the classical method for gene inactivation in *Brucella* (Halling *et al.*, 1991). Consistent with the essential feature of *ctrA* in *C. crescentus* and *S. meliloti*, only integrants and no deletants were obtained in each of our numerous attempts to construct a *B. abortus ctrA* deletion strain, even in the presence of a plasmidic rescue copy of wild-type *ctrA*. These results may be explained either by the essential character of *ctrA* in *B. abortus* or by a very low recombination frequency near the *ctrA* gene. In order to study the function of the *ctrA* gene, *B. abortus* was transformed with a plasmid allowing overexpression of *ctrA* from the *Escherichia coli lac* promoter. Overproduction of CtrA in this strain was checked by Western blot (data not shown), and the resulting morphology was examined using scanning electron microscopy (Fig. 1). The bacteria overproducing CtrA are branched and larger than the wild-type control. These phenotypes are consistent with a role for CtrA in the control of cell division in *B. abortus*.

To test whether the protein encoded by *B. abortus ctrA* can be phosphorylated, as expected for a response regulator, we performed a phosphorylation assay on *B. abortus* His_6–CtrA recombinant protein with MBP–EnvZ as the donor kinase, as described previously (Huang and Igo, 1996; Reisenauer *et al.*, 1999b) (Fig. 2). The phosphorylation was not observed in the *B. abortus* His_6–CtrAD51E mutant protein in which aspartate 51 had been replaced by glutamate. These data indicate that the *B. abortus* His_6–CtrA recombinant protein can be phosphorylated *in vitro* on a conserved aspartate, which was shown to be the phosphorylation site in *C. crescentus* CtrA (Quon *et al.*, 1996; Reisenauer *et al.*, 1999b).

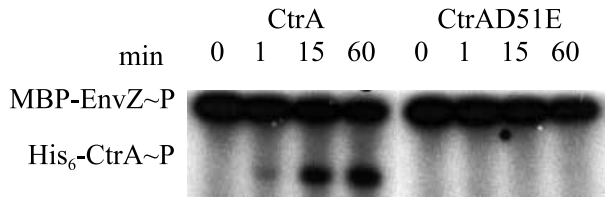

Fig. 2. *B. abortus* CtrA is phosphorylated at conserved aspartate 51 *in vitro*. Purified *B. abortus* His_6–CtrA and His_6–CtrAD51E mutant proteins were incubated with the MBP–EnvZ kinase in the presence of [γ-^{32}P]-ATP at 37°C as described in *Experimental procedures*. Reactions were stopped at the indicated times, and the samples were submitted to SDS–PAGE and autoradiography. After undergoing phosphorylation, MBP–EnvZ phosphorylated His_6–CtrA but not the His_6–CtrAD51E mutant protein, in which the conserved aspartate 51 has been mutated into glutamate.

Brucella abortus *CtrA binds to its own promoter in a phosphorylation-dependent manner*

As *ctrA* is autoregulated *in C. crescentus* (Domian *et al.*, 1999), we checked whether recombinant *B. abortus* CtrA was able to bind to its own promoter. As a first step in studying the *B. abortus ctrA* promoter, primer extension assays were performed on total RNA extracted from *B. abortus* to determine the *ctrA* transcriptional start site(s). We identified three transcripts (P1, P2′ and P2) that are initiated 287, 80 and 65 bp upstream of the *ctrA* translational start codon (Fig. 3A and B). Each transcriptional start site was confirmed with an additional primer (data not shown).

Sequence analysis of the 430 bp upstream of the *B. abortus ctrA* open reading frame (ORF) revealed putative CtrA-binding motifs, according to the *C. crescentus* TTAA-N_7-TTAAC 9-mer consensus (Fig. 3A). The binding of CtrA to its promoter was assessed by DNase I protection analysis (Fig. 3C). His_6–CtrA protected two regions, named $Fp1_{ctrA}$ and $Fp2_{ctrA}$ in Fig. 3C. In both cases, the protection was enhanced when His_6–CtrA was phosphorylated. $Fp1_{ctrA}$, located from nucleotides (nt) –354 to –311 regarding the predicted ATG, is 44 bp in size and overlaps the –35 region of the P1 promoter. This large protected region overlaps one complete CtrA box preceded by an orphan TTAA half-site, indicating that CtrA might bind as a monomer, as already demonstrated for *C. crescentus* CtrA (Ouimet and Marczynski, 2000). $Fp2_{ctrA}$, located from nt –106 to –81, is 26 bp in size and overlaps the –10 and –35 regions of the P2′ and P2 promoters respectively. The $Fp2_{ctrA}$ region is centred over a CtrA-binding motif composed of an upstream relaxed half-site and a per-

