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Van der Henst, Charles; Beaufay, François; Mignolet, Johann; Didembourg, Christian; Colinet, Julien; Hallet, Bernard; Letesson, Jean-Jacques; De Bolle, Xavier

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The Histidine Kinase PdhS Controls Cell Cycle Progression of the Pathogenic Alphaproteobacterium Brucella abortus

Charles Van der Henst, François Beaufoy, Johann Mignolet, Christian Didembourg, Julien Colinet, Bernard Hallet, Jean-Jacques Letesson, and Xavier De Bolle

Microorganisms Biology Research Unit (URBM), University of Namur (FUNDP), Namur, Belgium, and Institut des Sciences de la Vie, Division of Biochemistry and Molecular Genetics of Bacteria, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

Bacterial differentiation is often associated with the asymmetric localization of regulatory proteins, such as histidine kinases. PdhS is an essential and polarly localized histidine kinase in the pathogenic alphaproteobacterium Brucella abortus. After cell division, PdhS is asymmetrically segregated between the two sibling cells, highlighting a differentiation event. However, the function(s) of PdhS in the B. abortus cell cycle remains unknown. We used an original approach, the pentapeptide scanning mutagenesis method, to generate a thermosensitive allele of pdhS. We report that a B. abortus strain carrying this pdhS allele displays growth arrest and an altered DivK-yellow fluorescent protein (YFP) polar localization at the restrictive temperature. Moreover, the production of a nonphosphorylatable PdhS protein or truncated PdhS proteins leads to dominant-negative effects by generating morphological defects consistent with the inhibition of cell division. In addition, we have used a domain mapping approach combined with yeast two-hybrid and fluorescence microscopy methods to better characterize the unusual PdhS sensory domain. We have identified a fragment of the PdhS sensory domain required for protein-protein interaction (amino acids [aa] 210 to 434), a fragment sufficient for polar localization (aa 1 to 434), and a fragment (aa 527 to 661) whose production in B. abortus correlates with the generation of cell shape alterations. The data support a model in which PdhS acts as an essential regulator of cell cycle progression in B. abortus and contribute to a better understanding of the differentiation program inherited by the two sibling cells.

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Address correspondence to Xavier De Bolle, xavier.debolle@fundp.ac.be.

* Present address: Johann Mignolet, Department of Microbiology and Molecular Medicine, Faculty of Medicine/CMU, University of Geneva, Geneva, Switzerland.

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**TABLE 1** Primer sequences used for domain mapping

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
</tr>
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<tr>
<td>F1_Up</td>
<td>GGGGACAAGTTTGTGACAAAAAGCAGGCTCGATGGAGGCCGAGCGCGCCCTCCAA</td>
</tr>
<tr>
<td>F1_Down</td>
<td>GGGGACCACTTTGTGACAAAAAGCAGGCTCGATGCCCGTAAAAACACGCTCCGAA</td>
</tr>
<tr>
<td>F2_Up</td>
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</tr>
<tr>
<td>F2_Down</td>
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</tr>
<tr>
<td>F3_Down</td>
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</tr>
<tr>
<td>F3_Up</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGAGGCCGAGCGCGCCCTCCAA</td>
</tr>
<tr>
<td>F4_Down</td>
<td>GGGGACCACTTTGTGACAAAAAGCAGGCTCGATGCCCGTAAAAACACGCTCCGAA</td>
</tr>
<tr>
<td>F4_Up</td>
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</tr>
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</tr>
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<tr>
<td>CD_Down</td>
<td>GGGGACACCTTTGTGACAAAAAGCAGGCTGGTACGGCTTCTTTCATGATCAG</td>
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</table>

