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Clostridial DivIVA and MinD interact in the absence of MinJ

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ABSTRACT

One of the key regulators ensuring proper Z-ring placement in rod-shaped bacteria is the Min system. It does so by creating a concentration gradient of the MinC septation inhibitor along the cell axis. In *Escherichia coli*, this gradient is established by a MinE-mediated pole-to-pole oscillation of the MinCDE complex. In *Bacillus subtilis*, the creation of an inhibitory gradient relies on the MinJ and DivIVA pair of topological determinants, which target MinCD to the newly formed cell poles. Introducing the *E. coli* oscillating Min system into *B. subtilis* leads to a sporulation defect, suggesting that oscillation is incompatible with sporulation. However, *Clostridia*, close endospore-forming relatives of *Bacilli*, do encode oscillating Min homologues in various combinations together with homologues from the less dynamic *B. subtilis* Min system. Here we address the questions of how these two systems could exist side-by-side and how they influence one another by studying the *Clostridium beijerinckii* and *Clostridium difficile* Min systems. The toolbox of genetic manipulations and fluorescent protein fusions in *Clostridia* is limited, therefore *B. subtilis* and *E. coli* were chosen as heterologous systems for studying these proteins.

In *B. subtilis*, MinD and DivIVA interact through MinJ; here, however, we discovered that the MinD and DivIVA proteins of both *C. difficile*, and *C. beijerinckii*, interact directly, which is surprising in the latter case, since that organism also encodes a MinJ homologue. We confirm this interaction using both *in vitro* and *in vivo* methods. We also show that *C. beijerinckii* MinJ is probably not in direct contact with $DivIVA_{Cb}$ and, unlike *B. subtilis* MinJ, does not mediate the $MinD_{Cb}$ and $DivIVA_{Cb}$ interaction. Our results suggest that the *Clostridia* Min system uses a new mechanism of function.

1. Introduction

Bacterial cell division is a strictly regulated process requiring complex molecular machinery called the divisome [1]. The key component of the divisome is FtsZ, a highly conserved structural homologue of eukaryotic tubulin [2]. Currently, the first known event in bacterial cytokinesis is polymerization of FtsZ into a ring-like structure, the Z-ring, close to the cytoplasmic membrane at the future site of the division septum [3]. The Z-ring structure serves as a scaffold for the downstream components of the divisome, guiding the localisation and shape of the future septum [4]. Rod-shaped bacteria usually divide symmetrically, with the septum bisecting the long axis precisely in the middle of the cell. Proper mid-cell positioning is under strict spatial and temporal regulation by a number of FtsZ-interacting proteins and through at least two different molecular mechanisms, which serve as negative regulators of Z-ring assembly: the nucleoid occlusion (NO) system and the Min system (reviewed in Refs. [5–7]). The NO and Min systems were previously thought to be the major factors responsible for division site selection, but it was later shown that the main role of these two systems is preventing Z-ring formation at potential division sites rather than precisely positioning the division site [8]. NO prevents division septum formation over the nucleoid, while the Min system prevents additional septation close to the newly formed division septum and remains at the cell poles during the second division round; together these systems restrict septum formation to the central position.

In rod-shaped bacteria, such as the well-characterized model organisms *Escherichia coli* and *Bacillus subtilis*, the Min system regulates Z-ring assembly by creating a bipolar concentration gradient, with the lowest concentration of the inhibitor at mid-cell and the highest at the cell poles. This is achieved through the spatial regulation of MinC, the actual division inhibitory protein.

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MinC directly interacts with FtsZ, preventing the lateral association of FtsZ protofilaments [9,10]. MinC's inhibitory activity is triggered by its recruitment to the cytoplasmic membrane by an interaction with the ATPase MinD [11,12]. MinD, in turn, preferentially associates with the anionic phospholipids in the cell membrane via its amphipathic helix [13,14]. The MinCD inhibitory complex appears in many bacterial species, including the Gram-negative *E. coli* and the Gram-positive *B. subtilis,* serving the same regulatory function, but the way the concentration gradient is established differs between these two organisms, and relies on Min system components called topological determinants.

In *E. coli*, the concentration gradient is created by the topological regulation of the MinCD complex by the MinE protein. MinE interacts with MinD, stimulating its ATPase activity, leading to MinC release, MinD dissociation from the membrane, subsequent nucleotide exchange on MinD in the cytoplasm, and finally its re-association at the opposite cell pole [13,15]. MinE follows MinD towards the pole, resulting in an oscillation of MinCD and MinE from pole-to-pole [16]. This behaviour creates a bipolar gradient of the MinC inhibitor with the lowest concentration in the middle of the cell, leaving it available for Z-ring assembly [12,17].

In *B. subtilis*, the MinCD concentration gradient is formed in a remarkably different manner. No MinE homologue is present and the Min proteins are spatially regulated by the MinJ and DivIVA proteins. In *B. subtilis,* the key to MinCD polar localisation is the intrinsic ability of DivIVA to sense the negative membrane curvature [18] and an interaction between MinD and DivIVA, which is mediated by MinJ [19,20]. The strongest negative curvature occurs at the developing division septa, where DivIVA assembles into adjacent rings and remains as these sites are transformed into the new cell poles [21]. DivIVA recruits the intermediary protein MinJ, which links the MinCD complex to the DivIVA protein. Completion of division results in the creation of a MinCD bipolar gradient, protecting the daughter cell poles from division [21].

