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Morphological and functional asymmetry in α-proteobacteria☆

Régis Hallez, Anne-Flore Bellefontaine, Jean-Jacques Letesson and Xavier De Bolle

Unité de Recherche en Biologie Moléculaire (URBM), University of Namur, 61 rue de Bruxelles, B-5000 Namur, Belgium

The release of an increasing number of complete bacterial genomic sequences allows the evolutionary analysis of processes such as regulatory networks. CtrA is a response regulator of the OmpR subfamily, belonging to a complex regulatory network in the dimorphic bacterium *Caulobacter crescentus*. It coordinates the cell cycle with an asymmetric division, which is part of the adaptation of *Caulobacter* to poor-nutrient environments. CtrA is only found in α-proteobacteria, a group of bacteria encompassing genera with very distinct lifestyles, including host-associated bacteria. Analyses of CtrA regulatory networks and morphological examinations of some α-proteobacteria are presented. Our observations suggest that the core of the CtrA regulation network is conserved and that α-proteobacteria divide asymmetrically. We propose that the two daughter cells might be differentiated bacteria, each one displaying specific functions.

It is generally accepted that in eukaryotic cells, the cell shape allows specific functions to be performed. For example, a neuron displays a characteristic shape that is very different from an intestinal epithelial cell, and both cell types use these morphological features to perform different functions. In bacteria, there are also different cell shapes that have related functions but molecular mechanisms that are used to achieve such morphological specialization are poorly documented. The bacterial differentiation is a clear example of such a phenomenon, allowing *Myxococcus xanthus* [1] or *Bacillus subtilis* [2] to perform a complex development. *Caulobacter crescentus* is one bacterial model system that has been used to address this question because this bacterium divides asymmetrically to produce two morphologically and functionally different progeny cells. In this bacterium, the response regulator CtrA, which belongs to a complex regulatory network, acts as a molecular switch that allows the coordination of cell cycle progression and morphogenesis by controlling the expression level of ~100 genes [3–6]. Moreover, the dynamic positioning of signal transduction proteins allows temporal and spatial control of the activity (by phosphorylation) and presence (by proteolysis) of CtrA [7–11] and consequently determines the fate of each cell type. This network allows a mother *Caulobacter* cell to produce two genetically identical but morphologically and functionally different progeny cells, one being a smaller flagellated cell that is unable to replicate and the other a stalked cell that is competent for replication. Dimorphism results in an organism that is perfectly adapted to nutrient-poor environments because the flagellated form searches for nutrients and, when it finds them, differentiates into the stalked form that is responsible for the colonization of the niche by active replication. Thus, the CtrA regulatory network participates in the expression of an asymmetric genetic program leading to the dimorphism of *C. crescentus* and its adaptation to constant famine.

In addition to the free-living bacterium *C. crescentus*, the CtrA response regulator has been identified in other α-proteobacteria [12–14], for example, the plant symbionts *Mesorhizobium loti* and *Sinorhizobium meliloti*, the plant pathogen *Agrobacterium tumefaciens*, and the facultative and obligate mammalian pathogens *Brucella* spp. and *Rickettsia prowazekii*, respectively. The biological role of CtrA is therefore intriguing because most α-proteobacteria are not known to display a complex dimorphism, as occurs in *C. crescentus*, and, moreover, they present lifestyles radically different from each other. Here, we propose some common features between six α-proteobacterial species (representative of six α-proteobacterial genera) and discuss a potential conserved biological role of the CtrA network in the expression of an asymmetric genetic program. To characterize the CtrA regulatory network, we examined the CtrA regulon (i.e. genes directly regulated by CtrA) and the signal transduction system that controls CtrA, using genomic analysis. Electron microscopy observation of four α-proteobacterial species revealed asymmetric divisions very similar to those reported for *C. crescentus* and *S. meliloti* [11].

Partially conserved CtrA-dependent regulon

The DNA-binding domain of CtrA recognizes and binds to a well described consensus sequence in *C. crescentus*. This consensus sequence (9-mer TTAA-N_7-TTAAC) was found in many cell cycle-regulated promoters before the discovery of the *ctrA* gene itself [3,15]. DNA microarray and chromatin immunoprecipitation experiments allowed the description of the CtrA regulon in *C. crescentus* as well as the identification of a second CtrA-binding site consensus, the 8-mer TTAACCAT [4,16].

