Localization of the Outer Membrane Protein OmpA2 in *Caulobacter crescentus* Depends on the Position of the Gene in the Chromosome

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The outer membrane of Gram-negative bacteria is an essential structure involved in nutrient uptake, protection against harmful substances, and cell growth. Different proteins keep the outer membrane from blebbing out by simultaneously interacting with it and with the cell wall. These proteins have been mainly studied in enterobacteria, where OmpA and the Braun and Pal lipoproteins stabilize the outer membrane. Some degree of functional redundancy exists between these proteins, since none of them is essential but the absence of two of them results in a severe phenotype. *Caulobacter crescentus* has a different strategy to maintain its outer membrane, since it lacks the Braun lipoprotein and Pal is essential. In this work, we characterized OmpA2, an OmpA-like protein, in this bacterium. Our results showed that this protein is required for normal stalk growth and that it plays a minor role in the stability of the outer membrane. An OmpA2 fluorescent fusion protein showed that the concentration of this protein decreases from the stalk to the new pole. This localization pattern is important for its function, and it depends on the position of the gene locus in the chromosome and, as a consequence, in the cell. This result suggests that little diffusion occurs from the moment that the gene is transcribed until the mature protein attaches to the cell wall in the periplasm. This mechanism reveals the integration of different levels of information from protein function down to genome arrangement that allows the cell to self-organize.

The outer membrane (OM) of *Escherichia coli* and other Gram-negative bacteria is kept close to the cell through the action of several proteins that simultaneously interact with the OM and the peptidoglycan cell wall, serving as bridges between these structures (1). In *E. coli*, the Braun (Lpp) and Pal lipoproteins together with OmpA have this function (2–8). Lpp and OmpA are two of the most abundant proteins in the cell (9–11). Lpp is covalently attached to the cell wall, and it interacts with the outer membrane through a lipid moiety that is added to the protein while it is being translocated through the inner membrane (IM) (3, 12). The covalent bond between Lpp and the cell wall occurs between a lysine residue at the C terminus of the protein and the diaminopimelic acid present among the peptides of the cell wall (3). The attachment of Lpp to the cell wall is catalyzed by a family of peptidyl transferases (13), but this reaction does not occur at the same time as the cell wall expands, and up to one generation time may be necessary before Lpp attaches to newly incorporated peptidoglycan (14). Lpp is a small protein (78-amino-acid [aa] precursor and 58-aa mature protein in *E. coli*) that through bioinformatic techniques can only be found in gammaproteobacteria. However, it is possible that the small size of this protein limits its detection based on sequence similarity. In fact, a peptidoglycan composition analysis of several Gram-negative bacteria suggested that it may be present in other bacterial groups (15). OmpA has an integral outer membrane protein (OMP) domain at its N terminus and a periplasmic C-terminal domain (16–19). The contribution of OmpA to the stability of the OM comes from the binding of the C-terminal domain to the diaminopimelic acid present in the cell wall (20). The last protein involved in the stabilization of the OM is Pal. This protein interacts with the Tol proteins to form the Tol-Pal system (21). The TolA, -B, -Q, and -R proteins form a complex that expands from the IM through the periplasmic space (21–25). The Tol complex interacts with Pal through TolB (21, 26–28) and through TolA in a proton motive force-dependent manner (29, 30). During cell division, the Tol-Pal system is recruited to the division site, where it probably plays a relevant role in the stabilization of the OM (31). In *E. coli*, none of the proteins involved in OM stabilization is essential; however, cells of a pal*ompA* double mutant had an altered morphology and required supplementation with divalent cations for growth (2), and the lpp pal* mutant is likely not viable (27). Possible interactions of TolB with OmpA and Lpp have also been reported, suggesting that the three proteins involved in OM stability may act coordinately (27).

The Gram-negative bacterium *Caulobacter crescentus* can be isolated from freshwater environments and is capable of growing under low-nutrient conditions (32, 33). To colonize this type of environment, *C. crescentus* cells have different types of adaptations. At the biochemical level, the adaptation of *C. crescentus* to its environment can be observed in the lipid composition of the IM, which has a high content of glycolipids and a low concentration of phospholipids (34). In a similar way, the lipopolysaccharide (LPS) molecule of the OM lacks the terminal phosphates present in the classical LPS molecule (35). At the morphological level, when grown in low-phosphate-containing media, *C. crescentus* cells elongate their stalk (36, 37). The stalk is a compartmentalized polar structure that consists of a thin elongation of the cell envelope, and it has been proposed to help in the uptake of nutrients (38–41). The role of the OM of the stalk in this function has been confirmed by the presence of TonB-dependent transporters in this structure and by the uptake of phosphate analogs in isolated...
TABLE 1 Strains and plasmids