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** All Brucella strains used in this study were derived from *B. abortus* 544 Na† (a spontaneous nalidixic acid-resistant mutant) and were routinely cultivated in 2YT rich medium (1% yeast extract, 1.6% peptone, 0.5% NaCl). The *Escherichia coli* strain used for the molecular cloning experiments was DH10B (Invitrogen Life Technologies), except for the ccdB-containing plasmids that were propagated in the DB3.1 strain (Invitrogen Life Technologies). All *E. coli* strains were grown in LB broth. Antibiotics were used at the following concentrations when appropriate: *B. abortus* and *E. coli* (for liquid and solid cultures): nalidixic acid, 25 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml; rifampin, 50 μg/ml; and spectinomycin, 100 μg/ml. The *C. crescentus* strain used in this study was CB15N grown in PYE medium (10), and the antibiotic concentrations were 1 μg/ml for liquid cultures and 2 μg/ml for chloramphenicol and/or 15 μg/ml nalidixic acid for liquid cultures and 2 μg/ml chloramphenicol and/or 20 μg/ml nalidixic acid for solid cultures. The plasmids were mobilized from the *E. coli* S17-1 strain into *B. abortus* by conjugation. Mitomycin C was used at a final concentration of 2 μg/ml for 7 h in 2YT medium at 37°C with agitation.

**Essentiality assays.** The essentiality of divK and pdhS was tested using a previously reported procedure applied to demonstrate synthetic lethality (26). The absence of deletants for the strain harboring the *pdhS*Δ805A allele (*pdhS* with an H→A change at position 805) was significantly different from the frequency (7106) of deletion observed for the strain having a wild-type allele of *pdhS* (*P* = 0.0017 for a binomial variable, with *n* = 93 and a deletion probability of 7106). The low frequency of *pdhS* deletion in the presence of pBR1MCS-93 and a deletion probability of 7/106). The low frequency of deletants for the strain harboring the *pdhS*Δ805A allele was significantly different from the frequency of deletion (14/29) observed for the strain having a wild-type allele of *pdhS* (*P* = 2.6 × 10−11 for a binomial variable, with *n* = 37 and a deletion probability of 14/29).

**Molecular techniques.** The yeast two-hybrid (Y2H) assay was performed as previously described (36). The pVV213 plasmids (18) carrying the activating domain of Gal4p fused to PdhS or FumC were introduced in yeast (Saccharomyces cerevisiae) strain MaV103 and pVV212 plasmids carrying the binding domain of Gal4p fused to PdhS fused to FumC were introduced in yeast strain MaV103. For the mating, yeast strains were plated on yeast extract-peptone-dextrose (YPD) medium overnight and then replicated on selective medium (i.e., depleted in uracil or histidine with 100 mM 3-aminotriazole) when appropriate, as previously described (7). For the localization screen, Gateway cloning with pools of each coding sequence (CDs) was used, as previously described for pools of multiple CDs (9).

**Protein purification and autophosphorylation assay.** Purification of PdhS derivatives was performed as previously described (7). Briefly, a His6 tag was fused to the carboxy-terminal side of PdhS and PdhSΔ805A on a pET15B vector (Novagen). After 3 h of induction with 24 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) of the *E. coli* BL21(DE3) strain carrying pET15B-pdhS or pET15B-pdhSΔ805A, cells were lysed by sonication as previously described (35), and total cell extracts were placed on a Ni-nitrotriacetic acid (NTA) His Bind Superflow (Novagen). After successive washings with 25, 50, and 75 mM imidazole and elution with 100 mM imidazole, the elution buffer containing the purified proteins was replaced by an autophosphorylation buffer (50 mM Tris-HCl, 25 mM NaCl, 25 mM KCl, 5 mM MgCl2) using a NAP5 column (GE Healthcare). The autophosphorylation assay was performed as previously described (6, 39), with purified proteins being incubated for 5 or 30 min with 1 μl of radioactive 3,000 Ci/mmol [γ-32P]ATP (PerkinElmer) in a 50-μl reaction volume.

**Conserved motif prediction.** The first analysis of conserved domains in PdhS SD was performed using a simple BLAST (BLASTP) on the Conserved Domains Database (CDD). In the second analysis, three rounds of PSI-BLAST (i.e., two iterations after the first BLASTP) were made with the 661 first residues to generate a position-specific score matrix that was used for similarity search with the sequences extracted from the Protein Data Bank (PDB).