Aside from their role in proper Z-ring placement, the Min proteins are also involved in other processes. In response to starvation, *B. subtilis* can initiate a complex process of asymmetric division, which results in endospore formation. In the first stage of sporulation, two copies of the chromosome condense and form an elongated structure called the axial filament. It has been shown that DivIVA helps to tether the origin regions of this extended chromosome at the cell poles and plays a role in chromosome segregation during this initial stage [22]. In particular, DivIVA interacts with the sporulation-specific protein RacA, which preferentially binds the chromosome near the *oriC* region [23,24]. It has also been recently shown that a pair of Min system components, MinD and MinJ, together with the ComN competence regulator, also migrate the regions close to *oriC* toward the cell pole. While this polar-positioning depends on an interaction with DivIVA, it seems be RacA-independent, and to operate during both prespore development and vegetative division [25]. An increasing number of studies are extending our knowledge of how the Min system functions in both its *E. coli* oscillating form and its less dynamic *B. subtilis* one. An immediate question is why the two systems developed which carry out the same end but with different mechanisms. A hint came from our study in which the *E. coli* Min proteins were introduced into *B. subtilis* cells [26]. *E. coli* Min proteins have the intrinsic ability to oscillate [27] and they did so in *B. subtilis* cells [26]. Intriguingly, the oscillatory behaviour of $MinDE_{Ec}$ led to a sporulation defect in *B. subtilis*, indicating that *B. subtilis* sporulation is less efficient when the oscillating Min system is present [26]. Interestingly, close relatives of *Bacilli*, the endospore-forming *Clostridia,* harbour genes homologous to those of the oscillating Min system. *Clostridia* belong to the phylum *Firmicutes* together with *Bacilli,* and they contain homologues from both model Min systems in various combinations, making them an interesting group for studying Min system functions. *Clostridium difficile* encodes MinCDE homologues, and it was shown that its $MinDE_{Cd}$ exhibits oscillatory behaviour when expressed in *B. subtilis* [28]. Like the *E. coli* $MinDE_{Ec}$ proteins, the oscillating $MinDE_{Cd}$ proteins from *C. difficile* reduce *B. subtilis* sporulation efficiency [26,28]. It is not known whether the oscillating Min system interferes with sporulation in *C. difficile* or how these two processes can exist side-by-side. In addition to oscillating Min system homologues, *C. difficile* also encodes a DivIVA homologue [28]. Since no MinJ homologue in *C. difficile* was detected, the details of its DivIVA function during vegetative growth cannot be inferred from the *B. subtilis* model system.

Given that oscillation is a characteristic behaviour of MinDE proteins, that it seems to be independent of membrane composition [27], and that $MinDE_{Cd}$ from *C. difficile* exhibited oscillatory behaviour in *B. subtilis,* it seems very likely that *C. difficile* employs oscillation during at least the vegetative growth period of its life cycle [28]. However, it is not possible to claim that *Clostridia* universally use an oscillating Min system because some members of this class lack a MinE homologue. This raises the questions of whether the presence of proteins from both Min systems in *Clostridia* results in their co-operation or competition, and of how do they influence one another. For these reasons, we carried out further studies on Min proteins from *C. difficile,* as well as from *Clostridium beijerinckii*, which encodes all Min homologues (MinC, MinD, MinE, MinJ and DivIVA). Like most *Clostridia, C. beijerinckii* and *C. difficile* are strictly anaerobic, spore-forming bacteria. Despite anaerobic lifestyle of *Clostridia*, *B. subtilis* and *E. coli* have successfully proven to be suitable hosts for investigating the mechanism of action of *Clostridia* Min proteins [28]. Surprisingly, we discovered a direct interaction between DivIVA and MinD from both, *C. difficile* and *C. beijerinckii* and found that $DivIVA_{Cb}$ and $MinJ_{Cb}$ from *C. beijerinckii* likely do not directly interact. The results provided in this study extend previous findings on the functioning of clostridial Min proteins and raise new questions regarding division site selection and sporulation in *Clostridia*.

2. Material and methods

2.1. Construction of plasmids and B. subtilis strains

The *B. subtilis* and *E. coli* strains and plasmids used in this study, with a description of their construction and resulting genotypes, are given in Table 1 and Table S1. To construct plasmids harbouring monomeric C-terminal GFP fusions of the *C. beijerinckii* and *C. difficile* DivIVA proteins ($DivIVA_{Cb}$ and $DivIVA_{Cd}$) under the control of a P_{xyl} promoter, which were integrated into the *B. subtilis amyE* locus after transformation into *B. subtilis* cells, the genes encoding these proteins were amplified using PCR. The primer pair No. 1 (Table S2). with chromosomal DNA from *C. beijerinckii* NCIMB 8052 as a template (a kind gift from Prof. R. Bernier-Latmani), and the primer pair No. 2. (Table S2), with chromosomal DNA from *C. difficile* 630 as a template (a kind gift from Prof. N. Fairweather), were used in the PCR reactions. Amplified genes were cloned into the KpnI site of a pSG-GFP plasmid (a kind gift from Dr. H. Strahl von Schulten), resulting in plasmids pSG-IVA_{Cb}-GFP and pSG-IVA_{Cd}-GFP.

A YFP fusion of *C. difficile* $MinD_{Cd}$ was prepared previously [28]; to prepare a YFP fusion of *C. beijerinckii* $MinD_{Cb}$ and $MinJ_{Cb}$, the genes coding $MinD_{Cb}$ and $MinJ_{Cb}$ were amplified using the primer pairs No. 3. and No. 4 (Table S2). with chromosomal DNA from *C. beijerinckii* NCIMB 8052 as template. The amplified $minD_{Cb}$ or $minJ_{Cb}$ gene was cloned into the SalI and SphI sites of a pED962 plasmid (a kind gift from D. Rudner), enabling the expression of an N-terminal YFP fusion of the gene of interest under the control of an