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<tr>
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stalks (38, 42). Another adaptation to growth in diluted medium is the high number of TonB-dependent transporters that are encoded in the chromosome of this bacterium (43, 44). In contrast to E. coli, the stability of the OM of C. crescentus is mainly dependent on the Tol-Pal system, and it has been shown that these proteins are essential and that Pal, in addition of being recruited to the division site, also accumulates in the stalk (45–47). The relevance of the Tol-Pal system in the stability of the OM of C. crescentus can in part be explained by the absence of an Lpp protein in this bacterium (48). In addition to the Tol-Pal system, C. crescentus has two ompA-like genes that may be relevant for the integrity of the OM. In this work, we characterized the function and localization of one of these genes, ompA2. Our results indicate that this protein plays a minor role in the stability of the OM but that it promotes stalk growth. Characterization of the cellular localization of OmpA2 showed that this protein forms a gradient with a higher concentration at the stalked pole. Interestingly, the formation of this gradient is dependent on the position of the gene in the chromosome and is relevant for the function of the protein in promoting stalk growth. The localization mechanism and function of this protein are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of E. coli were grown in LB medium with the appropriate antibiotic at 37°C. Plasmids were maintained and purified from E. coli DH5α or TOPL10 strains. Strains of C. crescentus were grown at 30°C unless otherwise specified, in peptone-yeast extract (YPE) rich medium or Tris-based M5GG medium (49–51) supplemented with the appropriate amount of phosphate. Antibiotics were used at the following concentrations for E. coli: kanamycin, 50 μg ml⁻¹; spectinomycin, 50 μg ml⁻¹; tetracycline, 10 μg ml⁻¹; gentamicin, 20 μg ml⁻¹; nalidixic acid, 20 μg ml⁻¹. For C. crescentus, antibiotics were used at the following concentrations for liquid and solid media, respectively: kanamycin, 5 and 20 μg ml⁻¹; spectinomycin, 25 and 100 μg ml⁻¹; tetracycline, 3 and 3.3 μg ml⁻¹; gentamicin, 2 and 5 μg ml⁻¹. Strains and plasmids are listed in Table 1, and their construction is explained in the supplemental material.

Molecular biology techniques and Western blotting. Restriction enzymes and T4 DNA ligase were bought from New England BioLabs or Invitrogen. PCRs were done with TaKaRa PrimeSTAR-HS enzyme with high-GC buffer. For Western blot assays, SDS-PAGE-resolved proteins were transferred to nitrocellulose membranes, which were then incubated with a mouse red fluorescent protein (mRFP) polyclonal antibody raised against 6His-tagged mRFP. For detection, an alkaline phosphatase-conjugated anti-mouse antibody (Sigma) was used together with Tropix CDP-Star/Nitro-Block substrate. All samples were collected at an optical density at 660 nm (OD₆₆₀) of 0.3, and the protein amount in each extract was quantified via a Bradford assay. An equal amount of protein (3 μg) was loaded into every well.

Plasmolysis. A 1-ml aliquot of a culture with an OD₆₆₀ of 0.3 was centrifuged, and the supernatant was removed by aspiration. The cell pellet was resuspended in 100 μl 10 mM phosphate buffer (pH 7) with 2 M NaCl. A 1-μl aliquot of this sample was placed on a 1.5% agarose bed prepared with the same buffer, and the cells were immediately visualized.
Fluorescence microscopy. Fluorescence microscopy images were taken from a Nikon E600 microscope and a Hamamatsu ORCA-ER camera. Images were then processed with ImageJ as described below. Additional images that are shown for comparison were acquired with identical settings and processed in the same way. To determine the number of cells that showed a concentration gradient of the OmpA2-mCherry protein, aliquots from synchronized cultures of cells expressing the OmpA2-mCherry and MipZ-YFP protein fusions were imaged. The cells in the images were detected with MicrobeTracker (52), and the resulting meshes were manually inspected to discard from the analysis wrongly detected cells and, if necessary, cells with two MipZ-YFP fluorescent foci. The graphs of the fluorescence intensities along the cells were obtained using the MicrobeTracker tool inprofileall. If swarmer cells with a single chromosome were the subject of the experiment, then cells that were longer than 2.5 μm were discarded, since these cells were probably filamenting due to a problem in chromosome replication or segregation. The presence of a gradient was manually determined by inspection of the fluorescence intensity graphs. The criteria used to count a cell as having a polar gradient consisted of a maximal signal in the first or last quarter of the cell length and a constant decrease in the signal intensity toward the opposite pole. When required, cells were treated with 2 μg/ml of chloramphenicol and 100 μg/ml of nalidixic acid to stop DNA replication, translation, and transcription in swarmer cells.