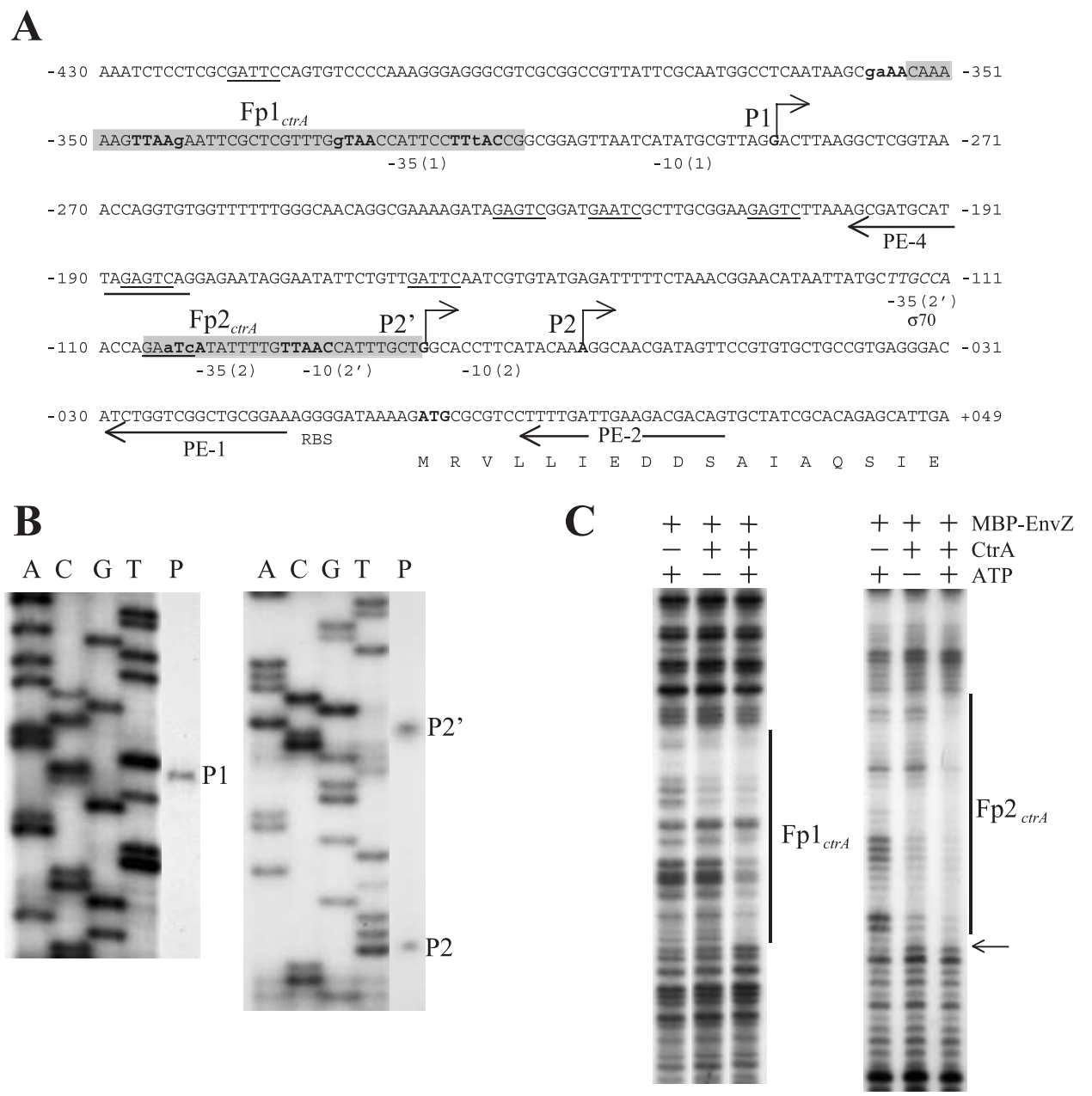

Fig. 3. Mapping of *ctrA* transcription start sites and identification of CtrA binding sites in the *ctrA* promoter.
A. 5′ non-coding region and beginning of *ctrA* coding sequence. The P1, P2′ and P2 start sites are shown by bent arrows. The corresponding –10 and –35 regions are marked below the sequence. Numbers 1, 2′ and 2 in brackets refer to P1, P2′ and P2 start sites respectively. The P2′ –35 element similar to the σ^{70} consensus sequence is in italic. The presumed translational start codon is in bold, and the ribosome binding site (RBS) is indicated. The CtrA recognition motifs are in bold, with lower case when diverging from the TTAA-N_7-TTAAC consensus. The two regions protected from DNase I by phosphorylated CtrA are shaded ($Fp1_{ctrA}$ and $Fp2_{ctrA}$). Oligonucleotides used for primer extension analysis (PE-1, PE-2 and PE-4) are indicated by arrows below the sequence. The CcrM recognition sites (GAnTC) are underlined.
B. Start sites of *B. abortus ctrA* transcription as determined by primer extension. Sequencing reactions (labelled A, C, G and T) are shown next to the primer extension products (lane P).
C. DNase I footprinting experiment on the *B. abortus ctrA* promoter with purified His_6–CtrA. Template DNA was labelled at either the upstream or the downstream end with regard to promoter orientation, as described in *Experimental procedures*. MBP–EnvZ was present in all the samples, whereas purified His_6–CtrA was omitted in the control lanes. Non-phosphorylated CtrA (–ATP) and phosphorylated CtrA (+ATP) were tested for binding to the *ctrA* promoter. The hypersensitive site is indicated by an arrow.

fect downstream half-site, regarding the *C. crescentus* consensus (Fig. 3A).

These *in vitro* data strongly support the hypothesis that phosphorylated CtrA regulates its own transcription in *B. abortus*, as already shown *in vivo* for *C. crescentus* (Domian *et al*., 1999) and *in vitro* for *S. meliloti* (Barnett *et al*., 2001). It is interesting to note that seven GAnTC sites are present in the *ctrA* promoter, one upstream of P1 and the other six upstream of P2′ and P2, suggesting that CcrM-dependent DNA methylation might also control *ctrA* expression.

CtrA binds to B. abortus ccrM *but not to* B. abortus divK*,* ftsZ *or the origin of replication, all known CtrA targets in* C. crescentus

In *C. crescentus*, CtrA was shown to control DNA methylation and cell division through the regulation of *ccrM* and *ftsZ* expression (Quon *et al*., 1996; Kelly *et al*., 1998) and to inhibit DNA replication initiation by binding to the replication origin of the unique *C. crescentus* chromosome (Quon *et al*., 1996; 1998; Domian *et al*., 1997). In *C. crescentus*, CtrA was also shown to regulate the pathway leading to its own activation by controlling *divK* expression (Laub *et al*., 2000). In order to study the putative conservation of these mechanisms in *Brucella*, we cloned the *B. abortus ftsZ* homologue as well as a putative chromosomal replication origin, as described in *Experimental procedures*. In contrast to *S. meliloti*, it is proposed that *Brucella* would display a unique *ftsZ* gene, as supported by similarity searches in *B. suis* and *B. melitensis* genomes as well as by Western blot analysis; indeed, a single band of about 70 kDa was detected in *B. abortus* total extracts using a polyclonal antibody against *C. crescentus* FtsZ (data not shown). As predicted by Brassinga *et al*. (2001), we identified a chromosomal origin of replication (*Bori*) in the *hemE*–RP001 region by autonomous plasmid replication assay. The *B. abortus ccrM* gene was cloned by Wright *et al*. (1997), and the *B. abortus divK* homologue was previously cloned in our laboratory (GenBank AF051940, unpublished).

DNase I protection assays were performed on the *B. abortus ccrM*, *divK* and *ftsZ* homologues and on *Bori*. Phosphorylated *B. abortus* His_6–CtrA protected two regions in the *ccrM* promoter (Fig. 4), as expected from the sequence analysis revealing two 9-mer motifs resembling putative CtrA boxes (Fig. 4A). $Fp2_{ccrM}$ is centred over the more proximal 9-mer motif, with regard to the transcriptional start site, and overlaps the –35 element. This region was shown previously to be DNase I protected by *C. crescentus* CtrA (Robertson *et al*., 2000). In addition, we observed a second DNase I protection (region $Fp1_{ccrM}$), 27 bp in size, centred over the more distal 9-mer motif. We assume that the use of the right protein/target homologous system has permitted the detection of this new protected region, compared with the similar experiment performed with *C. crescentus* CtrA (Robertson *et al*., 2000).

Sequences resembling putative CtrA boxes were also found in the *divK* homologue promoter and in *Bori* (Fig. 6), whereas no boxes were detected in the *ftsZ* promoter. The *C. crescentus divK*, *Cori* and *ftsZ* sequences contain one, five and one CtrA binding sites respectively. Using the same footprinting conditions as those applied to the *ctrA*

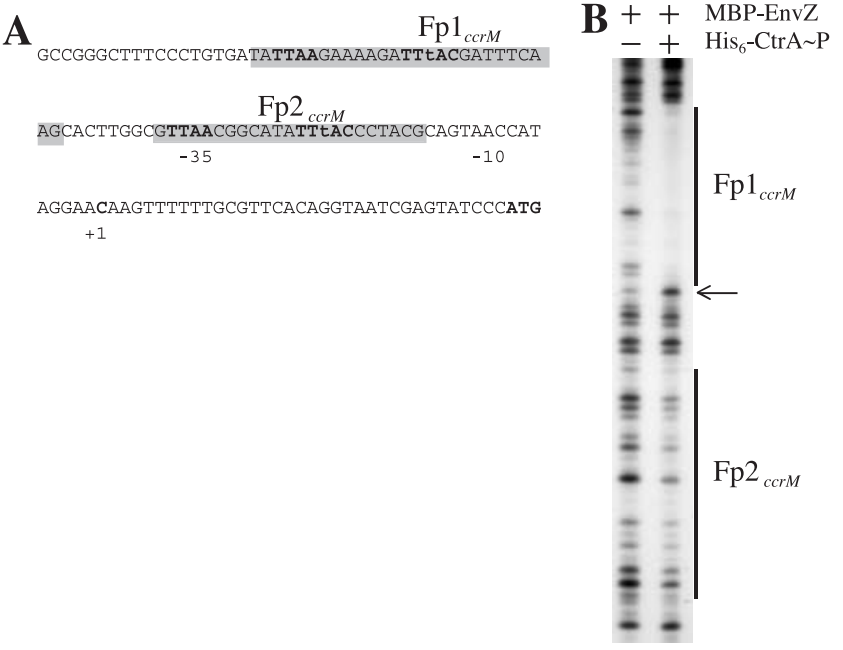

Fig. 4. *B. abortus* phosphorylated CtrA binds to two regions in the *ccrM* promoter.
A. Sequence upstream of the *ccrM* coding region. The transcriptional +1 site and the –10 and –35 elements are marked below the sequence (Robertson *et al*., 2000). The predicted translational start codon is in bold. The two CtrA recognition motifs are in bold, and the bases protected by phosphorylated His_6–CtrA are shaded.
B. DNase I protection of the *ccrM* promoter. Phosphorylated CtrA was omitted in the control lane. The vertical lines labelled $Fp1_{ccrM}$ and $Fp2_{ccrM}$ mark the protected regions. The hypersensitive site is indicated by an arrow.