**Domain mapping and PCR.** Fragments of the *pdhS* coding sequence (CDSs) were amplified using the primers reported in Table 1. The amplified fragments were compatible with Gateway recombinational cloning (Invitrogen). Each CDS fragment was then transferred (i) to the RK2-derived plasmid pRH008 for fusion with yellow fluorescent protein (YFP) (17) (9) or (ii) to pVV213 for the yeast two-hybrid assay (18). Each primer name corresponds to the CDS fragment to be amplified, with the “Up” primers hybridizing at the 5’ end of the CDS fragment and the “Down” primers hybridizing at the 3’ end of the CDS fragment.

**Microscopy.** Samples were examined with a Nikon 80i fluorescence microscope using a 100× objective, as previously described (18, 26). DAPI (4′,6′-diamidino-2-phenylindole) was used at 1 μg/ml to stain the DNA, and the mixture was incubated for 1 h at room temperature in phosphate-buffered saline (PBS) medium. The membrane-staining dye FM4-64 (Molecular Probes) was used at a final concentration of 1 μg/ml for 5 min in 2YT medium to observe global bacterial morphology. For the observations, bacteria were grown until they reached the stationary phase (optical density at 600 nm [OD600] of 1.2), and a culture sample (1.5 μl) was spread on the agarose cover with a sealed coverslip. Time-lapse microscopy was performed by placing strains on a microscope slide that was layered with a pad of 1% agarose containing 2YT medium (700 μl per
The culture was resumed under the microscope at 23°C, and images were taken every hour. For scanning electron microscopy (SEM), bacteria were fixed with paraformaldehyde 4% for 15 min at 37°C and then placed on a coverslip covered with poly-L-lysine (Sigma), coated at 50 g/ml, for 1 h at room temperature in water. Once the poly-L-lysine was removed, the samples were centrifuged at 1,500 rpm for 5 min and then dehydrated (successively for 5 min with 30, 50, 70, 85, and 100% ethanol. After the critical point was reached, gold particles were sprayed onto the samples with a metallizer (Balzers-Union). Scanning electron microscopic images were obtained using a JEOL device at 5,000 at 15 kV. When indicated, the average ± standard deviation was calculated from three independent experiments, with n representing the total number of counted bacteria.

Pentapeptide scanning mutagenesis. The previously reported pentapeptide scanning mutagenesis method (14) was applied to the generation of thermosensitive (TS) alleles for pdhS. We inserted the pdhS sequence by reverse BP (Invitrogen) from pVV212-pdhS (18) and pDONR223 to obtain the entry vector pDONR223-pdhS. This plasmid was used as a target for pdhS pentapeptide scanning mutagenesis. The pDONR223-pdhS plasmid was introduced in E. coli strain DH5α, which contains pFH395, a plasmid carrying the replicative transposon Tn4430 and a kanamycin resistance gene. Tn4430-mediated cointegrates between pFH395 and pDONR223-pdhS were selected by mating pooled DH5α transformants with the rifampin-resistant E. coli strain HB101R. Briefly, 3,000 donor colonies were mixed with the E. coli HB101R recipients for 2 h at 37°C on an LB agar plate without antibiotics to allow the mating. The bacteria were then resuspended in 1 ml of LB and spread on LB agar plates supplemented with rifampin and spectinomycin to select transconjugants. Plasmid DNA was extracted from a pool of ~30,000 transconjugants and transformed back into DH10B in order to isolate plasmids resulting from the resolution of the cointegrates by site-specific recombinations. Plasmid DNA from a pool of ~300,000 clones was digested with KpnI to excise the bulk of the Tn4430, ligated with T4 DNA ligase under dilution conditions promoting self-ligation, and used for transformation in DH10B. The KpnI excision leaves a scar of 15 nucleotides (nt) in the target sequence, corresponding to the insertion of 5 codons in the gene of interest (12). A total of 100,000 clones, containing about 75% of the expected self-ligated plasmids, were obtained and pooled for plasmid extraction. This pool of plasmids was enriched in pDONR223 derivatives carrying a mutated allele of pdhS (pdhS*). The resulting pool of pdhS* alleles was transferred by recombinational (LR) cloning to a Gateway-compatible plasmid derived from pJQ200-uc1 (30) (in which the Gateway cassette was amplified by PCR and cloned at the NotI restriction site present on the pJQ200-uc1) to generate the pJQ200-uc1-pdhS* library. About 3,000 pJQ200-uc1-pdhS* candidates were used to perform allelic replacement of the wild-type pdhS gene in B. abortus strain 544, as previously described for fumC allelic replacement (26). A total of 1,650 sucrose-insensitive clones were individually spotted on solid 2YT medium and incubated at 36°C and 42°C. Among the 1,650 clones tested, 137 candidates with different levels of thermosensitivity were identified, 20 of them being completely unable to generate colonies on 2YT agar plates at 42°C while retaining this capacity at 36°C.