Growth curves and competition assays. The growth curves were determined by inoculating 15 ml of the appropriate medium with 150 μl of a culture grown for a second time to saturation (OD₆₆₀ of approximately 1.2). Cultures were grown in 125-ml Erlenmeyer flasks and were incubated in a water bath at the appropriate temperature under constant shaking at 200 rpm. Samples of 1 ml were taken every 2 h, and the OD₆₆₀ was measured. When required, sodium deoxycholate (SDC) was added from a stock solution of 50 mg/ml in water.

For the competition assays, equal numbers of cells, determined by the optical density (9 × 10⁷ total cells for high-phosphate medium and 1.2 × 10⁷ total cells for low-phosphate medium) were mixed from stationary cultures in 3 ml of the appropriate medium. Cultures were incubated at 30°C under constant shaking at 200 rpm for 24 h, and the next culture was inoculated with 30 μl of the previous one. CFU were determined from the first mixture of cells and from the stationary cultures at 24 and 48 h.

Stalk measurements. Measurement of stalk sizes and percentages of stalked cells were performed by staining with FM 4-64FX at a final concentration of 17 ng/ml, working with aliquots from exponentially growing cultures at an OD₆₆₀ of 0.25 or from stationary cultures. Stalk sizes and percentages of stalked cells were determined using ImageJ.

Statistical tests. When required, a t test was performed to determine the significance of the difference between two sample sets. The test was performed using Microsoft Excel.

RESULTS
Caulobacter crescentus has two probable OmpA proteins. To identify the OmpA homolog protein in C. crescentus, a BLASTP search against the genome of this bacterium using OmpA from E. coli as query sequence was done. This search revealed several proteins that were only similar to the C terminus of OmpA. Since the N-terminal region of OmpA is essential for its function, the domain composition of the proteins with an OmpA C-terminal domain was examined. Although in none of the candidates was an OmpA N-terminal domain identified, two of the proteins, CC3494 and CC0747, contained an N-terminal domain predicted to be an integral OM β-barrel, suggesting that these two proteins could have a function similar to OmpA. In both proteins, the C- and N-terminal domains are connected through a linker with a high proline content (see Fig. S1A in the supplemental material).

Since OmpA is a very abundant protein in E. coli, it can be expected that a protein with a similar function would also be highly expressed in C. crescentus. For this reason, we verified the expression levels of these two genes in previously published global expression data (53, 54). While both genes are transcribed, the signal level of CC3494 was five times higher than that of CC0747 (see Fig. S1B and C), and when compared with the rest of the genes in the chromosome, CC3494 was one of the most highly expressed genes (data not shown). For this reason, we decided to determine the function of this gene, and we will refer to it as ompA2 to maintain consistency with a previous report in which CC0747 was referred to as ompA (55).

The ompA2 mutant has an unstable OM and shorter stalks. To determine the function of ompA2, we generated a mutant of this gene (LDG2) by replacing its coding sequence with an Ω56c cassette. In agreement with results of a recent high-throughput mutagenesis study aimed at determining the essential genes in C. crescentus (56), the mutant was viable and did not show any apparent phenotype. To better characterize this mutant, we compared the growth curves of the mutant under different conditions with those of the wild type. No differences in the lag phase or in the maximal OD₆₆₀ were observed (data not shown); however, the mutant showed a slower replication time in PYE, in M5GG defined medium supplemented with low phosphate (50 μM), and in PYE medium incubated at 37°C (Fig. 1A), suggesting that OmpA2 plays a more relevant role when the cell is under stress. A growth competition assay between the wild-type and LDG2 (ompA2 mutant) strains revealed that the mutant is at a disadvantage in high- and low-phosphate media (see Fig. S2 in the supplemental material), indicating that the OmpA2 protein is relevant for the general fitness of the cells. Mutants affected in the integrity of the OM become more susceptible to different types of substances in the growth medium (57); since OmpA2 is predicted to be relevant for OM stability, we tested the sensitivity of the ompA2 mutant to different antibiotics (chloramphenicol and nalidixic acid), detergents (Tween 20, SDS, and SDC), and lysozyme in a plate assay as previously described (58). In this test, we could not detect any difference between the mutant and the wild-type strain, indicating that the integrity of the OM was not severely affected (data not shown).

