

PdhS, an Old-Pole-Localized Histidine Kinase, Recruits the Fumarase FumC in *Brucella abortus*^{†‡}

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The bacterial pathogen *Brucella abortus* was recently demonstrated to recruit the essential cytoplasmic histidine kinase PdhS to its old pole. Here, we report identification of the fumarase FumC as a specific partner for the N-terminal “sensing” domain of PdhS, using an ORFeome-based yeast two-hybrid screen. We observed that FumC and PdhS colocalize at the old pole of *B. abortus*, while the other fumarase FumA is not polarly localized. FumC is not required for PdhS localization, and polar FumC localization is not FumA dependent. FumC homologs are not polarly localized in *Sinorhizobium meliloti* and *Caulobacter crescentus*, suggesting that polar recruitment of FumC by PdhS is evolutionarily recent.

It was previously reported that morphological asymmetry, here defined by the production of two sibling cells different in length at each cell cycle, is a common feature shared by at least four members of the alphaproteobacteria with diversified lifestyles, namely, the free-living model bacterium *Caulobacter crescentus*, the plant pathogen *Agrobacterium tumefaciens*, the plant symbiont *Sinorhizobium meliloti*, and the facultative intracellular pathogen *Brucella abortus* (5). In *B. abortus*, an essential cytoplasmic histidine kinase named PdhS is anchored to the old pole of the bacterium (6). PdhS interacts with the response regulator DivK (6).

PdhS is a rather atypical histidine kinase. It is cytoplasmic and contains an N-terminal “sensing” domain with no predicted function, except for a PAS domain that remains to be characterized. Nevertheless, the N-terminal part preceding the PAS domain of PdhS is sufficient for polar localization in *B. abortus*, as well as in the alphaproteobacteria *S. meliloti* and *C. crescentus* (6). This suggests that a molecular mechanism and/or polar structure required for PdhS targeting is conserved in these alphaproteobacteria.

FumC is identified as a PdhS binding protein in a yeast two-hybrid screen. Given that PdhS contains a long, soluble (predicted cytoplasmic) N-terminal domain, we hypothesized that this part of the protein could be involved in protein-protein interactions. We therefore carried out a yeast two-hybrid (Y2H) screen between the full-length version of PdhS and the majority (96.7%) of the 3,198 proteins predicted to be encoded in the *B. melitensis* 16M genome, whose coding sequences (CDS) are cloned in the ORFeome (2). A detailed description of the protocols used is provided in the supplemental material; all strains and plasmids are described in Table S1,

and primers are reported in Table S2. Four candidate clones were positive for at least two Y2H reporters. One of them was DivK (data not shown). We also identified two predicted hypothetical proteins (data not shown) and a predicted 463-residue protein (BAB2_0186; NCBI accession number YP_418415), renamed FumC here, that was positive for the three reporter genes used in the Y2H assay. We performed a second Y2H assay in which the activation domain (AD) and DNA binding domain (BD) fusions were switched, in the sense that AD-PdhS was tested against BD fusions to FumC or other proteins (Fig. 1A). The AD fusion with PdhS, as well as with its N-terminal (PdhS'N, amino acids [aa] 1 to 613) and C-terminal (PdhS'C, aa 611 to 1035) parts, was tested. As expected, BD-DivK was able to interact with AD-PdhS'C and AD-PdhS (Fig. 1A), and the interaction between BD-FumC and AD-PdhS was confirmed. In addition, an interaction between BD-FumC and AD-PdhS'N was observed, suggesting that the N-terminal domain of PdhS is sufficient to recruit FumC. The PdhS-FumC interaction discovered in the Y2H screen was further supported in this study by use of a distinct technical approach based on the particular localization of *B. abortus* PdhS when it is produced in *C. crescentus* (Fig. 1B). PdhS-CFP (cyan fluorescent protein) is able to form foci in *C. crescentus*, and when FumC-YFP (yellow fluorescent protein) is produced in the same strain, it systematically colocalizes with PdhS-CFP. A detailed description of these assays is provided in the supplemental material.