RESULTS
PdhS is required for bacterial growth. To observe phenotypes due to a loss of function of PdhS, we generated conditional alleles of the pdhS essential gene. We constructed a randomly mutated library of pdhS variants using the pentapeptide scanning mu-
tagenesis method (14, 19, 20). This method relies on the insertion of variable 5-aa peptides at random positions of a protein of interest resulting from the integration or excision of the bacterial transposon Tn4430 into the corresponding target gene (see Materials and Methods). Previous studies have shown that the method often generates mutants with increased functional and structural flexibility, correlated with different levels of thermosensitivity in the mutated proteins (19). We thus applied this method to isolate B. abortus strains that would be thermosensitive (TS) because they are mutated in the pdhS coding sequence. After allelic replacement of wild-type pdhS with the mutant library, 1,650 clones were screened for their failure to form colonies on rich medium at 42°C (restrictive temperature) while still being able to generate colonies at 36°C (permissive temperature). A total of 137 candidates showing different levels of thermosensitivity were identified, of which 20 were completely unable to grow at the nonpermissive temperature. One specific clone was retained for further characterization (Fig. 1). The complementation assay showed that the selected TS mutant was able to generate colonies at the restrictive temperature only when a low-copy-number plasmid (pMR10cat) carrying the wild-type copy of the pdhS gene was present (Fig. 1A). The presence of the pMR10cat empty vector did not complement the thermosensitive phenotype. This shows that the thermosensitive phenotype does not result from polar effects and that it is specifically due to mutation in the pdhS gene. We sequenced the pdhS coding sequence in the temperature-sensitive strain and found the expected insertion of 15 bp located upstream of the predicted PAS domain (Fig. 1B).

The optical density of an exponentially growing culture of the TS strain remained stable after being switched from the permissive temperature (36°C) to the restrictive temperature (42°C). This suggests that pdhS is required for normal growth. Viability of the wild type and the TS strain was not affected at the restrictive temperature for 90 min or 6 h. Indeed, at 42°C in rich medium, 99% (n = 512) and 99.3% (n = 440) of the wild-type bacteria were alive, while the proportions of live bacteria of the TS strain were 98.8% (n = 388) and 99.5% (n = 414) after 90 min and 6 h of incubation, respectively. Interestingly, at the restrictive temperature for 90 min, 3 h, or 6 h in liquid culture, the morphology of the TS strain is very similar to that of the wild-type strain (Fig. 1C) (data not shown); i.e., there were no morphological alterations. These data indicate that PdhS is required for growth, and its loss of function leads to a cell cycle arrest without generating aberrant morphologies in B. abortus, unlike in the pdhS overexpression strain (18).

PdhS controls DivK phosphorylation in vivo. We previously proposed that PdhS could be involved in the B. abortus cell cycle regulation through the control of the response regulator DivK (18). In the predivisional cell of C. crescentus, the polar localization of DivK relies upon its phosphorylation state (25). To study the effect of PdhS on DivK control in B. abortus, we used the TS strain to observe the dynamic localization of DivK-YFP under different conditions. We introduced the low-copy-number plasmid pMR10cat-divK-YFP into the TS strain. At permissive temperature (36°C), the resulting strain showed 11.3% ± 1.5% (average ± standard deviation; n = 107) of polar DivK-YFP fusion (Fig. 1C). However, after 90 min at the restrictive temperature (42°C), the DivK-YFP polar localization is strongly decreased (2% ± 1%; n = 107). When the same culture returned to the permissive temperature (36°C), the DivK-YFP fusion regained its polar localization in 10% ± 1% of the bacteria (n = 129). As a control, we introduced the pMR10cat-divK-YFP plasmid into the wild-type strain and showed that DivK-YFP polar localization was retained at 42°C (data not shown).

