Introduction
Gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli* are the most common model organisms used for studying cell division in rod-shaped bacteria. Bacterial cell division is a strictly controlled, binary fission process leading to the formation of two equal daughter cells. FtsZ, a tubulin-like protein, forms a structure termed the Z-ring, which marks the position of the future division septum and serves as a scaffold for downstream division proteins. The placement of the division septum at the midcell site is very precise and the details of how this is achieved are still unknown. Two different mechanisms which have a negative effect on Z-ring assembly have been described: nucleoid occlusion and the Min system (reviewed in Barák and Wilkinson 2007; Wu and Errington 2011; Rowlett and Margolin 2015). Recently, positive regulators of Z-ring placement have been reported – the SsgAB system found in *Streptomyces coelicolor* (Willemse and van Wezel 2009), PomZ in *Myxococcus xanthus* (Treuner-Lange et al. 2013), and MapZ in *Streptococcus pneumoniae* (Fleurie et al. 2014). The existence of a similar mechanism in *B. subtilis* has also been proposed (Monahan et al. 2014). It seems that Z-ring placement is controlled differently by different bacterial species; many of the proteins involved in these systems are not highly conserved.

The Min system efficiently blocks unwanted polar septation during vegetative growth by creating a concentration gradient along the cell axis and hence protecting the polar...
sites from Z-ring formation. The key component of the Min system is the MinC protein, which prevents Z-ring formation by preventing FtsZ polymerization and by inhibiting interactions between FtsZ protofilaments (reviewed in Adams and Errington 2009). MinC is recruited to the cytoplasmic membrane, thereby triggering its inhibitory activity, by interacting with MinD, which binds reversibly to organized groups of anionic phospholipids within the membrane (Hu and Lutkenhaus 2001; Hu et al. 2002; Barák et al. 2008). The specific action and localization pattern of the MinCD complex at polar sites depends on an interaction with a third component of the Min system, termed the topological determinant. It is MinE in E.coli and DivIVA/MinJ in B.subtilis.

The behavior of E.coli Min proteins is based on a finely tuned interaction between MinE and MinD, and is highly dynamic. Upon binding of MinE to MinD, the ATPase activity of MinD is stimulated, resulting in the dissociation of the MinCD complex from the membrane and its reassociation at an adjacent site. This is manifested as rapid oscillation of all three proteins from one pole to the other, creating a bipolar MinC gradient and leaving only one place at the midcell site for FtsZ polymerization (Hu and Lutkenhaus 1999; Raskin and de Boer 1999a,b). B.subtilis does not encode a MinE homolog, on the other hand, and the polar localization of MinC is achieved by an interaction with MinJ, which links the MinCD complex to the DivIVA protein (Bramkamp et al. 2008; Patrick and Kearns 2008). DivIVA stably localizes at the sites of septation based on its ability to bind to negatively curved membranes (Cha and Stewart 1997; Edwards and Errington 1997; Lenarcic et al. 2009; Eswaramoorthy et al. 2011), and it also persists at the cell poles. The preferential attraction of MinCD to the newly forming cell poles under the influence of MinJ/DivIVA blocks polar division in B.subtilis. This system is not entirely static: fast membrane dissociation and reassociation of B.subtilis MinD (MinD_{Bs}), which retains its ATPase activity, has been observed (Barák et al. 2008), but MinD_{Bs} does not drive the rapid oscillation of MinC as E.coli MinD (MinD_{Ec}) does in the presence of MinE_{Ec}.

One of the possible paths of the B.subtilis cell cycle is sporulation, which begins with asymmetric cell division. During vegetative cell division, DivIVA helps position MinCD at the cell poles, but during sporulation it plays a role in the proper segregation of chromosomes (Thomaides et al. 2001) by attracting the RacA protein (Ben-Yehuda et al. 2003). RacA recognizes the oriC region of elongated sister chromosomes and recruits these chromosomes to the cell poles (Thomaides et al. 2001). In addition, it was shown that DivIVA also interacts with SpoIIE, the most crucial protein for asymmetric cell division (Eswaramoorthy et al. 2014).

Min systems are not essential for cell viability, however, their absence has a clear effect on the cell phenotype. In min mutant strains, polar cell division produces mixtures of “mini” cells, which lack chromosomes, and extended rods containing multiple nucleoids (Adler et al. 1967; Reeve et al. 1973; de Boer et al. 1989). Furthermore, when E.coli MinD and MinE are introduced into B.subtilis, MinD_{Ec} oscillates just as it does in E.coli cells, and this oscillating system interferes with asymmetric septum formation during B.subtilis sporulation (Jamroškovic et al. 2012). Given the clear difference in the phospholipid composition of the E.coli and B.subtilis membranes (Kusters et al. 1991; López et al. 1998), this behavior was somewhat unexpected.