We further tested the sensitivity of the mutant to SDS, EDTA, and SDC by growing it in different concentrations of these substances and recording the OD₆₆₀ after 8 and 24 h; a difference was only observed with SDC (data not shown). A growth curve showed that in the presence of SDC the growth of the wild-type and mutant strains was similar and that they had the same replication time. In the presence of SDC, the growth of both strains arrested at one point in the growth curve; however, this arrest occurred earlier and was more pronounced in the mutant than in the wild-type strain (Fig. 1B).

To visually determine if the absence of OmpA2 resulted in an OM instability, wild-type and mutant cells were stained with the membrane stain FM 4-64FX. Observation of the cells immediately after addition of the stain revealed the presence of small vesicles that were 8 times more abundant in the ompA2 mutant than in the wild type (Fig. 1C, left panels). Examination of ompA2 mutant cells that had been incubated with the stain for a longer time revealed the presence of blurry fluorescent patches around them, suggesting that these cells were losing their membranes. A more detailed observation showed that this occurred 14 times more frequently for the ompA2 mutant than for wild-type cells (Fig. 1C, right panels).

Initial inspection of LDG2 cells grown in low-phosphate me-
medium suggested that these cells had shorter stalks than wild-type cells (Fig. 2A). To confirm this observation, the percentage of stalked cells and the average stalk lengths of cells from exponentially growing cultures in low-phosphate medium were determined (Fig. 2B). To facilitate the observation of short stalks, the cells were stained with FM 4-64FX. The cultures of the ompA2 mutant showed decrements of approximately 20% in stalk length and of 50% in the number of stalked cells. These differences were no longer present in stationary cultures, suggesting that the absence of OmpA2 produces a delay in the appearance and probably in the growth of the stalk that is compensated by the reduced division rate in the stationary phase.

OmpA2-mCherry forms a concentration gradient from the old pole and stalk to the new pole. To corroborate the high expression level of ompA2 suggested by the microarray data, a gene fusion of ompA2 with the gene coding for the fluorescent protein mCherry was generated. A plasmid carrying a 5′-truncated version of ompA2 fused at its 3′ end with the mCherry gene was inserted in the chromosome by single crossover. Integration of this plasmid results in a single full copy of the ompA2 gene fused to mCherry expressed under the control of the native promoter. The resulting strain (LDG1) was grown to an OD660 of 0.3 in high- and low-phosphate media, and cells were observed with a microscope (Fig. 3A). The fluorescence was readily observed even at short exposure times under all conditions, indicating that in accordance with its possible role as an OmpA analog, OmpA2 is an abundant protein. The observed peripheral fluorescence is consistent with that of a membranal or periplasmic protein. Surprisingly, OmpA2-mCherry was more abundant at the stalk and stalked pole, and its concentration gradually decreased toward the new pole, forming a gradient. The gradient was more clearly defined in stalked or predivisional cells, suggesting that it forms as the cell progresses through the cell cycle and that it becomes stronger as the cell ages, since it was more evident in cells with a longer stalk (Fig. 3A). A Western blot assay using anti-mCherry antibodies showed that the fusion protein was stable and that the fluorescence observed was due to the full protein (Fig. 4B). To verify that the fusion protein was inserted in the OM, cells grown in high-phosphate medium were plasmolyzed and observed with the microscope. It has been reported that under hyperosmotic shock, the inner membrane separates from the cell wall and compacts into plasmolysis bays (59). To validate the plasmolysing conditions, LDG1 cells expressing, under the control of a xylose-inducible promoter, the IM protein TolQ fused to yellow fluorescent protein (YFP) were used. Although no plasmolysis bays could be detected in the phase contrast images, the change in the distribution of the TolQ-YFP fluorescence indicated that plasmolysis did occur (Fig. 3B). As expected, the OmpA2-mCherry fluorescence remained at the periphery of the cell, indicating that this protein is integrated in the OM. Further support for the correct assembly of

FIG 1 The ompA2 mutant has an unstable OM. The growth rate, sensitivity to SDC, and outer membrane stability of the ompA2 mutant were determined. (A) Growth rates (expressed as generation time) of the wild-type (WT) and ompA2 mutant strains under different growth conditions. Error bars represent standard deviations, and significantly different growth rates are indicated with asterisks. (B) Growth curve for the wild-type and ompA2 mutant strains in the presence of SDC (SDC was present from the beginning of the experiment). (C) Wild-type and ompA2 mutant cells stained with FM 4-64FX. The arrow indicates a small vesicle. White bar, 1 μm.
OmpA2-mCherry in the OM was obtained after staining LDG1 cells with FM 4-64FX, which did not reveal any OM blebbing. In addition, the growth rate of strain LDG1 was the same as that of the wild type (data not shown), and in a competition assay against the wild-type strain the number of LDG1 cells did not decrease (see Fig. S2 in the supplemental material).