Here, we predict the CtrA regulon for α-proteobacteria, by searching the available genomic data for all promoter regions containing CtrA-binding sites, 9-mer and/or 8-mer.

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Corresponding author: Xavier De Bolle (xavier.debolle@fundp.ac.be).

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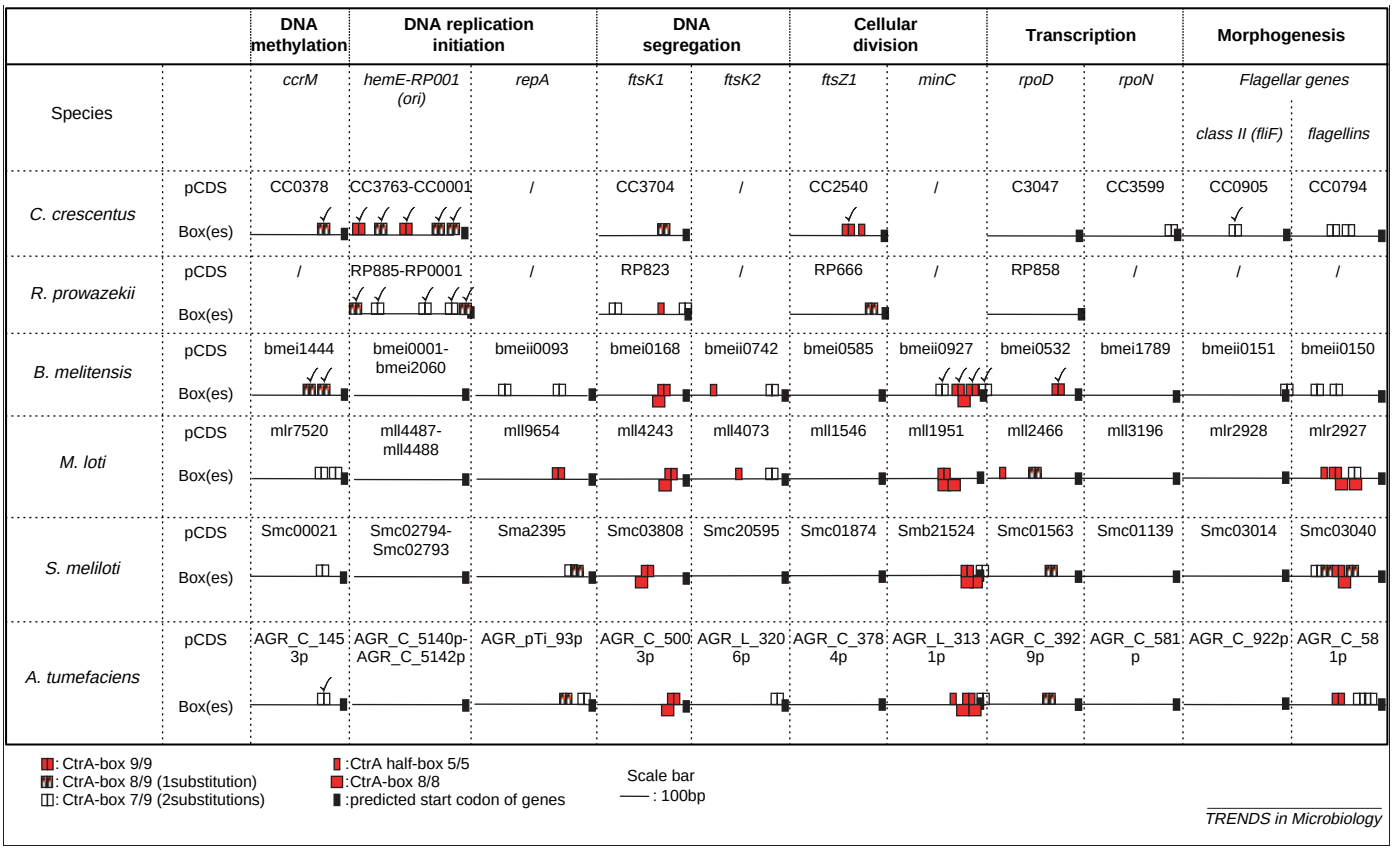