PdhS is encoded by an essential gene, while the two other HKs potentially involved in DivK phosphorylation are encoded by nonessential genes (i.e., pleC and divJ) (16). Thus, if the essential feature of PdhS relies on DivK phosphorylation state, the divK gene should be essential, like divK in C. crescentus, and the phos-
phosphorylation of PdhS and DivK should also be essential. We constructed a nonphosphorylatable version of PdhS in which the evolutionarily conserved histidine (H805) was replaced by an alanine residue (H805A). Using an autophosphorylation assay in the presence of $\gamma^{32}$P]ATP, we showed that the purified wild-type recombinant PdhS-His$_6$, protein was able to autophosphorylate (Fig. 2A). On the contrary, the purified PdhSH805A-His$_6$, protein did not incorporate radioactivity (Fig. 2A). This shows that PdhSH805A-His$_6$, lost its autophosphorylation capacity while being soluble, suggesting that it was folded. Using the rescue plasmid carrying the wild-type copy of pdhS (pBBR1MCS-pdhS), we were able to obtain pdhS deletion (ΔpdhS) strains, which was not the case with the empty vector pBBR1MCS or the rescue plasmid pBBR1MCS-pdhSH805A (Table 2), showing that PdhS and its phosphorylation are essential. divK deletion (ΔdivK) strains were obtained with a rescue plasmid carrying the wild-type copy of divK (pMR10cat-divK), and no deletion events were obtained with the empty vector, showing that divK is essential in B. abortus (Table 2). The divK$^{H805A}$ copy (16) carried on a pMR10cat cannot rescue a divK deletion strain, demonstrating the essentiality of DivK phosphorylation in B. abortus. Altogether, this shows that divK gene is essential and that phosphorylation of both PdhS and DivK proteins is essential under the tested conditions. Those data are consistent with a dominant-negative effect of pdhS$^{H805A}$-yfp on bacterial cell cycle progression, and particularly cell division, through alteration of the endogenous PdhS function (see Discussion).

**Defining putative subdomains in PdhS.** Because the dominant-negative effects on bacterial morphology were observed independently of the PdhS phosphorylation capacity, we attempted to identify the minimal PdhS portion that was still able to generate aberrant morphologies. The PdhS SD is unusually large compared to other HKs, being 795 aa long, whereas the SDs of PleC and DivI from B. abortus are 543 aa and 154 aa long, respectively (16). While searching for clearly conserved motifs in the SD, a PAS domain (20) was found (residues 661 to 727, E value, 1.13e−9) (Fig. 3). Thus, to better characterize PdhS in B. abortus, we attempted to identify functional subdomains in its SD using a domain mapping approach. We performed multiple sequence alignments with B. abortus PdhS homologs in other alphaproteobacteria (Brucella ovis, Ochrobactrum anthropi, Agrobacterium tumefaciens, Sinorhizobium meliloti, and Mesorhizobium loti), and we identified a total of 7 conserved regions flanked by unconserved regions (UCRs) of different lengths (from 46 to 68 residues) with many insertions or deletions (Fig. 3). For the domain mapping approach, each conserved domain was cloned with its two flanking regions (altogether forming a “fragment”) in order to increase the probability of correct folding and stability. The combinations of domains are named from the first domain to the last. (For example, F1-3 contains the domains 1, 2, and 3, as well as all UCRs flanking them.)