As a first step in characterizing the mechanism that allows OmpA2 to form a concentration gradient, the ompA2-mCherry allele was cloned in a low-copy-number plasmid and expressed from a vanillic acid-inducible promoter. The fluorescence level of cells carrying this plasmid was lower than that of cells expressing the same gene fusion from the chromosomal native promoter of the ompA2 gene, and this observation was confirmed by Western blotting (Fig. 4B). In this experiment, two bands were observed in all the lanes corresponding to extracts in which the OmpA2-mCherry protein was expressed from its own promoter; these bands probably corresponded to the full and the mature forms of the protein. The full protein was not detected in the extract corresponding to the plasmid-expressed protein, probably due to its lower concentration (Fig. 4B). The Western blot also showed the presence of proteins with a lower molecular weight than the processed OmpA2-mCherry protein in the extract corresponding to the strain expressing this protein from the replicative plasmid. Presumably, these proteins are the result of the proteolysis of OmpA2-mCherry, indicating that the protein expressed from the plasmid is less stable than that expressed from the chromosome, even though both alleles are the same.

A plasmolysis experiment confirmed that OmpA2-mCherry expressed from the plasmid was correctly inserted in the OM (data not shown), but this protein did not show the localization pattern previously observed, even in the absence of the wild-type protein (Fig. 4A), and it was not able to fully label the stalks (Fig. 4A, white arrows). A comparison of the fluorescence profiles of these cells with those expressing OmpA2-mCherry from its locus confirmed the differences of the localization patterns (see Fig. S3 in the supplemental material [compare the first two panels]). However, staining with FM 4-64FX did not reveal a defect in the stability of the OM of this strain (data not shown).

The localization of OmpA2 depends on the position of the gene on the chromosome. Since the fusion allele expressed from the plasmid and from the chromosome is the same, the absence of a protein gradient in the cells expressing OmpA2-mCherry from the plasmid suggested that this localization pattern is not an intrinsic property of the protein.

The chromosome of _C. crescentus_ cells is longitudinally organized, with the replication origin located at the old (stalked) pole and the terminus near the new pole. The _ompA2_ locus is near the origin, and its expected spatial location is close to the stalked pole, which is the pole with a higher concentration of the protein. This observation made us wonder if the position of the gene in the chromosome and, in consequence in the cell could be relevant for...
the localization of the protein. To test this idea, we changed the position in the chromosome of the ompA2-mCherry gene to approximately the midcell (xylR locus) or the opposite cell pole (vanR locus). The gene fusion was present as a single copy or as a second copy of ompA2 and in all cases under the control of the native promoter. The localization pattern of OmpA2-mCherry was then determined under high- and low-phosphate conditions (Fig. 5A).

Expression of the OmpA2 fluorescent fusion from the xylR locus as a single or second copy resulted in a mixed population of cells with an even distribution of fluorescence and cells with an inversed gradient (i.e., with a stronger signal at the new pole). In contrast, when ompA2-mCherry was expressed from the vanR locus, the majority of the cells had an inversed gradient. These observations were corroborated by quantifying the number of cells showing the wild-type gradient pattern, an inversed gradient, or an even distribution of fluorescence (Fig. 5B; see also Fig. S3 in the supplemental material). Regardless of whether ompA2-mCherry was present as a single or second copy, when the protein fusion was expressed from the xylR or vanR locus the amount of OmpA2-mCherry present in the stalk was lower than that observed when it was expressed from its native locus (Fig. 5A). A Western blot assay showed that the amount of protein present in all the strains in which the ompA-mCherry allele was present in a single copy was similar, irrespective of where in the chromosome it was expressed (Fig. 4B).

Since it was reported that the N-terminal domain of OmpA that does not interact with the cell wall does not show long-range diffusion (60), we decided to test if another integral OM protein coded by a gene present at one of the cell poles would also show a similar localization pattern as OmpA2. The gene CC1750 codes for a TonB-dependent transporter, and its spatial position should be similar to the vanR locus. A stable C-terminal fusion of CC1750 with mCherry (data not shown) showed that this protein was evenly distributed in the cell (Fig. 5C; see also Fig. S3), indicating that the presence or an OM β-barrel domain is not sufficient to generate the localization pattern of OmpA2. Alternatively, the localization pattern of OmpA2 could be the result of a constant incorporation of a large amount of OmpA2 from the position of the gene locus followed by a low diffusion rate of the protein. To test this idea, cells in which translation was inhibited with chloramphenicol (2 g/ml for 5 min) were spotted on a chloramphenicol-containing agarose pad and consecutively imaged. No differences were observed after 2 h of initial observation (data not shown), suggesting that OmpA2 maintains a stable localization which could contribute to the formation of the gradient.