Figure 1. CtrA-binding sites (CtrA boxes) found in promoters (upstream regions of coding sequences, because +1 sites are not predicted) potentially regulated by CtrA in at least one of the six species of α-proteobacteria. The cellular process-related genes are: *ccrM* for DNA methylation, *hemE-RP001* and *repA* for DNA replication initiation, *ftsK1* and *ftsK2* for DNA segregation, *ftsZ1* and *minC* for cellular division, *rpoD* and *rpoN* for transcription and class II flagellar genes and flagellins for morphogenesis. The six species of α-proteobacteria include *Caulobacter crescentus*, *Rickettsia prowazekii*, *Brucella melitensis*, *Mesorhizobium loti*, *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*. We have scanned genomes with these consensus sequences using 'genome-scale DNA-pattern' available from the Regulatory Sequence Analysis Tools website (http://rsat.ulb.ac.be/rsat/) [33]. The three consensus sequences of the CtrA boxes are TTAA-N_7-TTAAC (9/9), TTAAC (5/5) and TTAACCAT (8/8), respectively. The symbol '/' means that no strong homolog was found in the corresponding species. The tick (?) refers to experimental data published in the literature showing that these CtrA boxes are effectively bound by CtrA *in vitro*. The reference number of each predicted coding sequence (pCDS) in each species as available in the corresponding GenBank files are indicated in the 'pCDS' lines. GenBank accession numbers are: NC_002696 for *C. crescentus* [34]; NC_000963 for *R. prowazekii* [35]; NC_003317 and NC_003318 for *B. melitensis* [36]; NC_002678, NC_002679 and NC_002682 for *M. loti* [37]; NC_003047, NC_003037 and NC_003078 for *S. meliloti* [38]; NC_003062, NC_003063, NC_003064 and NC_003065 for *A. tumefaciens* [39].

Experimental data on some CtrA target genes demonstrated that these two consensus sequences are bound by CtrA in at least four α-proteobacterial species [12–14,16]. Figure 1 shows schematically the CtrA boxes found in the promoter of genes involved in several cellular and developmental processes, namely DNA methylation, DNA replication initiation, DNA segregation, cellular division, transcription and morphogenesis. The fact that several predictions of CtrA target genes were confirmed by *in vitro* footprinting (marked by a tick in Figure 1) strengthens the method of prediction [13,14,17–22].

Our observations suggest, on the one hand, that similar processes are regulated by CtrA through the control of either orthologous target genes (e.g. *ccrM* for DNA methylation) or distinct target genes. For example, CtrA controls the initiation of cell division by regulating transcription of the *ftsZ* gene encoding the tubulin-like cell division protein in *C. crescentus* [17,23] (and probably in *R. prowazekii*) and presumably by controlling the expression level of the *minC* gene encoding an inhibitor of Z-ring formation in the four other species. By contrast, some potential CtrA target genes appear to be more specific, as illustrated by the *tolQ* gene, the expression of which might be under CtrA control only in the four species associated with eukaryotic cells (See Table 1 in supplementary material online). In *Escherichia coli*, TolQ, TolR and TolA proteins form a complex which is probably involved in an energy dependent process necessary for the translocation of some outer membrane components [24]. Data presented in Figure 1 and supplementary Table 1 strongly suggest that, during evolution: (i) the control of several cellular and developmental processes by CtrA has been conserved through either the same or different target genes and (ii) some molecular mechanisms have been placed under CtrA control only in some species. Thus, the CtrA regulon appears to be conserved, at least partially, between the six studied α-proteobacterial species.

Conserved CtrA control system

In *C. crescentus*, at least two mechanisms are involved in the temporal and spatial control of CtrA (Figure 2). The first mechanism controls phosphorylation of CtrA through a phosphorelay involving the CckA hybrid histidine kinase (HK) [7] and a probable unidentified histidine phosphotransferase. The second mechanism controls proteolysis, and probably also the phosphorylation state of CtrA, which