**Production of PdhS portions containing intact F5 generates aberrant morphologies.** The presence of the nonphosphorylatable version of PdhS results in dominant-negative effects on bacterial morphology, such as branched cells. We thus hypothesized...
that a regulation domain present in PdhS SD could alter endogenous PdhS activity. We attempted to identify more precisely the region responsible for this phenotype by cloning the PdhS fragments fused to YFP on the low-copy-number vector pMR10cat in B. abortus. The production of fragments F1-5-YFP and F3-5-YFP generated aberrant bacterial shapes comparable to the altered morphologies resulting from the overexpression of pdhS gene, while fragments F1-4-YFP and F1-3-YFP had a wild-type phenotype (Fig. 2D) (data not shown), despite the polar localization of the expressed peptide (see Fig. 5). Moreover, the trimming of D5 in F1-5 (residues 1 to 613 instead of 1 to 661) did not generate numerous aberrant morphologies despite of its polar localization (18), further suggesting that intact or properly folded F5 could be necessary to modulate endogenous PdhS activity. Those data suggest that the presence of truncated PdhS proteins containing intact F5 correlates with the generation of altered morphologies and confirm the dominant-negative effect on PdhS that lead to cell division defects. We therefore propose that F5 contains a regulatory region of PdhS activity in B. abortus.

PdhS fragments interacting with PdhS and FumC. Using the yeast two-hybrid (Y2H) assay, a large portion of the PdhS SD (from residues 1 to 613) was previously shown to interact with the full-length PdhS protein (18) but also with the class II fumarase FumC (26). To better characterize the PdhS SD, we used the PdhS fragments to identify the minimal SD fragment required for these interactions. We fused all cloned PdhS fragments to the activating Gal4 activation domain, while the complete PdhS or FumC proteins were separately fused to the DNA binding domain of Gal4. The Y2H interactions were tested using HIS3 and URA3 reporters, on media lacking histidine or uracil, respectively (see Materials and methods).

The full-length PdhS (F1-CD) was used as a positive control. F3-CD, F2-5, F1-3, F2-PAS, F3-4, F3-PAS, and F2-3 gave a positive signal when PdhS itself was used as the prey in the Y2H assay (Fig. 4A). The common part of all of these fragments was F3, indicating that this portion of PdhS SD could be required for PdhS multimerization. However, when tested alone, F3 was negative in the Y2H assay (data not shown), a result that could be due to incorrect folding of the protein fragment or to the fact that F3 by itself is not sufficient for the interaction with PdhS.

For the PdhS-FumC interaction assay, peptides corresponding to F1-CD, F1-5, F3-CD, F3-5, F1-3, F2-3, F1-4, and F2-5 gave a positive interaction signal (Fig. 4B) which again identified the F3 as the common minimal fragment of PdhS that could be required to interact with FumC.

The RR DivK was shown to interact with the last 424 C-terminal residues of PdhS (18). We used this interaction as a positive control to test the production and the stability of truncated PdhS fragments in the Y2H assay (Fig. 4C). As expected, fragments containing the CD interacted with DivK, confirming that these fragments were properly produced and folded under the tested conditions. The fact that F1-CD and F3-CD retained the ability to interact with both PdhS and FumC while F4-CD and PAS-CD did not is consistent with the specific requirement of F3 for PdhS multimerization and FumC interaction.

PdhS minimal regions for polar localization. We have shown that the phosphorylation capacity of PdhS was not required for its polar localization in B. abortus and C. crescentus. In addition, a portion of PdhS SD (residues 1 to 613) fused to the YFP produced from a low-copy-number replicative plasmid (pMR10cat) is progressively localized in B. abortus (18), indicating that this fragment contains the required information sufficient for this specific subcellular localization of the protein. To further restrict the protein region involved in polar localization, PdhS fragments were fused to YFP and expressed in B. abortus strain 544 from the pMR10cat plasmid. YFP fusions carrying the F1-5, F1-4, and F1-3 PdhS fragments were polarly localized in B. abortus (Fig. 5), showing 68%, 65%, and 64% polar localization, respectively (n > 200). This shows that subdomains D4 and D5 and PAS domains are not required for polar localization in B. abortus. Less frequent polar localization patterns were also observed with the production of fragments F2-PAS-YFP and F3-5-YFP. Taken together, the data suggest that the minimal protein determinant for PdhS polar localization in B. abortus lies within the F1-3 or the F3-5 fragments.