Similarly to the ompA2 mutant, we noticed that the cells expressing ompA2-mCherry from the xylR or vanR locus showed shorter stalks than the strain that expressed the same allele from its native locus. For this reason, we decided to measure the average stalk length and the percentage of stalked cells in exponentially growing cultures in low-phosphate medium. Our results showed that when OmpA2 was expressed from the xylR or vanR locus as
single copies, both the average stalk length and the percentage of stalked cells decreased (Fig. 5D). However, when *ompA2-mCherry* was present as a second copy and the wild-type protein was expressed from its native locus, there was no effect on stalk growth. Although the correct localization of OmpA2 is required for its function in stalk growth, the presence of the protein is enough to stabilize the OM when OmpA2-mCherry is expressed from a plasmid. To test if the same is true when OmpA2 is expressed from...
different loci, the LDG1, -3, and -4 strains were stained with FM 4-64FX, and no differences in OM stability between the strains expressing OmpA2-mCherry from different positions in the chromosome were detected (data not shown), indicating that accumulation of OmpA2 in the stalked pole is not necessary for its role in OM stability. However, a competition assay between the strains expressing ompA2-mCherry from the vanR locus and from its native locus showed that the correct localization of this protein is important for growth in low-phosphate medium (see Fig. S2 in the supplemental material).

The OmpA2 gradient starts forming at the beginning of the cell cycle. Our previous results indicated that the position in the cell from which OmpA2 is expressed determines the localization of the protein and that this localization is required for its function in stalk growth. However, for at least half the cell cycle a C. crescentus cell has two full copies of its chromosome and two copies of the ompA2 gene, each located at different cell poles. To determine at what point of the cell cycle the OmpA2 gradient starts forming, we carried out a time-lapse experiment of synchronized cells expressing OmpA2-mCherry in which chromosome replication and segregation were followed as a marker of the end of the swarmer cell cycle stage. To be able to determine in which cells chromosome segregation had already occurred, a fusion of the MipZ protein with YFP was also expressed in these cells. The MipZ protein is involved in correctly positioning the FtsZ ring, and through its interaction with ParB it labels the parS locus located near the chromosome replication origin (61). Determining the localization of MipZ also allows the identification of the old and new cell poles in the early stages of the cell cycle. The time-lapse images showed that as the cells progressed through the cell cycle, the concentration gradient became more pronounced (Fig. 6A).

Our results suggested that the OmpA2-mCherry gradient becomes more defined as the cell progresses through the cell cycle and that this is a result of the time that the ompA2 locus has been at that polar position. To corroborate this idea, the total number of cells showing a polar gradient in a synchronized cultured was quantified at different time points. As a control we carried out the same analysis with cells expressing ompA2-mCherry from the vanR locus, since in these cells the appearance of the gradient should be delayed, as the arrival of the newly replicated ompA2 locus at its polar position occurs later (see Fig. S4 in the supplemental material). As can be observed in Fig. 6B, the number of cells that showed a polar gradient started to increase from the beginning of synchrony in the culture of cells expressing ompA2-mCherry from its native locus; in contrast, no significant increase occurred in the culture of the cells expressing ompA2-mCherry from the vanR locus.

Visual inspection of cells from the first time points of the time-lapse experiment suggested that the gradient starts forming early in the cell cycle, since cells showing a concentration gradient could be detected just 10 min after synchronization, before segregation of the chromosome had occurred (Fig. 6A and C, red arrow and trace, respectively), which is a clear indicator that a cell is still at the swarmer stage of the cell cycle. However, not all the cells showed this localization pattern (Fig. 6A and C, green arrow and trace, respectively). Since new OmpA2-mCherry is constantly being added and fluorescent proteins mature slowly, it was possible that there was a delay between the appearance of the gradient and its detection. To compensate for the maturation time of the fluorescent protein, swarmer cells were treated immediately after synchronization with nalidixic acid and chloramphenicol to stop DNA replication, transcription, and protein synthesis and were visualized after 45 min. To rule out a possible artifact in the visualization of the still-weak gradient at this early stage of the cell cycle, the number of cells with a single MipZ focus showing an accumulation of the fluorescent signal at the new pole (data not shown) or old pole (Fig. 6C, red trace) or with an even distribution (Fig. 6C, green trace) was determined. This analysis showed that approximately 60% of the cells with a single MipZ focus had an old pole gradient, while only 20% and 10% had an inverse pattern or an even distribution, respectively (Fig. 6D), indicating that after approximately 80 min from the time that the ompA2-mCherry gene arrives at the cell pole (see Fig. S4 in the supplemental material), more than half of the cells have an OmpA2 gradient. When the same experiment was done with the strain expressing OmpA2-mCherry from the vanR locus, a similar number of swarmer cells with an old and new pole gradient were found. Since the stalked cells of the same strain have in their majority a new pole gradient (Fig. 5B), the high proportion of swarmer cells with an old pole gradient indicated that in the cells expressing ompA2-mCherry from the vanR locus the establishment of the gradient was delayed, in agreement with the late arrival of the vanR locus at the future new pole position (see Fig. S4).