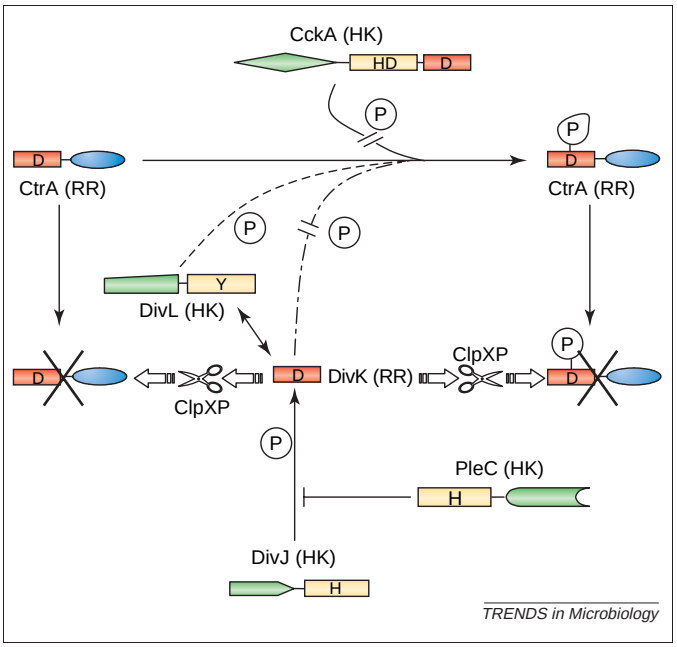

Figure 2. Schematic representation of the CtrA control system proposed in *Caulobacter crescentus*. The sensor and transmitter domains of histidine kinases (HK) are indicated in green and yellow, respectively. The receiver domain of response regulators (RR) is in red. H, Y and D represent phosphorylated residues, histidine and tyrosine for HK and aspartate for RR, respectively. The DNA-binding domain of CtrA is illustrated in blue. Arrows denote the flow of phosphoryl groups, but do not indicate that the HK and CtrA directly interact. Given that CckA and DivK possess a receiver domain with a conserved transphosphorylated aspartate (D), it is probable that the phosphotransfer between one of these factors and CtrA is indirect, probably involving an unidentified histidine phosphotransferase. Dashed lines illustrate a phosphate transfer between DivL and CtrA, only demonstrated *in vitro* [27]. The double arrow connecting DivL and DivK illustrates a physical interaction shown by a yeast two-hybrid screen [28]. DivK is able to stimulate the proteolytic degradation of CtrA-P and CtrA [10,25] and probably also to control the phosphorylation state of CtrA, as illustrated by the dashed line between DivK and CtrA-P [6]. Antagonistic activities of DivJ and PleC on the DivK phosphorylation state are illustrated. The pair of scissors symbolizes the proteolytic machinery ClpX/ClpP, responsible for the degradation of CtrA.

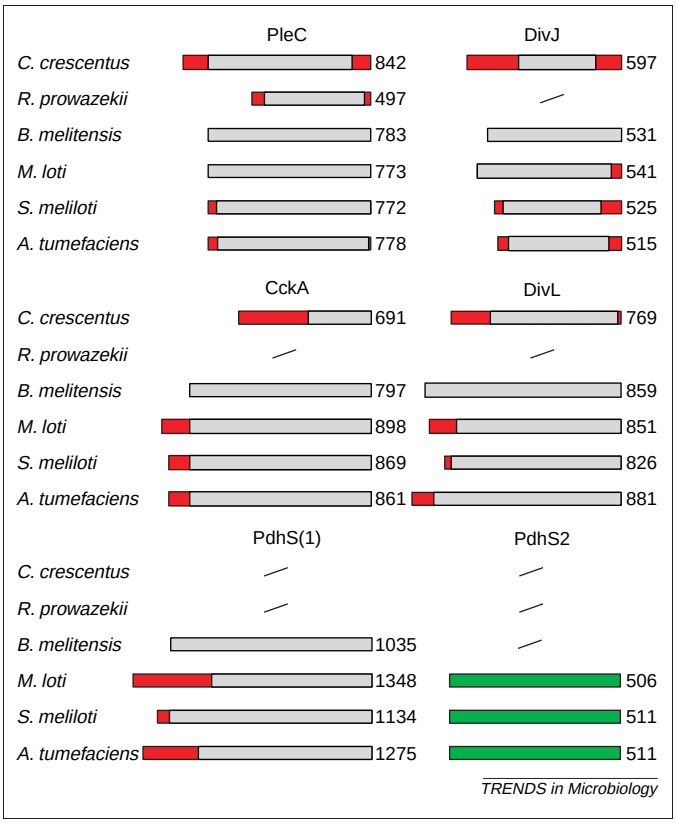

Figure 3. Schematic representation of orthologs of the sensors involved in the control of CtrA found in six α-proteobacterial genomes: *Caulobacter crescentus, Rickettsia prowazekii, Brucella melitensis, Mesorhizobium loti, Sinorhizobium meliloti* and *Agrobacterium tumefaciens*. The portion of the proteins with similarity to the *B. melitensis* homolog (considered here as a reference) is indicated in grey, illustrating that it is often the C-terminal portion of the protein that is conserved. The portion of PdhS2 orthologs with high similarity is represented in green. The length (in amino acids) of each protein in each species is indicated on the right. The '/' symbol indicates that no strong homolog was found in the corresponding species. The reference number of each gene encoding these proteins is indicated in Table 2 of the supplementary material online. CtrA and DivK are not shown because they are very well conserved over their whole length, except for the C-terminal peptide of CtrA [40].