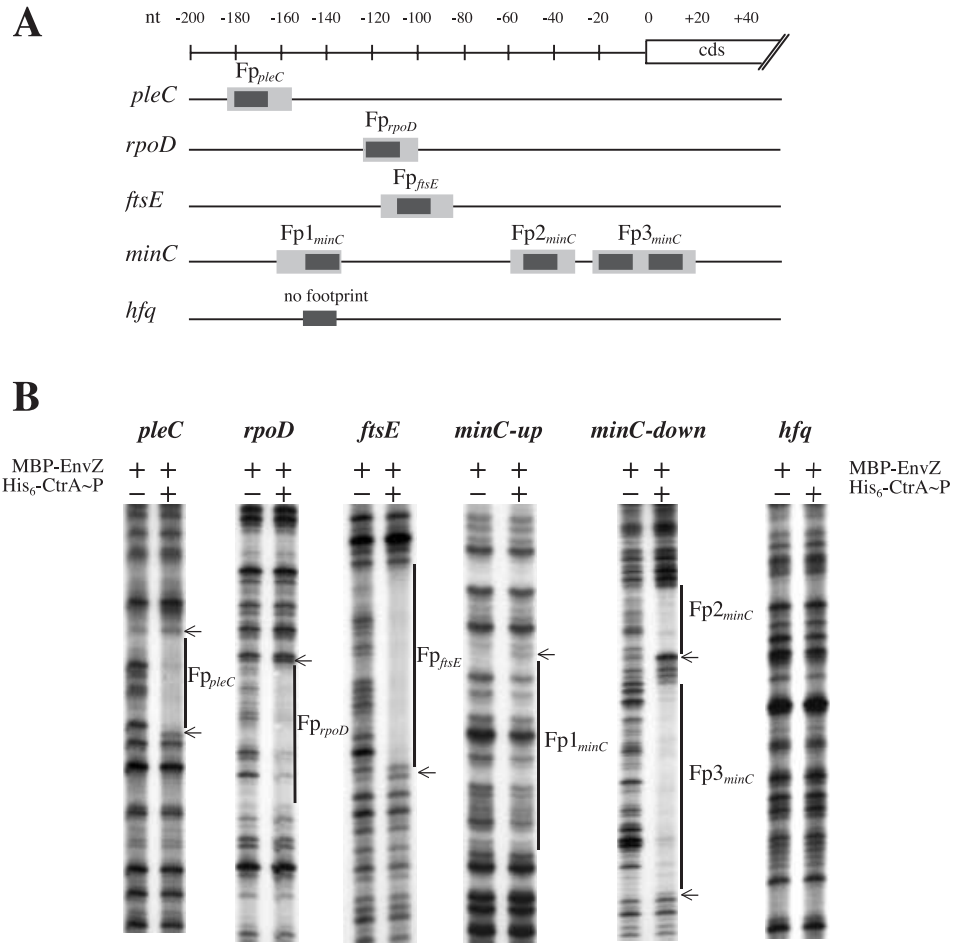

Fig. 5. Binding of His$_6$–CtrA to new *B. abortus* targets.
A. Diagram of the promoter regions of the *B. abortus pleC*, *rpoD*, *ftsE*, *minC* and *hfq* genes. The putative CtrA recognition motifs are shown as dark grey boxes, and the regions protected from DNase I digestion by phosphorylated CtrA as light grey boxes.
B. DNase I protection assays with phosphorylated CtrA on *pleC*, *rpoD*, *ftsE*, *minC* and *hfq* promoters. Phosphorylated CtrA was omitted in the control lanes. The vertical lines (labelled Fp) mark the protected regions. Arrows indicate hypersensitive sites.

and *ccrM* promoters, no protected regions were observed in the *B. abortus ftsZ* and *divK* homologues and *Bori* templates (data not shown). This result suggests that *ftsZ* and *divK* homologues and *Bori* may not be regulated by CtrA in *B. abortus*, at least directly, contrary to what is described in *C. crescentus*. This is the first indication that the pathway downstream from CtrA, besides partial conservation through the regulation of *ccrM* and *ctrA*, may vary from one organism to another.

CtrA binds to different target genes in B. abortus *and* C. crescentus

To explore further the hypothesis that CtrA could regulate target genes in *B. abortus* other than those already described in *C. crescentus* (Laub *et al.*, 2000), we looked for *Brucella* genes containing the CtrA binding consensus in their promoters. We followed two approaches to select *B. abortus* promoters to be tested in DNase I protection assays for CtrA binding.

First, we identified a priori CtrA target candidates on the basis of the literature and using *Brucella* genomes. As the DivK response regulator is part of a phosphorelay involving the HPKs PleC and DivJ in *C. crescentus* (reviewed by Martin and Brun, 2000), we looked for *pleC* and *divJ* homologues in the *Brucella* genomes available (*B. melitensis* 16M at the University of Scranton and *B. suis* 1330 at TIGR). We identified three *Brucella* sequences encoding putative HPKs of 783, 1035 and 609 residues. We chose HPK$_{783}$ for further studies, because it is a close homologue of PleC and exhibits eight of the nine nucleotides forming the canonical CtrA box as established in *C. crescentus* (Fig. 6). The *B. abortus hfq* gene, encoding HF-I (host factor I), also retained our attention. It is expressed only in the stationary phase, is necessary to *B. abortus* virulence and has been proposed to be CtrA

A Sequences positive for CtrA binding

	1 5 10 15 20
Fp1$_{ctrA}$-up	agc gaAA caaaaag TTAAg aat tcgc
Fp1$_{ctrA}$-down	tcgt ttg gTAA ccattcc TTtAC cgg
Fp2$_{ctrA}$	aga aTcA tattttg TTAAC cat ttgctg
Fp1$_{ccrM}$	ata TTAA gaaaaga TTtAC gat ttcaagc
Fp2$_{ccrM}$	gcg TTAA cggcata TTtAC cct acgc
Fp$_{pleC}$	cg att TTTA cgcctcg TTAAC gaa ttcact
Fp$_{rpoD}$	agg TTAA tccgcca TTAAC agg aaat
Fp$_{ftsE}$	ggcg aga TTAA ccgacg TTAAC cat agggga
Fp1$_{minC}$	cctttta tcg TgAA taatccc TTAAa gtg
Fp2$_{minC}$	g aaa TTAA ccttccg TTAAC cat tccgcc
Fp3$_{minC}$-up	c ctc TTAA cttgccg TTAAC caa
Fp3$_{minC}$-down	aga TgAA tcaagtg cTAAC aga aactcg

B

	1 5 10 15 20
***B. abortus* consensus**	ann TTAA C/t nnnnng TT A/t AC nat
C. crescentus consensus	TTAA nnnnnnn TTAAC

C Sequences negative for CtrA binding

	1 5 10 15 20
divK promoter	tca aTgA aatggag TTAAa ttg
hfq promoter	ctg TTAA ccgaagc TaAAg agc
Bori-1	aaa TgAA actgatc TTAAt ccc
Bori-2	caa TTgg taaccta TTAAa tta

Fig. 6. A. Alignment of 12 *B. abortus* CtrA-binding motifs identified in six *B. abortus* promoters that were protected from DNase I digestion by phosphorylated His$_6$–CtrA. The nucleotides that are underlined were not CtrA protected in DNase I assays. Asterisks in the Fp2 region of the *ctrA* promoter indicate the nucleotides that were mutated (TTAA becoming AATT) to abolish CtrA binding, as a negative control (data not shown). Both Fp1$_{ctrA}$ and Fp3$_{minC}$ cover two CtrA boxes; therefore, they were split into Fp$_x$-up and Fp$_x$-down with regard to promoter orientation. The last base in Fp1$_{ctrA}$-up and the first base in Fp1$_{ctrA}$-down are successive; the last base in Fp3$_{minC}$-up is the first base in Fp3$_{minC}$-down.
B. Alignment of the deduced *B. abortus* CtrA binding consensus with the *C. crescentus* consensus. The *B. abortus* CtrA binding consensus was established by collecting the bases that were abundant (present in at least half the sites protected) at a given position.
C. Alignment of the putative CtrA boxes found in *divK*, *Bori* and *hfq* templates that were negative for CtrA binding.

regulated on the basis of a putative CtrA box overlapping the –10 element in its promoter (Figs 5A and 6A; Robertson and Roop, 1999).