DISCUSSION

The mechanisms involved in OM stabilization have been mainly studied in enterobacteria, where their relevance and interactions between each other have been investigated. However, in other bacterial groups the relative relevance and the interaction between these mechanisms may have been adapted to different cell growth patterns or environmental conditions. This seems to be the case for C. crescentus, where the relevant Braun lipoprotein is missing. In this bacterium the Pal lipoprotein is essential, but other proteins with a domain composition that suggest a function in OM stabilization are also present. This is the case with OmpA2, which has a domain composition similar to that of OmpA of E. coli. Inactivation of the ompA2 gene resulted in a strain that did not show any clear growth defects under optimal growth conditions but had a slower growth rate under different physiological stresses and was at a disadvantage when competing with the wild-type strain. A direct link between OmpA2 and the growth rate under the stress conditions tested was not evident, but it is possible that the main OM stabilization mechanism may be under additional strain when the cells grow at a faster rate or when the stalk is synthesized. When the stability of the OM is severely compromised, the cells become more susceptible to compounds that destabilize the OM (detergents or EDTA) or to substances for which it is normally not permeable (antibiotics or bile salts). When the susceptibility of the ompA2 mutant to different substances was tested, we did not detect any major changes, indicating that the OM of this strain remains essentially intact under normal growth conditions. However, the ompA2 mutant was more susceptible to the presence of SDC, and staining with FM 4-64FX showed that the stability of the OM was affected. Further work will be required to determine the relationship between OmpA2 and the essential Pal lipoprotein and with other proteins that might be involved in OM stabilization.

Unexpectedly, when OmpA2 was fused to a fluorescent protein it was observed that this protein formed a concentration gradient from the old to the new pole. A similar localization pattern has
been described previously for the OM protein IcsA and other autotransporters (62, 63). In comparison with the localization pattern of OmpA2, the polar gradient formed by IcsA is more tightly defined. The IcsA localization mechanism depends on targeting of the cytoplasmic IcsA protein to the pole, the presence of a complete LPS, and proteolysis by an inversely localized protease (64, 65). When *ompA2-mCherry* was expressed from a plasmid, the concentration gradient was not observed, which suggested that...

FIG 6 The OmpA2 gradient forms before chromosome segregation. The localization of OmpA2-mCherry was followed during a cell cycle of a synchronized culture. (A) Independent time-lapse images of cells, grown in high-phosphate medium, that expressed MipZ-YFP and OmpA2-mCherry from its native or *vanR* locus. Green arrow, a cell with an even fluorescence distribution; red arrow, a cell with a fluorescence gradient. (B) Percentages of cells in a synchronized culture, demonstrating the fluorescence gradient. The black line indicates the percentages of cells with two MipZ-YFP foci. (C) The OmpA2-mCherry fluorescence intensity profile along the cell. The red and green profiles correspond to the cells marked by the red and green arrows in panel A. (D) Percentages of antibiotic-treated swarmer cells that showed a normal, inverse gradient or an even fluorescence distribution. The bars in all cases represent the averages obtained from three independent experiments in each of which at least 150 cells were analyzed. Error bars represent the standard deviations from three independent experiments.
the localization of OmpA2 is not dependent on its interaction with another protein or on the recognition of a chemical signal present in the cell wall. Instead, changing the position of the ompA2-mCherry gene in the chromosome and, in consequence, its spatial localization within the cell affected the localization of the protein in a similar way, indicating that the localization of OmpA2 depends on the position of the gene locus. This result indicates that there is very limited diffusion of OmpA2 from the moment when it is translated until the time that the processed protein can stabilize its position. This would presumably occur when OmpA2 is inserted in the OM, or when it binds to the cell wall. It has been shown that in C. crescentus the mRNA colocalizes with the gene locus from which it was transcribed (55, 66). If the diffusion of the resulting protein were hindered, this could result in a gradual accumulation of the protein. This idea corresponds with the intensification of the gradient as the cell progresses through the cell cycle and as it ages. In addition, the gradient may form faster if the transcription of the gene increases before chromosome segregation and as it ages. In addition, the gradient may form faster if the transcription of the gene increases before chromosome segregation, as may be the case for ompA2 (see Fig. S1B in the supplemental material). It has been shown that translocation of exported proteins across the IM to the periplasm occurs after translation, although this may not be the case for all exported proteins (67, 68).