is mediated by the monodomain response regulator (RR) DivK [10,25] whose phosphorylation level and localization are controlled by at least two homologous HKs, DivJ and PleC [8,9,11,26]. Although DivJ and PleC are able to phosphorylate DivK, it appears that these two histidine kinases have antagonistic activities on the cellular concentration of phosphorylated DivK *in vivo* [8,11]; this suggests that DivJ effectively phosphorylates DivK, and PleC probably dephosphorylates it. Another protein, known as DivL, homologous to classical HKs but with a tyrosine in place of the conserved histidine, is able to phosphorylate CtrA *in vitro* but not DivK [27]. However, DivL was isolated in a yeast two-hybrid screen using DivK as the bait, strongly suggesting that these two proteins can physically interact [28]. One of the most surprising features of these regulating factors is their subcellular localization, which changes during the cell cycle [7–9,11,29]; the subcellular localization of DivL, however, is still unknown.

We have identified orthologs of these proteins in five other α-proteobacterial species. Orthologs of each of the five signaling proteins (DivK, DivJ, PleC, DivL and CckA) were found in all genomes analyzed (Figure 3), except for *R. prowazekii*, which only possesses homologs of the *pleC* and *ctrA* genes (respectively called *barA* and *czcR*, [14]). Interestingly, as illustrated in Figure 2, PleC is an upstream factor in a cascade controlling CtrA activity. As is the case for most obligate pathogens, *R. prowazekii* has a much reduced genome size (1.1 Mb compared with more than 3 Mb for the other species). Surprisingly, it appears that *R. prowazekii* did lose the intermediate factors (DivJ and DivK) during evolution, maintaining only the upstream and the downstream components of this pathway. Therefore, in this organism, CtrA activity is either controlled by an unidentified signaling pathway, or it is directly controlled by PleC, in contrast to what is reported for *C. crescentus*, and probably occurring in the four other species. Another interesting observation is that the interacting domains (i.e. transmitter domains of HK [28]) of the partners are much more conserved than their putative sensor domains (Figure 3), suggesting that a similar integration process might be controlled by different signals from one bacterium to the other.

We have also identified new putative members of the CtrA control pathway in the genomes of *Brucella melitensis*, *M. loti*, *S. meliloti* and *A. tumefaciens*. These genes are called *pdhS* for '*pleC/divJ* homolog sensor' because they present a high similarity with both so-called

genes involved in CtrA control in *C. crescentus*. The fact that these new two-component signal transduction proteins were identified in facultative host-associated bacteria might reflect their ability to sense and adequately respond to diversified signals, in particular those encountered during their host-associated stages.

To summarize, on the one hand, the main differences between orthologs of proteins controlling CtrA activity in each of the six analyzed α-proteobacterial species are found in the N-terminal extremity of HK, where sensor domains are usually located. By contrast, the four host-associated species present new potential CtrA-controlling factors. Taking both of these observations together, and knowing that α-proteobacterial species display very different lifestyles, it is conceivable that this bacterial group might have kept a common regulatory network to adapt to very different ecological niches during evolution.

Asymmetric division of α-proteobacteria

Few bacterial species are known to divide asymmetrically. Nevertheless, a dividing *Caulobacter* mother cell produces two daughter cells that differ in size and morphology: a larger stalked cell and a smaller flagellated cell. Recently, Lam *et al.* [11] have reported that *S. meliloti* is also able to divide asymmetrically and therefore produces two progeny cells of different size [11]. As illustrated in Figure 4, scanning electron micrographs taken on the α-proteobacterial species *C. crescentus*, *Brucella abortus*, *S. meliloti* and *A. tumefaciens* strongly suggest that asymmetric division is a common feature in the α-proteobacterial group. Indeed, bacteria at the end of septation are about to produce two daughter cells of different size. This particular phenomenon is probably the result of a common molecular mechanism ensuring the correct location of the division machinery. Interestingly, *C. crescentus* does not have orthologs of *minC*, *minD* and *minE* genes, in contrast to *Brucella*, *S. meliloti* and *A. tumefaciens*. In *E. coli*, these genes are involved in the correct equatorial localization of the septum, by inhibiting the formation of septal rings at polar sites [23,30]. Thus, the presence of Min proteins in α-proteobacteria displaying an asymmetric division suggests that this system might be involved in the inhibition of septation at cell poles, but would not be required for the correct positioning of the septum next to the centre of the mother cell.