Secondly, we searched for the 9-mer consensus motif TTAA-N$_7$-TTAAC in the *B. melitensis* genome. This search led to the identification of more than 30 putative binding sites and, among them, three putative CtrA targets were selected: (i) an *rpoD* homologue encoding a putative protein of 672 residues sharing 82%, 68% and 48% identity with the principal sigma factor of RNA polymerase in *S. meliloti*, *C. crescentus* and *E. coli* respectively; (ii) a *minC* homologue, part of a conserved *minB* operon (with *minD* and *minE* homologues), that codes for a putative protein of 248 residues displaying 40% and 31% identity with the *Mesorhizobium loti* (Kaneko *et al*., 2000; http://www.kazusa.or.jp/rhizobase/) and *E. coli* MinC cell division inhibitors respectively; (iii) an *ftsE* homologue encoding a putative protein of 219 residues sharing 83%, 59% and 46% identity with the FtsE cell division ATP-binding protein in *M. loti, C. crescentus* and *E. coli* respectively. *Brucella rpoD*, *ftsE* and *minC* homologues present one, one and two canonical 9-mer motifs, respectively, in their 5′ non-coding regions. In addition, the *minC* homologue promoter contains two additional imperfect 9-mer motifs (Figs 5A and 6A). It is worth mentioning that there are no detectable CtrA boxes in the *C. crescentus ftsE* and *rpoD* promoters (Laub *et al*., 2000), whereas *minC* is absent from the *C. crescentus* genome (Nierman *et al*., 2001; Quardokus *et al*., 2001).

DNase I footprinting experiments were thus performed on the five *B. abortus* genes of interest (*pleC*, *rpoD*, *minC*, *ftsE* and *hfq*) with phosphorylated His$_6$–CtrA. DNA fragments of about 300 bp, covering the 5′ non-coding regions, were generated by polymerase chain reaction (PCR) from *B. abortus* genomic DNA (primers are listed in Table 1). We observed CtrA protections on all the DNA templates with the exception of *hfq* (Fig. 5B). This result suggests that *hfq* is not a direct CtrA target in *B. abortus*. In the promoters of *B. abortus pleC*, *rpoD* and *ftsE* homologues, the protected region was centred, as expected, over the predicted CtrA boxes (Figs 5A and 6A). On the more complex *minC* promoter bearing four putative CtrA-

binding motifs, we observed three protected regions (Fig. 5). $Fp1_{minC}$ and $Fp2_{minC}$ are 25 bp and 27 bp in size, respectively, and overlap the two distal CtrA-binding motifs, whereas $Fp3_{minC}$, the third protected region, is 48 bp in size and overlaps the last two predicted CtrA boxes (Fig. 6A). Interestingly, the fourth CtrA box overlaps the predicted translational start site of the *B. abortus minC* homologue.

Figure 6 presents the alignment of all the regions protected by CtrA from DNase I digestion that were identified in this work as well as the *B. abortus* sequences containing putative CtrA boxes that were negative in footprint (*divK*, *hfq* and *Bori*). Comparative analysis of these sequences has permitted us to deduce a *B. abortus* CtrA binding consensus (annTTAA$^{C}/_{t}$-N_5-gTT$^{A}/_{t}$ACnat) that highlights the relative importance of nucleotides that are located outside the canonical *C. crescentus* CtrA box and were identified by their abundance in the bound templates (Fig. 6A) and their absence in the negative ones (Fig. 6C).

These footprinting data, which will be discussed further, are consistent with the hypothesis that, in *B. abortus*, CtrA specifically binds to and regulates the *ctrA*, *ccrM*, *rpoD*, *pleC*, *minC* and *ftsE* genes.

Discussion

In *C. crescentus*, the CtrA transcriptional regulator plays a key role in controlling many cell cycle-related events, including DNA replication initiation, cell division initiation, DNA methylation and flagellar biogenesis (Quon *et al*., 1996; 1998; Kelly *et al*., 1998; Reisenauer *et al*., 1999b). CtrA homologues are described in *R. capsulatus* (Lang and Beatty, 2000), *S. meliloti* and *A. tumefaciens* (Barnett *et al*., 2001), bacteria that all belong to the alpha subfamily of proteobacteria.

The data presented here strongly support the hypothesis that the gene characterized in this study is the *B. abortus* functional homologue of the well studied *C. crescentus* and *S. meliloti ctrA* genes. This is based

Table 1. Vectors and primers used in this study (excluding those indicated in *Experimental procedures*).

Plasmids	Description	Reference or source
pSK-Km	*Brucella* suicide vector, kan^{R}, pSK– oriT derivative	Tibor *et al*. (1995); I. Danese *et al*. (unpublished)
pSK-Bori	replicative in *B. abortus*, 497 bp *Bam*HI–*Sal*I fragment from pGEM-Bori in pSK-Km	This study
pET15bctrA	*ctrA* coding sequence in pET15b (Novagen)	This study
pET15bctrAD51E	pET15bctrA carrying *ctrA*D51E mutation	This study
pGEM-Bori	469 bp PCR fragment containing *hemE*–RP001 intergenic region in pGEM-T (Promega)	This study
pBBR1MCS	Broad-host-range cloning vector, chloramphenicol resistant and replicative in *Brucella*	Kovach *et al*. (1994)
PBBplac-*ctrA*	pBBR1MCS with a 0.7 kb *Bam*HI fragment containing the *B. abortus ctrA* coding region cloned under the control of lac promoter	This study

Primers for DNase I protection assays	Sequences[a]	Comments[b]
ctrA-for	5′-CCGGAAGCTTAACCAGTGTGAATG-3′	*ctrA* promoter (–472 to –21)
ctrA -rev	5′-TTTCCGGATCCGACCAGATGTC-3′	
ccrM-for	5′-ATGCCGATTTGCCTCCCGGGAA-3′	*ccrM* promoter (–431 to –1)
ccrM-rev	5′-GGGATACTGGATCCCCTGTGAAC-3′	
ftsZ-for	5′-AGCGGCCGCGTCGACCGGACATATCGTCGGCAA-3′	*ftsZ* promoter (–394 to +1)
ftsZ-rev	5′-GCGGGATCCTTGGTCCTTGTTCCTCTTAA-3′	
divK-for	5′-CCGGAAGCTTACGCCAGCCAGAAA-3′	*divK* promoter (–344 to –4)
divK-rev	5′-CGGGATCCAAATCCCTGGCCCTTA-3′	
ftsE-for	5′-AGCGGCCGCGTCGACGTTTGCGTCGGCTT-3′	*ftsE* promoter (–239 to +1)
ftsE-rev	5′-CTAGTCTAGACCGGGTCAGCTTCTCTT-3′	
minC-for	5′-AGCGGCCGCGTCGACTGATCCCGTTCATT-3′	*minC* promoter (–264 to +94)
minC-rev	5′-CTAGTCTAGAGCCGTCGAGCGGAAGTT-3′	
rpoD-for	5′-AGCGGCCGCGTCGACCACGATTGTCCGAA-3′	*rpoD* promoter (–293 to –14)
rpoD-rev	5′-CTAGTCTAGAGGCGACCCCGGTCGCTT-3′	
pleC-for	5′-AGCGGCCGCGTCGACCACTCTTCCGGCTT-3′	*pleC* promoter (–294 to –22)
pleC-rev	5′-CTAGTCTAGATGGAACAGCCCTGCGAA-3′	
hfq-for	5′-AGCGGCCGCGTCGACCCGGAGTTTATCGGTTTT-3′	*hfq* promoter (–366 to –29)
hfq-rev	5′-GCGGGATCCCCAGCTCGCGATATGATAAA-3′	
ori-for	5′-CCATCGATTCATTGGTTGTCCAT-3′	*Bori* (*hemE*–RP001 intergenic region)
ori-rev	5′-CGGGATCCTTTGTTGCCCTCCTGTT-3′	

a. Restriction sites are underlined.
b. Positions are indicated relative to the putative translational start site.