However, in B. abortus, endogenous polar PdhS may recruit the tested F1-3-YFP and F3-5-YFP since they contain the F3 segment, which was proposed to allow interaction with PdhS (Fig. 4A). To circumvent this possibility, we took advantage of the fact that B. abortus PdhS SD fused to YFP is polarly localized in C. crescentus (18), which does not have a homologue with an SD similar to PdhS. To test the minimal PdhS part required for polar localization in an in vivo background devoid of endogenous PdhS, we thus determined the localization of PdhS fragments in C. crescentus. Similarly to B. abortus, fragments F1-5-YFP, F1-4-YFP, and F1-3-YFP were polarly localized in C. crescentus. However, no polar fluorescent foci were observed when fragment F3-5-YFP was produced in C. crescentus. This indicates that the minimal portion of PdhS that is still able to be polarly localized in B. abortus and C. crescentus lies in the F1-3 fragment. The polar localization of F3-5-YFP in B. abortus but not in C. crescentus could be attributed to the presence of the F3 multimerization segment, allowing its recruitment by the endogenous PdhS. Additionally, fragment F2-5-YFP was polarly localized in C. crescentus. That fragment F2-PAS is polarly localized in B. abortus suggests the D1 domain is not strictly required for polar localization. We thus propose that the minimal portion of PdhS that polarly localized the protein in both B. abortus and C. crescentus resides within the F2-3 fragment, while the F1, F4, F5, and PAS fragments of the SD are not strictly required for polar localization. Altogether, the data generated by the domain mapping approach contribute to a better characterization of the PdhS SD.

**DISCUSSION**

We used the pentapeptide scanning mutagenesis method on an essential gene present in a class III pathogen to obtain a validated TS strain. We observed PdhS loss of function and a dominant-negative effect in B. abortus. The data presented here show that PdhS is involved in the control the bacterial cell cycle progression by regulating growth and division processes in B. abortus.

The aberrant morphologies generated by the presence of the nonphosphorylatable version of PdhS (PdhS<sup>H805A</sup>) can be due to the residual phosphatase activity since the mutation of the phosphorylatable conserved histidine has been shown to disrupt kinase activity but not the phosphatase activity of several HKs (22, 25). Moreover, the fragments containing F5 and the PdhS<sup>H805A</sup> form could titrate PdhS partners in the cell. This dominant-negative effect can be correlated with the presence of the interacting fragment 3 that can permit the mutated protein to multimerize with endogenous wild-type protein. Overexpression of pdhS and production of fragment F1-5 or the H805A form all resulted in vari-
ous morphological alterations, including the formation of Y-shaped bacteria (Fig. 2), which highlights a dominant-negative effect on cell division. Indeed, this shape is compatible with a cell division defect (37) without alteration of the growth capacity, since mitomycin C, a compound known to indirectly block cell division by cross-linking DNA, produced this effect as well (Fig. 2B). This observation is consistent with the previously reported formation of branches and swelling when cell division is impaired in Rhizobium meliloti (24). The TS strain at the restrictive temperature does not display these aberrations, probably because the growth problem (Fig. 1) prevents the generation of morphological alterations under these conditions. The mechanism

FIG 4 Interaction assay using the yeast two-hybrid (Y2H) approach. PdhS fragments that gave a positive interaction result are shown in black lines scaled to the PdhS primary structure. (A) PdhS fused to the DNA binding domain of the transcription factor Gal4 interacts with F1-CD (whole PdhS protein used as a positive control, from domain 1 to the catalytic domain), F1-3, F2-5, F2-3, F3-CD, F2-PAS, F3-4, and F3-PAS. The common fragment to all interacting fragments was F3 (residues 210 to 434). (B) FumC fused to the DNA binding domain of Gal4 interacts with the whole PdhS (F1-CD) and F1-3, F2-5, F2-3, F3-CD, F1-5, and F1-4. The common portion of the PdhS fragments interacting with FumC is F3 (residues 210 to 434). (C) PdhS fragments tested for their interaction with the response regulator DivK, reported to bind to the catalytic domain (18).
allowing PdhS to control cell division is unknown, but one could predict that if PdhS controls DivK function, it could also indirectly control CtrA abundance and/or activity. In agreement with this hypothesis, CtrA abundance is decreased in a pdhS overexpression strain (18). Moreover, biochemical studies showed that CtrA−P is able to bind to the minCDE promoter (1), and the MinD-YFP fusion protein was shown to oscillate in B. abortus (17), suggesting that it acts as an oscillator for FtsZ positioning, like in E. coli (8). Thus, by controlling DivK, CtrA, and Min, PdhS could indirectly control cell division in B. abortus (Fig. 6C). The recent report of unipolar growth in B. abortus and several other alphaproteobacteria (4) allows the generation of a simple model for the interpretation of the effect of mitomycin C and (over)expression of pdhS alleles. In this model (Fig. 6D), instead of having two growing poles in two sibling cells (thus one per cell, at the new pole, after cell division), the two growing foci remain in the nondividing mother cell, which starts to grow at two different places in the same cell, preferentially at the new pole (opposite to PdhS) (Fig. 6A, B, And D), therefore generating the Y-shaped bacteria.