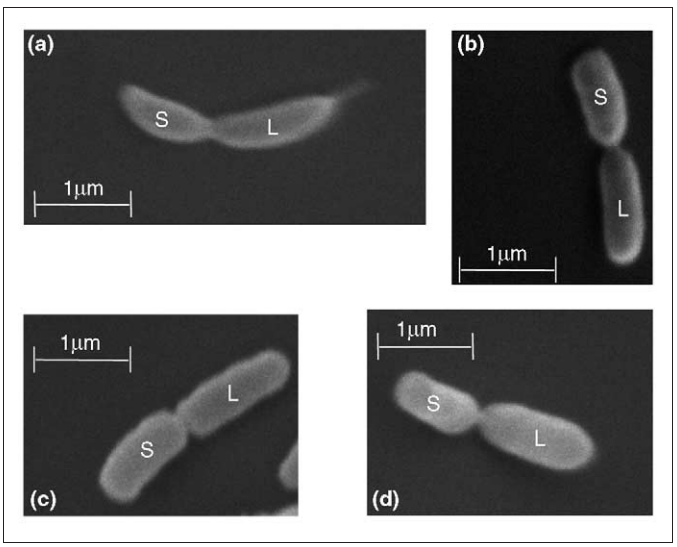

Figure 4. Scanning electron micrographs of α-proteobacterial cells just before septation. The species observed are **(a)** *Caulobacter crescentus*, **(b)** *Brucella abortus*, **(c)** *Sinorhizobium meliloti* and **(d)** *Agrobacterium tumefaciens*. The large and small cells are called L and S, respectively.

To summarize, we have underlined some common features between species of the highly diversified α-proteobacterial group. The six species mentioned above: (i) possess a homolog of the essential *ctrA* gene, experimentally shown as functional in at least four of these six species; (ii) present a conserved CtrA regulon, with some specificities that might be related to the lifestyle of these bacteria; (iii) possess orthologs of genes involved, in the case of *C. crescentus*, in the temporal and spatial control of CtrA and (iv) display a clear asymmetric division, at least for the four observed species. However, it is possible that CtrA regulons in some α-proteobacteria might have strongly diverged from those reported here, as is the case for *Rhodobacter capsulatus*. Indeed, in this species, the *ctrA* and *cckA* genes are not essential for viability [31,32] and do not appear to control essential cell cycle processes, as suggested by genomic analysis (data not shown).

There is no polar organelle, such as a stalk or a flagellum, described for *Sinorhizobium* or *Brucella*. However, recent experiments performed on *S. meliloti* demonstrated that the response regulator DivK presents an asymmetric subcellular localization similar to the one described in *C. crescentus* [11]. Thus, the polar localization of the CtrA-controlling factors could be another feature commonly used by several α-proteobacterial species, which might allow the differential regulation of genes in small versus large cells. We propose that the cellular differentiation morphologically observed in *C. crescentus* could be present at the molecular level in the other α-proteobacteria.

In conclusion, we propose that specific functions of the two cell types should be examined in several α-proteobacteria. These specific functions could, for example, be their capacity to accomplish one of the steps involved in their symbiotic or parasitic relationship with the eukaryotic host. Moreover, it is also possible that other bacteria develop forms of primitive differentiation that remain to be discovered.

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Microbiological conferences scheduled for October 2004

ASM Conference on Functional Genomics & Bioinformatics Approaches to ID Research
6–9 October, Portland, Oregon, USA

Frontiers of Cellular Microbiology and Cell Biology: Spatial and Temporal Dynamics of the Endomembrane System
16–21 October, San Feliu de Guixols, Spain

11th International Symposium on Staphylococci & Staphylococcal Infections
24–27 October, Charleston, South Carolina, USA

44th Interscience Conference on Antimicrobial Agents & Chemotherapy
30 October–2 November, Washington, USA