on the following arguments: (i) *B. abortus* CtrA is highly similar to *S. meliloti* and *C. crescentus* CtrA proteins (93% and 81% sequence identity respectively); (ii) antibodies to *B. abortus* CtrA cross-react with *C. crescentus* CtrA (data not shown); (iii) His_6–CtrA is phosphorylated *in vitro* on an aspartate residue in position 51 that is highly conserved among response regulators (Volz, 1993; 1995) and was shown to be the phosphorylation site in *Caulobacter* CtrA (Quon *et al*., 1996; Reisenauer *et al*., 1999b); (iv) in *B. abortus*, the *cis* elements bound by CtrA correspond to the CtrA binding consensus established in *C. crescentus* (see Fig. 6); (v) His_6–CtrA binds to its own promoter, suggesting that *ctrA* transcription is autoregulated in *B. abortus*, as already demonstrated *in vivo* for *C. crescentus* and *in vitro* for *S. meliloti* (Quon *et al*., 1996; Barnett *et al*., 2001); (vi) attempts to disrupt the *B. abortus ctrA* gene were unsuccessful, suggesting that the expression of this gene is essential for cell viability, as is the case for *C. crescentus* and *S. meliloti ctrA* genes (Quon *et al*., 1996; Barnett *et al*., 2001); (vii) overexpression of *ctrA* in *B. abortus* leads to a spectacular phenotype, suggesting strongly that, as demonstrated in *C. crescentus*, CtrA is involved in the control of cell division.

Analysis of B. abortus ctrA *promoter (Fig. 7)*

Our results show that the *B. abortus ctrA* promoter contains three transcriptional start sites that are located 287, 80 and 65 bp upstream of the first coding ATG (P1, P2′ and P2 respectively). Interestingly, the 222 bp distance between *B. abortus* P1 and P2 is identical to the distance between P1 and P2 in the *S. meliloti ctrA* promoter (Fig. 7; Barnett *et al*., 2001). In the primer extension experiment, we have detected a third band, with a similar intensity to P2, that is separated from it by only 15 bp (as shown in Fig. 3). This P2′ transcript was confirmed with an additional primer. In addition, the sequence TTGCCA found at position –35 of *B. abortus* P2′ (Fig. 3A), which is close to the consensus –35 element of *E. coli* σ^{70} (Hawley and McClure, 1983), argues in favour of transcription initiation from the P2′ promoter. It is worth mentioning that the *B. abortus* P2′ –35 element is conserved in the *S. meliloti ctrA* promoter at the same position (Fig. 7); it would therefore be interesting to study *S. meliloti ctrA* transcription in various conditions in order to see whether this P2′ transcript is also present. One can assume that this particular arrangement of two overlapping promoters might be relevant for the proper control of *ctrA* transcription. In *E. coli*, it has been shown that the NtrC factor can regulate its own expression either positively or negatively by binding to two overlapping promoters (Ptashne, 1986; Collado-Vides *et al*., 1991).

On the one hand, considering the distance between the P1 and P2 transcriptional start sites, the *B. abortus ctrA*

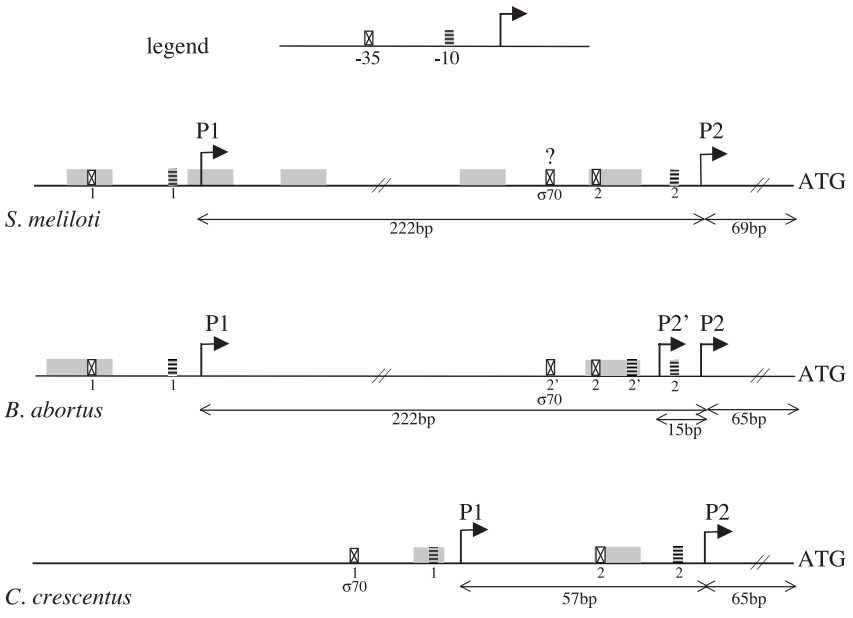

Fig. 7. Comparative diagrams of the *ctrA* promoter regions in *B. abortus*, *S. meliloti* (Barnett *et al*., 2001) and *C. crescentus* (Domian *et al*., 1999). Locations of the transcriptional start sites are marked by bent arrows, and the –35 and –10 elements are represented as indicated in the key at the top of the figure. The regions protected from DNase I digestion by phosphorylated CtrA are shown as grey boxes. The distances between the start sites as well as the distance to the first coding ATG are indicated. The numbers 1, 2′ and 2 indicated below the –10 and –35 elements refer to P1, P2′ and P2 start sites respectively. The question mark indicates the position of the putative σ^{70} –35 element that is conserved in *S. meliloti*.

promoter is more similar to the *S. meliloti* than to the *C. crescentus ctrA* promoter, the latter displaying two transcriptional start sites 57 bp away from each other (Fig. 7). On the other hand, the *B. abortus ctrA* promoter contains two CtrA protected regions, whereas the *S. meliloti ctrA* promoter displays five CtrA binding sites (Barnett *et al*., 2001), suggesting that the control of *S. meliloti ctrA* transcription may be more complex. In particular, the *S. meliloti* P1 transcriptional start site itself is bound by CtrA, which is not the case in *B. abortus*.

Analysis of B. abortus *CtrA targets*

It was shown previously that the CcrM methyltransferase and the CtrA response regulator are conserved in α-proteobacteria (Quon *et al*., 1996; Stephens *et al*., 1996; Wright *et al*., 1997; Lang and Beatty, 2000; Barnett *et al*., 2001; Kahng and Shapiro, 2001). In addition, CtrA autoregulation and CtrA regulation of *ccrM* expression seem conserved in these bacteria (Quon *et al*., 1996; Domian *et al*., 1999; Robertson *et al*., 2000; Barnett *et al*., 2001; Kahng and Shapiro, 2001; this study). Furthermore, all the footprinting experiments performed in these different organisms have shown that the promoters bound by CtrA contain motifs corresponding to the TTAA-N_7-TTAAC 9-mer consensus originally established in *C. crescentus*, suggesting that this consensus might be a conserved feature in α-proteobacteria.

The *C. crescentus* origin of replication (*Cori*) is localized in the *hemE*–RP001 intergenic region (Marczynski and Shapiro, 1992). It exhibits five motifs, similar to the CtrA-binding 9-mer consensus, which were all shown to be selectively bound by CtrA *in vitro* (Quon *et al*., 1998). Further *in vivo* experiments confirmed these *in vitro* footprintings; indeed, the analysis of a modified strain with altered CtrA activity and point mutations in the CtrA binding sites in *Cori* demonstrated that CtrA directly binds and represses the replication origin (Quon *et al*., 1998; Siam and Marczynski, 2000). Surprisingly, when looking at the *B. abortus hemE*–RP001 intergenic region that contains a functional origin of replication (*Bori*), besides the expected DnaA boxes, we only found two questionable CtrA 9-mer motifs (Fig. 6C) that were not bound by phosphorylated CtrA (data not shown). Thus, our data indicate that, in *B. abortus*, CtrA does not regulate *Bori* directly, although it might be indirectly involved in the control of replication through the regulation of *ccrM* expression (Robertson *et al*., 2000), as suggested by the abundant GAnTC methylation sites in *Bori*. This difference in replication regulation between *C. crescentus* and *B. abortus* may be related to the presence of two chromosomes in *Brucella* (with the exception of *B. suis* biovar 3; Michaux *et al*., 1993; Jumas-Bilak *et al*., 1998) and/or the symmetrical division of these organisms.