The *B. abortus* genome encodes two fumarases, FumC and FumA. BAB2_0186 is the homolog of *Escherichia coli* FumC (see Fig. S1 in the supplemental material), which belongs to the family of class II fumarate hydratases (also called fumarases) and is involved in the tricarboxylic acid (TCA) cycle, in which it reversibly converts fumarate into L-malate (7). The *E. coli* genome also contains two genes sharing a high percentage of identity (89%) and named *fumA* and *fumB*, encoding, respectively, FumA and FumB, belonging to the family of class I fumarate hydratases (8). In *B. abortus*, the protein BAB1_0977 (NCBI accession number YP_414395) shares 66 and 65% identities (80% and 79% similarities) with *E. coli* FumA and FumB, respectively. BAB1_0977 was therefore named FumA. FumA

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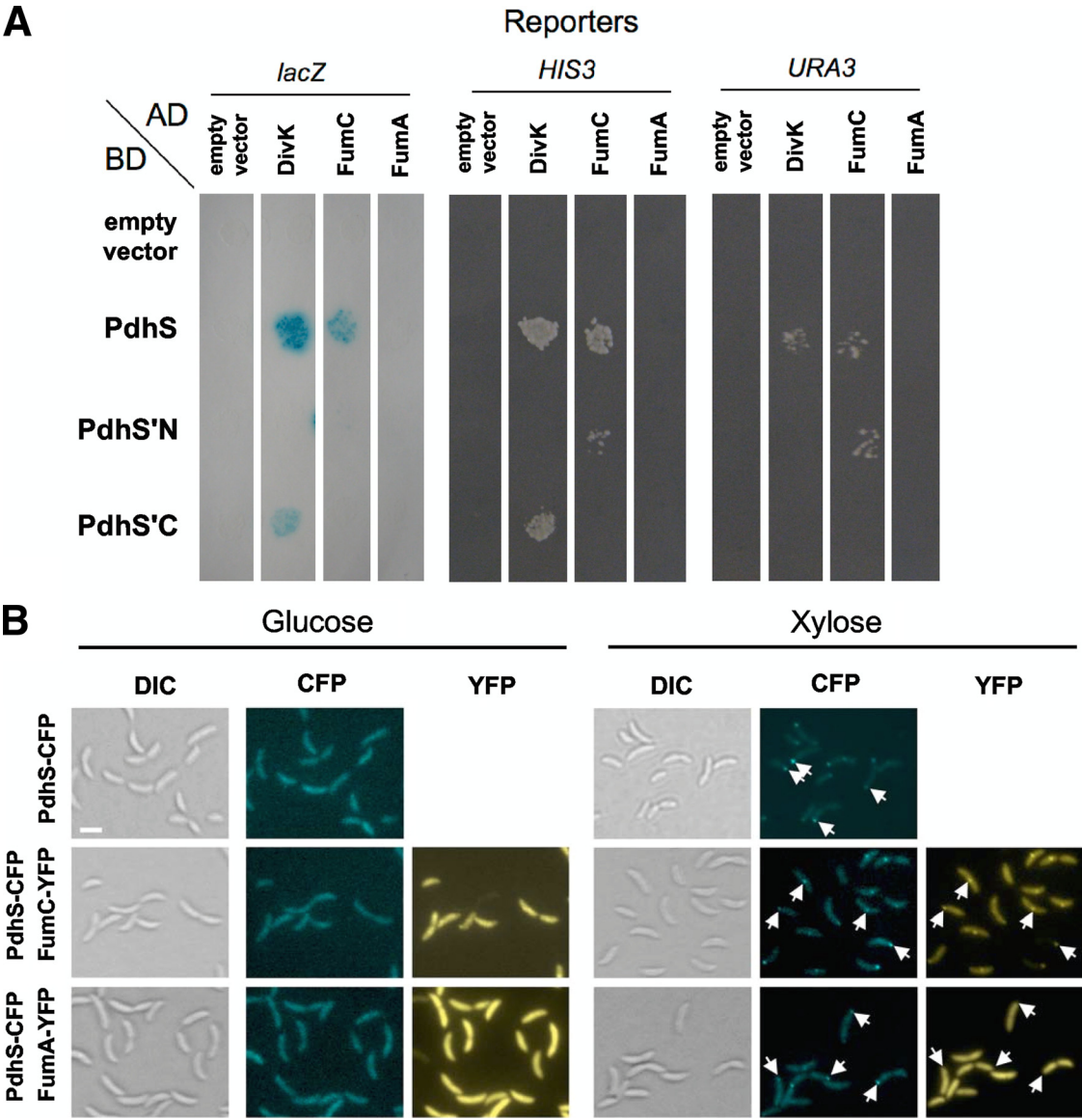