Many spore-forming bacteria from the phylum Firmicutes, including the Clostridia, contain homologs from both MinCDE and MinCDJ/DivIVA systems. For example, the gram-positive pathogenic spore-former Clostridium difficile harbors homologs of MinC, MinD, MinE, and also DivIVA. Exactly which homologs are present varies according to the organism (Stragier 2002; Jamroškovic et al. 2012; this study) and it is not known whether they form a Min system which behaves as either of the two described. It is not even known whether all of these homologs are functional.

Because of the nature of C.difficile anaerobic lifestyle and its confined genetic toolbox, we have decided to address these questions by investigating the mechanism of action of the C.difficile Min proteins (Min_{Cd}) in a heterologous B.subtilis host. We found that the Min proteins of C.difficile are functional in a heterologous host B.subtilis and can affect its vegetative division. We also found that the C.difficile MinD and MinE proteins exhibit oscillation, meaning that oscillating Min proteins are not confined only to gram-negative bacteria. Oscillation of a YFP-MinD_{Cd} fusion protein was observed in B.subtilis cells in the presence of MinE_{Cd}. The same behavior can also be seen by combining the MinD and MinE proteins from E.coli and C.difficile, which opens interesting questions about the evolution of Min systems and the origins of gram-positive and gram-negative bacteria. Finally, we noted that the sporulation efficiency of those strains where oscillation was observed was diminished, indicating that either Min_{Cd} proteins or their oscillation interferes with the process of sporulation in B.subtilis.

**Experimental Procedures**

**Culture conditions and bacterial strains**

Strains were grown in LB (Luria-Bertani) medium (Ausubel et al. 1987) or in DSM (Difco sporulation medium) (Schaeffer et al. 1965) at 37°C. DNA manipulations and
E. coli transformations were performed according to Sambrook et al. (1989). The B. subtilis strains used in this work are derivatives of B. subtilis MO1099, and were prepared by transformation using plasmid or chromosomal DNA following the method of Harwood and Cutting (1990). All B. subtilis and E. coli strains used in this study are listed in Table 1 and details of their construction are in Table S1. When required, media were supplemented with ampicillin (100 μg mL⁻¹), spectinomycin (100 μg mL⁻¹), erythromycin (1 μg mL⁻¹), lincomycin (25 μg mL⁻¹), kanamycin (10 μg mL⁻¹ or 30 μg mL⁻¹), chloramphenicol (5 μg mL⁻¹), and tetracycline (5 μg mL⁻¹). To induce the expression of proteins under the control of the P xyl and P hyperspank promoters, the media were supplemented, respectively, with xylose at t₀ to a final concentration of 0.02–0.5% (w/v) and IPTG (isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 0.1–0.5 mmol/L.

Construction of recombinant plasmids

Plasmids were constructed using standard methods and propagated in E. coli MM294; their construction is described in Table S1. Primers used in the study are listed in Table S2. All PCR fragments were amplified from the chromosomal DNA of the C. difficile 630 strain (kind gift from Prof. Neil Fairweather).

Table 1. Bacterial strains used in this study.