We have also identified other genes, *rpoD*, *pleC*, *minC* and *ftsE* homologues, which we propose to be CtrA regulated in *B. abortus* on the basis of DNase I protection assays, an *in vitro* technique whose biological relevance for direct CtrA binding has already been proved in *C. crescentus* (Quon *et al*., 1998). Interestingly, although none of these genes has been described as a direct CtrA target in *C. crescentus*, they are all somewhat related to processes regulated by CtrA in this organism, suggesting that, in *B. abortus*, CtrA might control similar cellular events through the regulation of different target genes, as discussed below.

The global analysis of gene expression during the *C. crescentus* cell cycle using DNA microarrays (Laub *et al*., 2000) has shown that the expression of the *rpoD* gene, encoding the major sigma subunit of RNA polymerase, was cell cycle regulated contrary to a previous study suggesting that *rpoD* was equally expressed throughout the cell cycle (Malakooti and Ely, 1995). In addition, expression levels of *rpoD* were drastically reduced in a *C. crescentus ctrA* loss-of-function mutant. As there is no CtrA box in the *C. crescentus rpoD* promoter, Laub *et al*. (2000) proposed that CtrA was indirectly involved in *rpoD* regulation. We have found a CtrA-binding motif in the *B. abortus rpoD* homologue that is bound *in vitro* by phosphorylated CtrA, strongly supporting the view that *rpoD* expression might be under direct CtrA control in *B. abortus*.

On the one hand, we have shown that *B. abortus* CtrA does not bind to the promoters of *divK* and *ftsZ* homologues *in vitro*, suggesting that these genes may not be under direct CtrA control in *B. abortus*, contrary to what is described in *C. crescentus* (Fig. 8). On the other hand, the *B. abortus pleC*, *minC* and *ftsE* homologues are bound *in vitro* by phosphorylated CtrA, and are therefore proposed to be directly regulated by CtrA in this organism. In *C. crescentus*, it has been shown that the PleC–DivJ–DivK pathway leads to the phosphorylation of CtrA (Fig. 8; Wu *et al*., 1998). In this phosphorelay, the HPK PleC acts genetically upstream of the HPK DivJ to regulate the phosphorylation of the DivK response regulator (Wheeler and Shapiro, 1999), the latter being transcriptionally regulated by CtrA in *C. crescentus* (Laub *et al*., 2000). On the basis of the CtrA protection recorded on the *B. abortus pleC* homologue, we propose that, in *B. abortus*, CtrA might also regulate its own phosphorylation pathway through the control of *pleC*, instead of *divK* as is the case in *C. crescentus*. It must be stressed that there is no CtrA-binding motif in the *C. crescentus pleC* promoter, whereas putative CtrA boxes are detected upstream of three *M. loti* PleC/DivJ homologues (mlr0562, mll6540 and mlr7649; http://www.kazusa.or.jp/rhizobase/). In this context, it will be interesting to characterize further the function and reg-

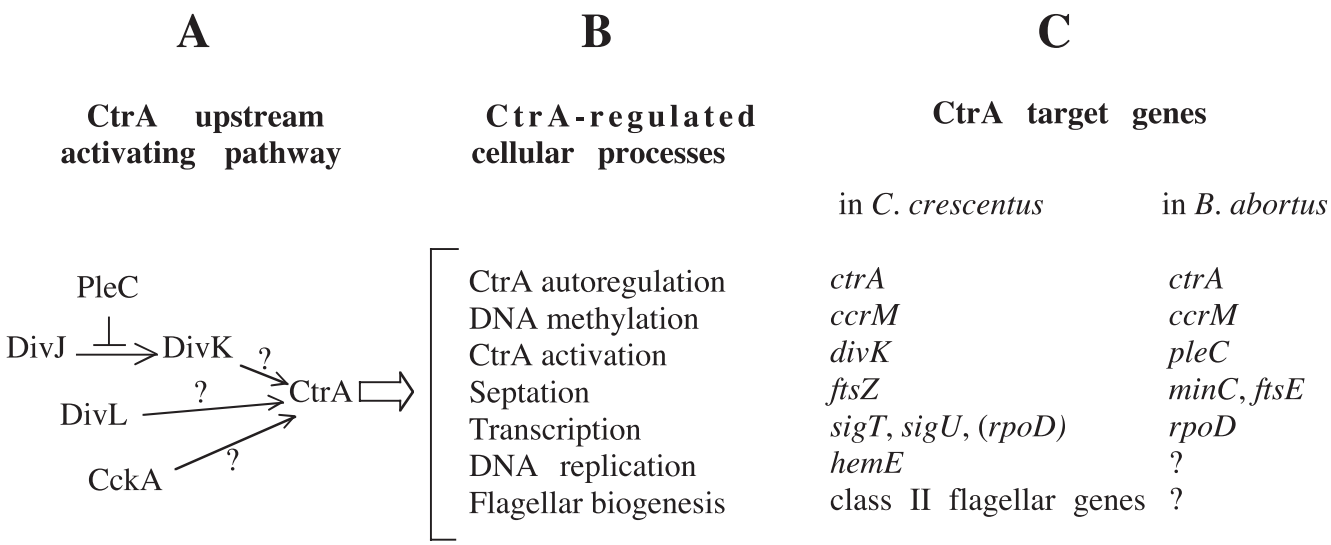

Fig. 8. In *C. crescentus* and *B. abortus*, CtrA regulates similar cellular processes through the regulation of distinct target genes.
A. Schematic of CtrA upstream activating pathway in *C. crescentus*. Arrows represent phosphorelays, and question marks point to missing components or unknown phosphotransfers.
B. The main cellular processes that are CtrA regulated in *C. crescentus* are indicated in parallel with the corresponding CtrA target genes.
C. Comparative diagram of direct CtrA target genes in *C. crescentus* with possible direct CtrA targets in *B. abortus* as determined by DNase I protection assays. The *C. crescentus sigT* and *sigU* genes, which encode alternative sigma factors of RNA polymerase, are proposed to be direct CtrA targets, contrary to *C. crescentus rpoD* (in brackets) that would be indirectly regulated by CtrA (Laub *et al*., 2000).

ulation of the two other *Brucella* kinases homologous to both DivJ and PleC (HPK_{1035} and HPK_{609}) that present putative CtrA-binding motifs in their 5′ non-coding regions.

Our footprinting data on the promoters of *minC* (Fig. 5B) and *ftsZ* homologues suggest that, in *B. abortus*, CtrA is also involved in the regulation of cell division initiation as well as in proper localization of the septum by controlling the expression of the *minC* division inhibitor, instead of the *ftsZ* division initiator. In *C. crescentus*, whereas the *minB* operon (including *minC*) is absent, transcription of *ftsZ* is repressed by CtrA in non-replicative swarmer cells and is activated, in parallel to initiation of DNA replication, in stalked cells that are deprived of CtrA (Domian *et al*., 1997; Kelly *et al*., 1998). In *E. coli*, MinC, MinD and MinE, the products of the *minB* operon, prevent Z-ring formation at any membrane site other than the proper one at mid-cell (de Boer *et al*., 1989). MinC is a division inhibitor that blocks Z-ring formation *in vivo* (de Boer *et al*., 1990; Bi and Lutkenhaus 1993) and FtsZ polymerisation *in vitro* (Hu *et al*., 1999; Hu and Lutkenhaus, 2000). In addition to *minC*, we have also shown that phosphorylated CtrA binds to a *B. abortus ftsE* homologue at a conserved 9-mer motif that is absent in the regulatory region of *C. crescentus ftsE* (GenBank AAK24187). In *E. coli*, an *ftsE* null mutant exhibits filamentous growth and requires high salt medium, indicating that it is directly or indirectly involved in cell division and/or salt transport (de Leeuw *et al*., 1999). The exact function and substrate specificity of this putative ATP-binding component of an ABC (ATP binding cassette) transporter are still unknown.