FIG. 1. *B. abortus* PdhS interacts with FumC. (A) Yeast two-hybrid assay for the PdhS-FumC interaction. The fusions to either the AD or the BD of Gal4p are indicated next to the pictures. Three reporters were assayed for activation. PdhS'N and PdhS'C correspond to the N-terminal (613 aa) and C-terminal (425 aa) regions of PdhS, respectively. The PdhS-FumC interaction is positive for *lacZ*, *HIS3*, and *URA3* reporters. The PdhS'N-FumC interaction is positive for *HIS3* and *URA3* reporters, suggesting that FumC interacts with the N-terminal part of PdhS. The *divK* and *fumA* CDS were used as positive and negative controls for the PdhS-FumC interaction, respectively. Another set of negative controls corresponded to the plasmids ("empty vector") coding for either the Gal4p AD or the Gal4p BD, without insert. (B) Reconstruction of FumC recruitment by PdhS in *C. crescentus*. Three strains of *C. crescentus* carrying a *pdhS-cfp* fusion (XDB1166) under the control of the *xylX* promoter were constructed. In two of them, an additional fusion, either *fumC-yfp* (pJM080) or *fumA-yfp* (pJM095; negative control), is carried on a replicative plasmid. The cells were grown overnight in PYE (peptone-yeast extract) with glucose to reach a starting optical density at 600 nm (OD_{600}) of 0.05. The medium was then supplemented with glucose (no induction) or xylose (induction) for 5 h, and the cells were subsequently immobilized on agarose pad slides and visualized by differential interference contrast (DIC) microscopy and in CFP and/or YFP channels. The fusions produced by each strain are indicated on the left. The type of observation, either DIC microscopy (Normarski) or CFP or YFP typical fluorescence, is also indicated for each culture medium. White arrows indicate the positions of some PdhS-CFP foci. All pictures are shown with the same magnification. Bar, 2 μ m.

is not able to interact with PdhS in a Y2H assay (Fig. 1). A demonstration of fumarase activity by recombinant FumA and FumC is provided in the supplemental material.

We constructed an in-frame deletion of *fumA* or *fumC* CDS in the *B. abortus* 544 Nal^r strain (named, respectively, XDB1160 or XDB1157) by using an allelic-replacement pro-

cedure. The two single deletions did not alter overall cell morphology or cell growth compared to that for the wild-type strain (data not shown). However, *fumA* and *fumC* deletions were synthetic lethal (see the supplemental material). From these results, we concluded that *fumA* and *fumC* are functionally redundant, at least under the culture conditions used here,