It is possible that the diffusion of the OmpA2 precursor could be reduced if its translocation occurred while it is transcribed. Once OmpA2 is inserted in the OM, its diffusion may be slowed by the reduced fluidity of the OM (60) or by its efficient interaction with the cell wall. Several of these mechanisms must be responsible for the localization of OmpA2, since another OM protein encoded by a gene that should be located at the cell pole did not show a localization pattern similar to that of OmpA2.

In addition to a reduced OM stability, the ompA2 mutant showed a decrease in the number of stalked cells and in the length of the stalks. A similar phenotype was reported for a mutant in an accessory protein of the essential BAM complex, in which the integrity of the OM was more severely affected than in the ompA2 mutant (58). The conserved BAM complex is required for the assembly of OM integral β-barrel proteins, and its absence has pleiotropic effects (69–71). The stalk growth defect in the ompA2 mutant more clearly suggests a link between the OM and the growth of this structure. Lipoproteins located in the OM were recently shown to be important for cell wall growth in E. coli (72, 73). It is possible that in C. crescentus similar lipoproteins are involved in cell wall growth at the base of the stalk. The OmpA2 protein may help to maintain the appropriate contact between the OM lipoproteins and the proteins required for synthesis of the cell wall at the base of the stalk.

Interestingly, the strains with a mislocalized OmpA2 protein also showed a defect in stalk growth. These cells had normal levels of OmpA2, and although the protein was present in the stalk, it was present at a lower concentration than in the wild-type strain. These results indicated that OmpA2 needs to be in the stalk at a minimal concentration to stimulate stalk growth. Coexpression of OmpA2 from its native locus and OmpA2-mCherry from a different position in the chromosome restored normal stalk growth, showing that the presence of the protein in a different position of the cell does not compete with the function of the protein in the wild-type location. This suggests that OmpA2 does not recruit the proteins required for stalk growth but that it stimulates them in a direct or indirect fashion.

The facts that the stalk is not completely labeled in the cells expressing OmpA2-mCherry from the multicopy plasmid together with the lower concentration of OmpA2-mCherry in these cells suggest that in part the presence of this protein in the stalk depends on its concentration. The concentration of OmpA2-mCherry in the stalks of the cells expressing the protein from other chromosomal loci is similar to that present in the stalk poles, suggesting that the protein is carried away from the base of the stalk as it grows from its base (74) and that there is no active mechanism for accumulation of OmpA2 in the stalk.

By reducing the diffusion of OmpA2, C. crescentus cells are able to produce a localization pattern that is relevant for the function of the protein. A side effect of this simple mechanism is that it could also constrain the plasticity of the genome organization, since cells in which the ompA2 gene was moved to a different position in the chromosome were at a disadvantage in competing with wild-type cells (see Fig. S2 in the supplemental material). If other proteins use a similar mechanism, they could have a stabilizing effect on the overall gene order in the chromosome. It will be interesting to determine if other proteins rely on a similar mechanism as OmpA2 does for their localization.

Proteins similar to OmpA2 can be found in other bacteria, including other stalked bacteria, where they could be important for stalk growth and in consequence may have the same localization mechanism as in C. crescentus. An analysis of the position of the ompA2 gene in these bacteria showed that the position of ompA2 is in general conserved among the Caulobacteraceae, as it is within the Hyphomonadaceae, but at a different position (see Fig. S5 in the supplemental material), indicating that the role of OmpA2 may be different in these bacteria. Interestingly, in some cases the position of the ompA2 gene suggests a similar role as in C. crescentus. Further work will be required to confirm a conserved function and localization mechanism for OmpA2 in other bacteria.

ACKNOWLEDGMENTS

This work was supported by grants from the Consejo Nacional de Ciencia y Tecnologia (SEP-CONACYT 178685) and DGAPA/UNAM (IN2102511).

We thank Sebastian Arriaga for the CC1750-mCherry fusion strain and the Molecular Biology Unit of the Instituto de Fisiología Celular UNAM for DNA sequencing.

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