Beside these footprinting data obtained *in vitro* on *minC* and *ftsE*, we observed that *ctrA* overexpression in *B. abortus* led to cells with an aberrant morphology, i.e. enlarged and branched cells. Similar phenotypes were correlated with blocked cell division in *S. meliloti* (Latch and Margolin, 1997) and were also observed under *ccrM* overexpression in *S. meliloti* (Wright *et al*., 1997) and *B. abortus* (Robertson *et al*., 2000). These data are consistent with our hypothesis that CtrA is directly involved in cell cycle regulation in *B. abortus*.

In conclusion, our data support the hypothesis that, in *B. abortus*, CtrA does not directly regulate the initiation of replication, contrary to the situation described in *C. crescentus* (Quon *et al*., 1998). Besides, we propose that cell division and the CtrA upstream phosphorylation pathway are under CtrA control in *B. abortus*, as already described in *C. crescentus*, although the CtrA target genes are not the same in these two bacteria. A distinct distribution of CtrA binding sites in *B. abortus* compared with *C. crescentus* is consistent with a plasticity of the CtrA regulation network among alpha-proteobacteria. In addition, similar distribution of CtrA boxes upstream of *M. loti*, *A. tumefaciens* and *S. meliloti* homologues to some of the newly identified CtrA targets in *B. abortus* strongly suggests that the whole regulation network involving CtrA is very similar among members of the Rhizobiaceae group, compared with *Caulobacter*. Indeed, there are no CtrA boxes upstream of *ftsZ* in *M. loti* and *S. meliloti*, whereas both organisms display putative CtrA boxes upstream of cell division genes such as *minC* and *ftsE*.

Besides the fact that these members of the Rhizobiaceae group are closely related, the similarities in the regulation network involving CtrA might be related to a common lifestyle, i.e. the interaction of the bacteria with a eukaryotic host. Particularly, intracellular trafficking may require a specific cell division control. It would therefore be interesting to analyse *ctrA* regulation and CtrA target expression in *Brucella* infection models such as cultured HeLa cells and macrophages. Finally, the increasing number of partial or whole bacterial genomes available in the public databases, in particular those belonging to the alphaproteobacteria, will permit the comparative analysis of the CtrA-dependent genetic network and will thus contribute to our understanding of the CtrA paradigm first established in *C. crescentus*.

Experimental procedures

Bacterial strains and plasmids

Escherichia coli strains were grown at 37°C in Luria–Bertani (LB) broth. *E. coli* DH10B (Gibco BRL), S17-1 (Simon *et al*., 1983) and BL21DE3 (Novagen) were used for cloning, plasmid mobilization in *B. abortus* and protein overexpression respectively. The *C. crescentus* (ATCC 33532) and *B. abortus* 544 (Nal^R derivative) strains were grown as described by Ely (1991) and Lestrate *et al*. (2000) respectively. Whole-cell extracts of *Brucella* strains were prepared by J.-M. Verger and M. Grayon (Institut National de la Recherche Agronomique, Laboratoire de Pathologie Infectieuse et Immunologie, Nouzilly, France) as described previously (Cloeckaert *et al*., 1990).

Antibiotics were used at the following concentrations: nalidixic acid, 25 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; and ampicillin, 100 μg ml^{-1}. Vectors pGEM-T (Promega), pBluescript-II SK– (Stratagene) and pZErO-2 (Invitrogen) were used for cloning, pET15b (Novagen) for protein overexpression in *E. coli* and pBBR1MCS (Kovach *et al*., 1994) for *ctrA* overexpression in *B. abortus* 544 Nal^R.

Protein purification, generation of antibodies and immunoblot analysis

MBP–EnvZ was prepared as described previously (Huang and Igo, 1996). The six-histidine His_6–CtrA and His_6–CtrAD51E fusion proteins were overexpressed in *E. coli* BL21(DE3) (Novagen). For this purpose, the *ctrA* ORF was amplified with primers *ctrA*-ATG (5′-GGATAACATAT GCGCGTCCTTTTG-3′) and *ctrA*-stop (5′-GGATCCTCA GGCGCTTTCGCGCAT-3′), cut out with *Bam*HI–*Nde*I and ligated into the corresponding sites in pET15b to give pET15bctrA. A point mutation was introduced in *ctrA* wild-type ORF by PCR using, in parallel with oligonucleotides *ctrA*-ATG and *ctrA*-stop, complementary mutagenic primers (5′-CATTCTGCTGGAGCTCAATC-3′ and 5′-GATTGAGCT CCAGCAGAATG-3′) introducing a *Sac*I site and replacing aspartate 51 with a glutamate to generate pET15bctrAD51E. Protein purification was performed on a 2.5 ml nickel chelation resin column (His-Bind; Novagen) in 6 M urea as recommended by the manufacturer, followed by renaturation of the proteins by two successive dialysis steps [buffer 1: 1× PBS, pH 8, 0.4 M sucrose, 1 mM EDTA; buffer 2: 20 mM Tris, pH 8, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. Protein samples in buffer 2 were concentrated up to 4 mg ml^{-1} using a 4 ml Centricon column (Millipore) and finally stored at –20°C in 50% glycerol.

Specific antibodies directed against *B. abortus* CtrA were obtained after three intramuscular immunizations of a rabbit, every 4 weeks, with 50 μg of purified recombinant His_6–CtrA protein adjuvanted with QuilA (Spikoside, Isotec product).

For immunoblot analysis, proteins were subjected to electrophoresis on 12% SDS–PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech) by the semi-dry transfer technique (Ausubel *et al*., 1991). The blots were probed with anti-CtrA polyclonal antibodies used at a 1:2500 dilution. Bound antibodies were detected by chemiluminescence with peroxidase-conjugated secondary antibodies and the ECL Western blotting detection kit (Amersham Pharmacia Biotech).

Phosphorylation of His_6–CtrA

His_6–CtrA was phosphorylated mainly as described previously for *C. crescentus* CtrA (Reisenauer *et al*., 1999b). Briefly, purified MBP–EnvZ (1.7 μM) was incubated in phosphorylation buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 20 mM $MgCl_2$, 1 mM DTT, 0.4 mM ATP and 10 μCi of [γ-^{32}P]-ATP from ICN) in a total volume of 30 μl. After 5 min at 37°C, a 6 μl aliquot was removed and added to SDS–PAGE loading buffer. Purified His_6–CtrA or His_6–CtrAD51E (9.6 μM each) was added to the rest of the reaction, which was incubated further at 37°C. Samples were collected after 1, 15 and 60 min and subjected to 12% SDS–PAGE followed by autoradiography.

Primer extension analysis

Experiments were carried out with *B. abortus* total RNA (40 μg) extracted from log-phase cultures grown in 2YT using the RNeasy Midi kit from Qiagen. The primers PE-1 (5′-CTTTCCGCAGCCGACCAGAT-3′), PE-2 (5′-GCACGTCGT CTTCAATCAA-3′) and PE-4 (5′-CTGACTCTAATGCATCGC TT-3′) are complementary to regions located, respectively, from bp –30 to –11, +12 to +31 and –201 to –182 with respect to the translational start site (Fig. 3A). The three primers were 5′ end-labelled with [γ-^{32}P]-ATP (ICN) using T4 polynucleotide kinase (Roche) and ethanol precipitated with 0.02% glycogen. Primer extension reactions were conducted mainly as described elsewhere (Sambrook *et al*., 1989), using Superscript II reverse transcriptase (Gibco BRL). Sequencing reactions using dideoxy chain termination (Sanger *et al*., 1977) with primers PE-1, PE-2 and PE-4 were loaded next to the corresponding extension products to determine the exact positions of the start sites.