We contributed to the characterization of the B. abortus essential HK PdhS using a domain mapping approach. We identified new parts in the SD required for (i) PdhS multimerization, (ii) PdhS interaction with the fumarase FumC, (iii) B. abortus cell cycle perturbation, and (iv) PdhS polar localization in B. abortus and C. crescentus (Fig. 7). This illustrates the complexity of the molecular functions of PdhS. A first observation of the domain mapping is the abundance of UCRs proposed to be linkers between domains and their length. This is striking for UCRs flanking D3, which are 50 and 46 aa long. It is of course not excluded that UCRs, and not only conserved domains, play a functional role. We could not rule out that a fragment folded in one assay is also folded in another one. This is why only PdhS fragments positive by at least one assay were taken into account for data interpretation, in an attempt to eliminate incorrectly folded or proteolyzed fragments that could give false-negative results.

Fragment F3 (residues 210 to 434) is proposed to play a major role since it is required for PdhS multimerization, interaction with FumC, and localization. If the association with a polar structure mediates localization, then fragment 3 could be seen as an interaction platform in PdhS. The structure of D3 is unknown, but a weak similarity (E value of 10−3 after PSI-BLAST) was found with the PAS-B domain of the Mmso redox sensor of Methylococcus capsulatus (34). In Mmso, fladin adenine dinucleotide (FAD) is bound to the PAS-A domain but not to the PAS-B domain (34). Because PdhS is involved in B. abortus cell cycle regulation, the interaction between this HK and the citric acid cycle enzyme FumC is interesting since it could link cell cycle progression with the metabolic state of the bacterium. However, up to now this hypothesis has not been supported by experimental data. The presence of the fragments containing F5 correlates with the generation of altered morphologies in B. abortus. The D5 domain is weakly similar (E value of 4 × 10−3) to the LOV1 domain of Phot1 from Chlamydomonas reinhardtii, a flavin mononucleotide (FMN) binding domain (11). However, none of the residues involved in FMN binding are conserved, suggesting that D5 could have a similar fold without being able to bind FMN. The D5 domain is
also weakly similar (E value of $10^{-3}$) to the 3MJQ structure in Protein Data Bank, an uncharacterized PAS domain with a fold resembling the one of the LOV1 domain. Structural predictions identified D3 and D5 as weak PAS-like domains, suggesting that there are at least three PAS-like domains in the SD of PdhS. Functional prediction is difficult because PAS-like domains fulfill highly diversified functions, therefore preventing the proposition of testable functional hypotheses about these domains.

The data presented here indicate that PdhS is involved in \textit{B. abortus} cell cycle progression by regulating cell growth and division and highlight the complexity of this HK by associating functions with subdomains within the SD. The morphological asymmetry and the regulatory network involving the RR DivK and CtrA are conserved among several alphaproteobacteria (2, 3, 15, 33). Interestingly, the symbiotic bacterium \textit{Sinorhizobium meliloti} contains a PdhS homolog named CbrA (12, 13). PdhS and CbrA share similarities in the SD, and suggested functions for PdhS activities and subdomains could be used to generate hypotheses about functions in CbrA. Because PdhS is asymmetrically localized at the time of division in \textit{B. abortus}, being concentrated at the old pole of the mother cell (16), the two daughter cells could display differences regarding cell growth and cell division control.

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