| Strain or plasmid | Genotype or description | Construction | Reference or origin |
|-------------------|------------------------|--------------|---------------------|
| B. subtilis PY79  | Prototrophic derivative of B. subtilis 168 |              | Youngman et al. (1984) |
| MO649             | thrC::cat               |              | Guérout-Fleury et al. (1996) |
| MO1099            | amyE::erm               |              | Guérout-Fleury et al. (1996) |
| IB220             | spo0A::kan              |              | Schmeisser et al. (2000) |
| IB1056             | minD bi::cat, amyE::erm |              | Barák et al. (2008) |
| IB1059             | minD bi::cat, amyE::P xyl-gfp-minD bi spc |              | Pavlendová et al. (2010) |
| IB1111             | minD bi::cat, amyE::P hyperspank yfp-minD ec spc |              | Pavlendová et al. (2010) |
| IB1112             | minD bi::cat, divIVA::tet amyE::P hyperspank yfp-minD ec spc |              | Pavlendová et al. (2010) |
| IB1141             | minC bi::kan            |              | Jamrošková et al. (2012) |
| IB1242             | minD bi::cat, divIVA bi::tet, amyE::P hyperspank yfp-minD ec spc, thrC::Pyyl-minE ec spc |              | Jamrošková et al. (2012) |
| IB1362             | minD bi::kan            |              | Jamrošková et al. (2012) |
| IB1371             | minCD bi::kan           |              | Jamrošková et al. (2012) |
| IB1410             | thrC::Pyyl-minE cd spc  | MO649::pNP-minE cd | This study |
| IB1412             | minD bi::cat, amyE::P hyperspank yfp-minD ec spc, thrC::Pyyl-minE cd spc | IB1111::chr DNA IB1410 | This study |
| IB1413             | minD bi::cat, divIVA bi::tet, amyE::P hyperspank yfp-minD ec spc, thrC::Pyyl-minE cd spc | IB1112::chr DNA IB1410 | This study |
| IB1415             | amyE::P hyperspank yfp-minD ec spc | MO1099::pED yfp minD cd | This study |
| IB1416             | minD bi::cat, amyE::P hyperspank yfp-minD ec spc | IB1056::chr DNA IB1415 | This study |
| IB1533             | minC bi::kan            | IB1415::chr DNA IB1362 | This study |
| IB1418             | amyE::P hyperspank yfp-minD ec spc, thrC::Pyyl-minE ec spc | IB1415::chr DNA IB1410 | This study |
| IB1419             | minD bi::cat, minC bi::kan, amyE::P hyperspank yfp-minD ec spc | IB1416::chr DNA IB1362 | This study |
| IB1545             | minD bi::cat, minC bi::kan | IB1056::chr DNA IB1362 | This study |
| IB1546             | minD bi::cat, minC bi::kan, amyE::P hyperspank yfp-minD ec spc, thrC::Pyyl-minE cd spc | IB1419::chr DNA IB1410 | This study |
| IB1549             | amyE::Pyyl-minC ec spc  | MO1099::pSG minC cd | This study |
| IB1550             | minC bi::kan, amyE::Pyyl-minC ec spc | IB1549::chr DNA IB1141 | This study |
| IB1552             | amyE::Pyyl-minC ec mGFP spc | MO1099::pSG minE ec mGFP | This study |
| IB1562             | minD bi::cat, minC bi::kan, amyE::Pyyl-gfp-minD bi spc, thrC::Pyyl-minE ec spc | IB1059::chr DNA IB1362::chr DNA IB1410 | This study |

E. coli

MM294  F⁻ endA1 hsdR17 (rk-, mk) supE44 thi-1 recA1

BTH101  F⁻ cya-99 araD139 galE15 galK16 rpsL1(str6) hsdR2 mcrA1 mcrB1

C. difficile

C. difficile 630  kind gift from Prof. Neil Fairweather

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Oscillation of *C. difficile* Min system

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Fairweather, Imperial College London). *C. difficile* has been recently renamed to *Peptoclostridium difficile* (Yutin and Galperin 2013), but we continue to use its traditional name here.

### Sporulation efficiency

The sporulation efficiency assay was performed as described in Harwood and Cutting (1990). Sporulation was induced by nutrient exhaustion in liquid DSM supplemented with 0.5 mmol/L IPTG, 0.5% xylose (w/v), and half the dose of the appropriate antibiotics at 37°C for 24 h after inoculation. Afterward, aliquots of the culture were serially diluted and plated on to LB plates before and after heat treatment (85°C, 15 min). Colonies formed from nontreated samples contain viable cells, those formed from heat-treated samples contain cells that were able to sporulate. The sporulation efficiency was defined in terms of colony-forming units (CFU) formed from nontreated samples (viable cells) and heat-treated samples (spores), and was normalized against the sporulation efficiency of the originating strain. Each strain was assayed at least three times. The agar plates for photography were prepared by resuspending a single colony in 100 μL of liquid DSM and applying 10 μL of this suspension to DSM plates supplemented with 0.1 mmol/L IPTG and 0.02% xylose (w/v). These plates were sealed and incubated for 14 days at room temperature.

### Fluorescence microscopy and cell length determination

*Bacillus subtilis* strains were inoculated from a fresh overnight plate to an OD	extsubscript{600} of 0.1 and grown as liquid cultures in DSM to the desired phase. Protein expression was induced at t\textsubscript{0} by the addition of IPTG and/or xylose to a final concentration of 0.1–0.5 mmol/L and 0.02–0.3% (w/v), respectively. A small amount of culture was examined microscopically on freshly prepared poly-L-lysine-treated slides or transferred to microscope slides covered with a thin layer of 1% agarose in LB. If necessary, cells were concentrated by centrifugation (3 min at 2300 g) and resuspended in a small volume of supernatant prior to microscopic examination. Cells and septal membranes were visualized by staining the cell cultures with FM 4–64. The cell length was taken to be the axis length from one cell pole to the other as measured using ImageJ (http://imagej.nih.gov/ij/). The average cell length was determined for at least 500 cells from each sample. Minicells were not included in the calculations of the average cell lengths.