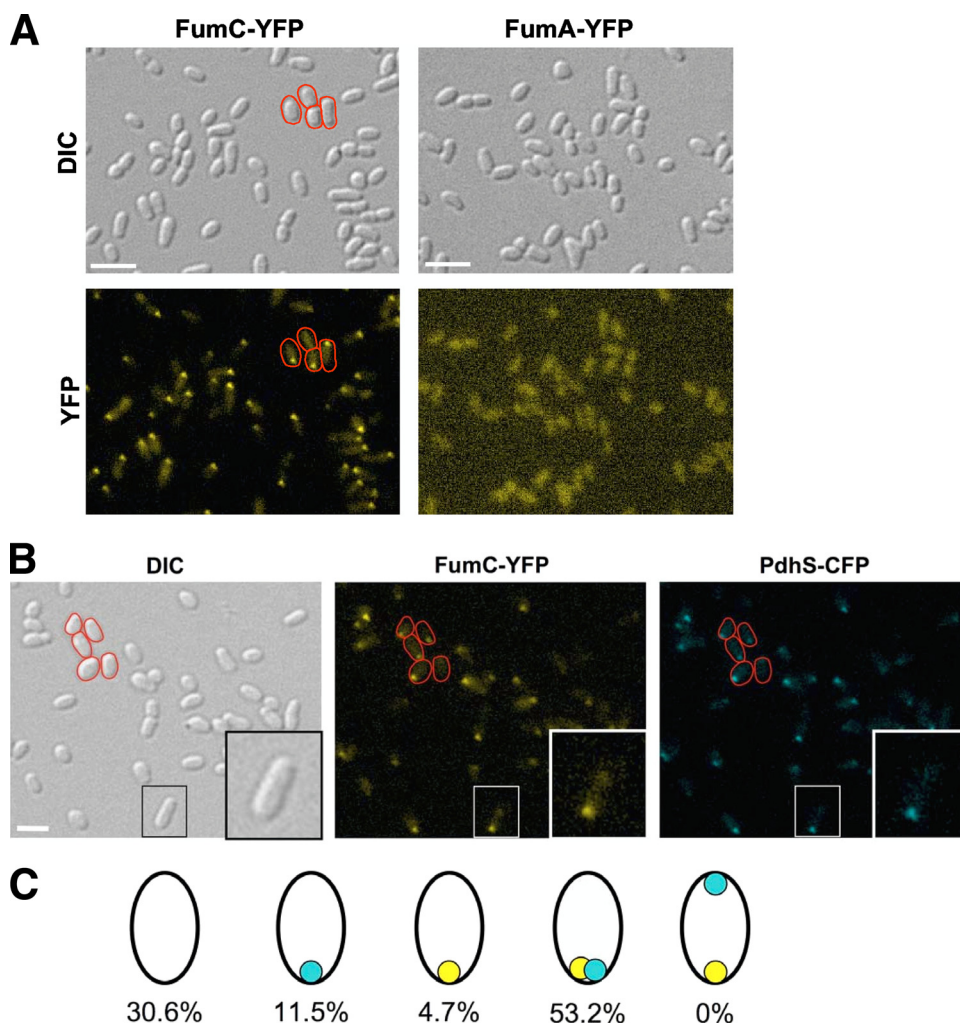


FIG. 2. FumC-YFP is localized at the old pole in *B. abortus*. (A) Localization of FumC-YFP and FumA-YFP in *B. abortus*. The XDB1150 and XDB1153 strains, producing, respectively, FumC-YFP and FumA-YFP from fusions integrated into the *B. abortus* 544 chromosome, were observed during the exponential growth phase by DIC microscopy (Normarski) and for the typical fluorescence spectrum of YFP. (B) Colocalization of PdhS-CFP with FumC-YFP in *B. abortus*. A *fumC-yfp* fusion was integrated by homologous recombination at the *fumC* locus in the chromosome of XDB1155, a *B. abortus* strain in which *pdhS* was replaced by a *pdhS-cfp* allele. Observations were made by DIC microscopy (Normarski) and for typical fluorescence of YFP and CFP, as indicated. Some bacteria are surrounded with a red line in the pictures to show that the FumC-YFP and PdhS-CFP signals are polar. Bar, 2 μ m. (C) Schematic drawing illustrating the proportions of cells that exhibit a localization pattern for PdhS-CFP and/or FumC-YFP. A total of 425 bacteria were examined. The polar foci of PdhS-CFP and FumC-YFP are shown in blue and yellow, respectively. It is striking that all 226 bacteria (53.2%) for which a CFP focus and a YFP focus were detectable have colocalized signals and that none present foci labeling the opposite poles. A quite large fraction of the population (30.6%) does not display any focus, whether CFP or YFP. This could be due to a low expression level of the fusions, resulting in poor sensitivity for the fluorescent signal.

and that translational fusions of *fumC* and *fumA* to *yfp* are at least partially functional, because they rescued the lethality of the *fumA fumC* double deletion.

FumC localizes at one pole in *B. abortus*. We generated strains carrying a chromosomal fusion of *fumC* CDS with *yfp*, *cfp*, and mCherry gene CDS (strains XDB1150, XDB1151, and XDB1152, respectively) in the *B. abortus* 544 Nal^r background. The *fumC* CDS is not predicted to be cotranscribed with the downstream CDS (data not shown). By fluorescence microscopy, we observed that FumC-YFP accumulated at one pole in 74% ($n = 431$) of the XDB1150 cells, reminiscent of the localization pattern of PdhS (Fig. 2). Similar results were observed for FumC-CFP and FumC-mCherry fusions, suggesting that the nature of the fluorescent protein is not responsible for

the polar localization. This localization pattern was also observed when a *fumC-yfp* fusion was expressed from the low-copy-number plasmid pJM063 introduced into either the wild-type or the Δ *fumC* strain (data not shown). An inactive version of FumC (with the H188N substitution [see the supplemental material]) was still able to be recruited to a pole when it was fused to YFP (data not shown). In contrast, FumA-YFP was homogeneously scattered throughout the cytoplasm whether it was produced from the chromosomal *fumA* locus (XDB1153 strain) (Fig. 2A) or from the low-copy-number plasmid pJM043 in *B. abortus* (data not shown). Western blot analysis performed on every strain indicated that the fusion proteins were stable (data not shown). We wondered whether the absence of one fumarase, either FumA or FumC, has an impact