DNase I protection experiments

Footprinting experiments (DNase I protection assays) were

performed mainly as described previously (Reisenauer *et al*., 1999b) using DNase I (Boehringer) at 1:2000 to 1:2500 dilution. Purified *B. abortus* His_6–CtrA was first incubated with MBP–EnvZ for 30 min at 37°C in non-radioactive phosphorylation buffer with or without 5 mM ATP for phosphorylated or unphosphorylated CtrA respectively. For each footprinting reaction, His_6–CtrA was used at a final concentration of 85 $\mu g\ ml^{-1}$. DNA templates were obtained as follows: the promoters of interest were generated by PCR on *B. abortus* genomic DNA using the oligonucleotides listed in Table 1 and inserted in either pBluescript-II SK– or pGEM-T vectors. The resulting plasmids (pSK*pctrA*, *-pftsZ*, *-phfq*, *-pminC*, *-ppleC*, *-pftsE*, *-prpoD*, pGEM-T*pdivK* and pGEM-T*pccrM*) as well as pGEM-Bori were linearized with a first restriction endonuclease (BioLabs or Gibco BRL) and end-labelled with [α-^{32}P]-dCTP (Amersham) and Klenow (large fragment of DNA polymerase I; Gibco BRL). The plasmids were then subjected to a second digestion before 1% agarose gel electrophoresis and gel purification (DNA extraction kit; MBI Fermentas) of the fragment of interest. Depending on the first restriction enzyme used, the DNA templates obtained in this way were labelled at either the upstream or the downstream promoter end with regard to gene orientation.

The DNase I digestion products were phenol extracted, ethanol precipitated and finally separated on 6% polyacrylamide–7 M urea sequencing gels. A sequencing ladder generated with universal primer and M13mp18 vector as template (T7 DNA polymerase sequencing kit; USB) was used to position the protected regions.

The nucleotide sequences of the *B. abortus pleC, rpoD, ftsE, minC, divK, ctrA* and *ccrM* promoters have been deposited in the GenBank database and assigned successive accession numbers (AF411565 to AF411571).

Cloning of the B. abortus ftsZ *gene and chromosomal origin of replication*

The ftsZ *gene.* DNA fragments obtained from partially *Sau*3A1-digested *B. abortus* 544 genomic DNA were cloned in the *Bam*HI-digested pZErO-2 vector (Invitrogen). The resulting *B. abortus* 544 genomic library has the following characteristics: inserts of 3–11 kb with an average size of 6 kb; 6400 independent recombinant clones were obtained. Primers *ftsZ*-Up (5′-ACCGAGCTGAAGCCGCGTAT-3′) and *ftsZ*-Down (5′-ATGATGTTGGCTTCCGGAT-3′) directed towards a conserved region of *ftsZ* in *C. crescentus* (GenBank U40273; Quardokus *et al*., 1996), *S. meliloti* (GenBank AF024660; Margolin *et al*., 1991) and *A. tumefaciens* (GenBank AF024659; Ma *et al*., 1997) were used to amplify an 860 bp segment (corresponding to bp 31–890 on *C. crescentus ftsZ* coding sequence) on *B. abortus* genomic DNA by PCR. The product of this reaction was sequenced and used to screen the *B. abortus* genomic library. Two positive clones were isolated and characterized. A 642 bp overlap between these clones was detected and shown to be located within the *B. abortus ftsZ* ORF. The sequence of this assembled ORF encodes a putative protein of 566 residues exhibiting 70%, 69% and 53% identity with FtsZ from *A. tumefaciens*, *S. meliloti* and *C. crescentus* respectively. The nucleotide sequences of the *B. abortus ftsZ* and its gene product have been deposited in the GenBank database and assigned accession number (AF360732). The deduced peptidic sequence of the FtsZ homologue contains the conserved glycine-rich motif (GGGTGTG) that is required for GTP binding (RayChaudhuri and Park, 1992). We have used an affinity-purified polyclonal antibody raised against *C. crescentus* FtsZ (Din *et al*., 1998; Quardokus *et al*., 2001) to identify the *B. abortus* FtsZ protein. The detection of a single cross-reactive band on *B. abortus* total extracts (data not shown) suggests that a unique FtsZ homologue is expressed in *B. abortus*.

The putative origin of replication. The *C. crescentus hemE*–RP001 cluster, proposed as an 'ori signature' (Brassinga *et al*., 2001), was compared with the *B. melitensis* 16M and *B. suis* 1330 genomic sequences (available at the University of Scranton and TIGR respectively). A similar cluster was found in these two genomes. Primers *ori*-for and *ori*-rev (listed in Table 1) flanking the intergenic region between the *B. melitensis and B. suis hemE* and RP001 homologues were used to amplify the corresponding region on *B. abortus* genomic DNA.

This 469 bp fragment was cloned in pGEM-T (Promega) and sequenced. There is only 1 bp difference between the *B. abortus* sequence and the corresponding *B. suis* and *B. melitensis* regions. This substitution is located outside any putative CtrA binding site. Next, this DNA fragment was cloned in a *B. abortus* suicide (i.e. non-replicative) vector, a kan^R derivative of pSKoriT (Tibor *et al*., 1995; I. Danese *et al*., unpublished), and the resulting vector, pSK-Bori, was transferred by conjugation in *B. abortus*. In order to test whether this vector was able to replicate in *B. abortus*, plasmid extractions were performed as described elsewhere (Takahashi and Nagano, 1984), and the resulting DNA was transformed in *E. coli* DH10B (Gibco BRL) and analysed by restriction. As pSK-Bori was able to replicate in *B. abortus*, the *B. abortus hemE*–RP001 intergenic region contains all the attributes of a functional chromosomal origin of replication. The nucleotide sequence of the *B. abortus* origin of replication has been deposited in the GenBank database and assigned accession number AF411572. As expected for a chromosomal origin of replication, it contains several potential binding sites for the DnaA replication initiator that are similar to the consensus sequence $TT^A/_T$TNCACA established in *E. coli* (Zyskind and Smith, 1986; Messer and Weigel, 1996).

Sequencing reactions were performed at the automated sequencing service of FUNDP (Belgium) using the ABI Prism Big Dye Terminator sequencing chemistry and an ABI Prism 377 DNA sequencer.

Acknowledgements

We thank D. Devos for cloning the *B. abortus ctrA* and *divK* homologues as well as scanning electron microscopy pictures, M. Dewez-Jadin for technical assistance in cloning and protein purification, and A. Tibor and J.-Y. Paquet for fruitful discussions. We also thank J.-M. Verger and M. Grayon for providing *Brucella* strains extracts, K.-J. Huang and M. Igo (University of California, Davis, CA, USA) for providing pKJH5 used for overexpressing the MBP–EnvZ fusion protein, Y. Brun and E. Quardokus (Department of

Biology, Indiana University, IN, USA) for the gift of polyclonal antibody against *C. crescentus* FtsZ, and R. Wright for sharing unpublished sequence data and for providing pRW154 carrying the *ccrM* promoter and the beginning of the coding sequence. We are particularly grateful to A. Reisenauer (Stanford University, CA, USA) for sharing unpublished protocols on DNase I footprinting and primer extensions assays. Finally, we are indebted to A. Reisenauer, G. Robertson and C. Jacobs for helpful discussions. Preliminary sequence data from the *B. melitensis* 16M genome was obtained from the Institute of Molecular Biology and Medicine, University of Scranton, Scranton, PA, USA. Preliminary sequence data from the *B. suis* 1330 genome was obtained from The Institute for Genomic Research (website at http://www.tigr.org). A.-F. Bellefontaine was supported by a specialization grant from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA, Belgium). This work was partially supported by FRFC grant no. 2.4501.98 (Fonds pour la Recherche Fondamentale Collective, Belgium).

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