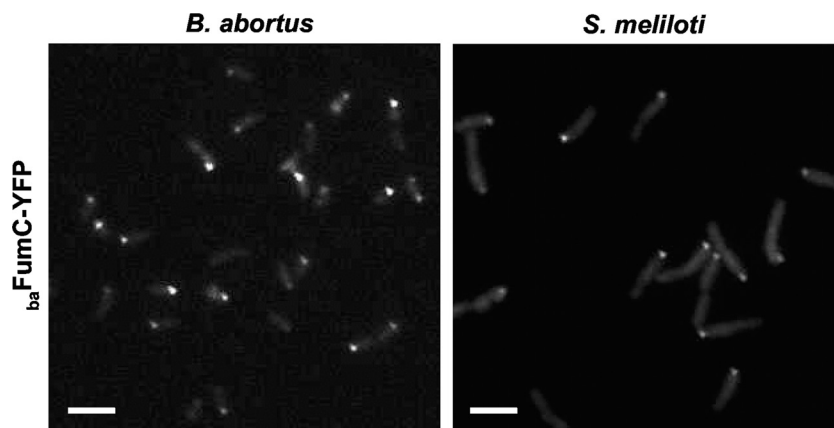


FIG. 3. Localization of $_{ba}$ FumC in *B. abortus* and *S. meliloti*. For the two species, the localization of the *B. abortus* FumC-YFP fusion was observed in the exponential growth phase of the culture. The *fumC-yfp* fusion is carried on a low-copy-number plasmid (pJM063). Bar, 2 μ m.

on the subcellular localization of the other. However, FumA-YFP and FumC-YFP fusions retained localization patterns in Δ *fumC* and Δ *fumA* mutants, respectively, that were similar to those found with the wild-type strain (see Fig. S2 in the supplemental material). These data suggested that FumC localization, at least under the conditions tested here, does not depend on the presence of FumA and that the FumA-YFP localization pattern is not altered when FumC is absent. We tested the localizations of other TCA cycle enzymes fused to the N or C terminus of the mCherry fluorescent reporter but did not detect polar localization (C. Van der Henst and M. Deghelt, unpublished data), suggesting that no TCA cycle enzyme is localized at the old pole in *B. abortus*.

FumC and PdhS colocalize at the old pole of *B. abortus*. As FumC and PdhS display similar unipolar localization patterns and physically interact in Y2H assays, we reasoned that they may colocalize in *B. abortus*. Therefore, a *B. abortus* 544 strain (XDB1155) in which the wild-type chromosomal *pdhS* allele was replaced by a *pdhS-cfp* fusion was constructed. PdhS-CFP, as the only copy of PdhS that supported viability, did not impair cell morphology and displayed a localization pattern consistent with that reported previously (6). A *fumC-yfp* fusion was then integrated at the *fumC* locus, as described for the XDB1150 strain. The FumC-YFP localization pattern in this *pdhS-cfp* background strain (XDB1156) was similar to those described above. As depicted in Fig. 2B and C, 92% ($n = 246$) of the cells for which a FumC-YFP polar focus was detectable exhibited a corresponding PdhS-CFP polar focus at the same localization. Moreover, although 16% of the entire bacterial cell population ($n = 425$) exhibited either a PdhS-CFP or a FumC-YFP polar focus, cells with two foci located at opposite poles were never observed. These data strongly argue in favor of a model in which FumC and PdhS colocalize at the old pole of *B. abortus*.

FumC is not required for PdhS polar localization. The discovery of a new PdhS partner, FumC, prompted us to explore the impact of the absence of FumC on PdhS subcellular distribution. A *fumC* deletion was therefore constructed in the *B. abortus* 544 *pdhS-cfp* strain (XDB1155) to generate the XDB1158 strain. The XDB1155 and XDB1158 strains displayed indistinguishable PdhS-CFP localization patterns, with,

respectively, 76% and 75% of cells exhibiting a single polar focus, strongly suggesting that FumC alone is not required for PdhS polar targeting (see Fig. S3 in the supplemental material).

***B. abortus* FumC does not share its localization property with $_{cc}$ FumC and $_{sm}$ FumC, its orthologs in *C. crescentus* and *S. meliloti*.** The sequence conservation among FumC homologs in alphaproteobacteria led us to investigate whether polar localization was a common trait of FumC homologs. To address this question, two alphaproteobacteria were selected: *C. crescentus*, which does not possess any close PdhS homolog, and *S. meliloti*, which possesses two close PdhS homologs (5), PdhS1 (or CbrA) (3, 4) and PdhS2. The localization of FumC homologs in these organisms (*C. crescentus* FumC [$_{cc}$ FumC] and *S. meliloti* FumC [$_{sm}$ FumC]) was examined. In *C. crescentus*, the production of $_{cc}$ FumC-YFP from a chromosomal fusion under the control of the $_{cc}$ *fumC* promoter did not lead to the detection of foci but displayed a diffuse localization pattern (see Fig. S4 in the supplemental material). An $_{sm}$ FumC-YFP fusion was produced from the low-copy-number plasmid pJM106 in *S. meliloti*. The $_{sm}$ FumC-YFP fusion did not induce a major alteration of cell morphology and appeared to be distributed diffusely throughout the cytoplasm (see Fig. S4 in the supplemental material). Based on these data, we propose that FumC polar recruitment is not a common feature shared by all alphaproteobacteria.

***B. abortus* FumC is polarly localized in *S. meliloti* but not in *C. crescentus*.** The PdhS property to localize at poles in *S. meliloti* and *C. crescentus* suggested the conservation of a polar structure among several alphaproteobacteria (6). We thus investigated whether *B. abortus* FumC ($_{ba}$ FumC), $_{cc}$ FumC, and $_{sm}$ FumC have the ability to be recruited at one pole in other alphaproteobacteria. For this, pJM063 was first introduced into *C. crescentus* and *S. meliloti* to monitor $_{ba}$ FumC-YFP localization. The $_{ba}$ FumC-YFP fusion was diffusely distributed throughout the *C. crescentus* cytoplasm (see Fig. S5 in the supplemental material), but when it was produced in *S. meliloti*, polar foci with a localization pattern similar to that observed for *B. abortus* were detected (Fig. 3). Then pJM105 (a low-copy-number plasmid carrying a $_{cc}$ *fumC-yfp* fusion) and pJM106 (a low-copy-number plasmid carrying an $_{sm}$ *fumC-yfp*

fusion) were separately introduced into the *B. abortus* Δ *fumC* (XDB1157) strain to ensure that the $_{cc}$ FumC-YFP and $_{sm}$ FumC-YFP fusions, respectively, did not compete with endogenous $_{ba}$ FumC. The $_{cc}$ FumC-YFP fusion was not recruited to a cell pole of *B. abortus* (see Fig. S5 in the supplemental material), whereas the control $_{ba}$ FumC-YFP fusion was (see Fig. S6 in the supplemental material). Polar foci corresponding to the $_{sm}$ FumC-YFP fusion were observed for only 7% of the *B. abortus* cell population ($n = 1,330$). $_{cc}$ FumC-YFP in *S. meliloti* and $_{sm}$ FumC-YFP in *C. crescentus* did not display polar localization, although $_{sm}$ FumC-YFP presented a patchy localization that could be due to its overproduction (see Fig. S5 in the supplemental material). Altogether, these results indicated that $_{ba}$ FumC contains structural information allowing for its polar localization, a characteristic that is absent or altered in its $_{cc}$ FumC and $_{sm}$ FumC homologs. Mapping of the substitutions between $_{ba}$ FumC and $_{sm}$ FumC sequences on the three-dimensional structure of *E. coli* FumC reveals that a belt of substitutions surrounds the FumC homotetramer (see Fig. S7 in the supplemental material). These substitutions do not directly affect the catalytic site (see Fig. S8 in the supplemental material) but may be involved in the generation of an interaction surface on the $_{ba}$ FumC structure that is absent in $_{sm}$ FumC. This $_{ba}$ FumC interaction surface could be involved in binding to PdhS and possibly PdhS homologs.

In conclusion, the data we report here strongly support that PdhS is able to directly recruit an important metabolic enzyme at the old pole in *B. abortus*, thereby suggesting that it could play multiple roles in the segregation of different functions between sibling cells. Moreover, $_{ba}$ FumC localization suggests that the old pole could be a distinctive site of recruitment for various proteins involved, for example, in signaling or some aspects of metabolism. This is particularly relevant for *B. abortus*, since this facultative intracellular pathogen is able to inhibit its replication before reaching an endoplasmic reticulum-like compartment (1), and it is suspected that metabolic rearrangements are crucial for *Brucella* species adaptation and survival inside mammalian cells (1a). The links between metabolism, asymmetric division, and intracellular survival thus deserve further study.

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