### Bacterial two hybrid system

Fusions of MinD\textsubscript{Cd} to the T18 and T25 fragments of adenylate cyclase were constructed for the bacterial adenylate cyclase two-hybrid (BACTH) system (Karimova et al. 1998). The MinD\textsubscript{Cd} sequence was PCR-amplified using the respective primer pairs (Table S2) with the chromosomal DNA of *C. difficile* 630 as a template. These PCR fragments were then cloned into the BamHI/EcoRI sites of the pUT18, pUT18C, pKT25, and pKNT25 plasmids. Fusions of Min\textsubscript{C}\textsubscript{Bs}, Min\textsubscript{D}\textsubscript{Bs}, and Min\textsubscript{J}\textsubscript{Bs} in the BACTH system had been previously prepared (Jamroškovický et al. 2012). To test protein–protein interactions, the adenylate cyclase-deficient *E. coli* BTH101 strain was cotransformed with various plasmid combinations and plated onto LB plates supplemented with X-gal (40 μg mL\textsuperscript{-1}), IPTG (0.1 mmol/L), ampicillin (100 μg mL\textsuperscript{-1}), and kanamycin (30 μg mL\textsuperscript{-1}), and grown for 24–72 h at 30°C. Constructs were tested for autoinduction with the originating vectors containing only individual fragments of adenylate cyclase. The β-galactosidase activity was measured as described by Miller (1972).

### Bioinformatic analysis

The NCBI’s PSI-BLAST program (Altschul et al. 1997) was used to search for homologs using the default threshold of 0.005 and to evaluate identity and similarity of homologous sequences. The sequences of the following strains were used in queries and alignments: *B. subtilis* (Bacillus subtilis PY79; taxid: 1415167), *C. difficile* (Peptoclostridium difficile 630; taxid: 272563), and *E. coli* (E.coli str. K-12 substr. MG1655; taxid: 511145). Specific strains of clostridia were selected based on the availability of their whole genome sequence. The positions amphipatic helices were predicted using AmphipaSeek (Sapay et al. 2006). Multiple alignment of protein sequences was done using ClustalW plugin of CLC Sequence Viewer 7.6 software (CLC Bio, Cambridge, MA).

### Results

**Clostridium difficile** Min proteins can influence *B. subtilis* cell division

Our first question to address was whether the proteins of *C. difficile* are functional and could affect *B. subtilis* cell
division. It was previously shown that higher expression of MinC<sub>E</sub> and MinD<sub>E</sub> in <i>B.subtilis</i> has a negative effect on bacterial cell division, resulting in elongation of the cells (Marston and Errington 1999). This effect was also observed when the <i>E.coli</i> MinC<sub>E</sub> and MinD<sub>E</sub> proteins were heterologously overexpressed in <i>B.subtilis</i> cells (Pavlendová et al. 2010). The average cell length of these elongated cells was 4 μm. To examine the effect of the <i>C.difficile</i> Min proteins on <i>B.subtilis</i> cells, we placed the corresponding genes under the control of inducible promoters. The resulting strains are listed in Table 1. Measurements of cell length were performed with no inducer and with both low and high concentrations of inducer (low = 0.1 mmol/L IPTG and/or 0.02% xylose; high = 0.5 mmol/L IPTG and/or 0.3% xylose); the results are summarized in Table S3 and are illustrated in Figure 1. Additionally, the average cell length of the wild-type strain (MO1099) was measured with and without the addition of xylose, to exclude its effect on cell division (not shown).

To explore the effect of <i>C.difficile</i> MinC (<i>MinC<sub>Cd</sub></i>) on <i>B.subtilis</i> cell division, we placed the gene under the control of a xylose-inducible promoter (P<sub>xyl</sub>) into an amyE locus. The cell length of the resulting IB1549 strain was measured without xylose and with of 0.3% xylose. This strain showed increased average length reaching 3 μm and occurrence of cells longer than 4 μm under both conditions up to 11% (Table S3). As the cell length also increased in the uninduced sample, it may be inferred that the cell division system of <i>B.subtilis</i> is so sensitive to MinC<sub>Cd</sub> that even the low concentrations of it produced by a leaky P<sub>xyl</sub> promoter (Vavrová et al. 2010) are enough to cause cell elongation. Leaky expression affected cell length in a previous Min system study as well (Pavlendová et al. 2010).

We also investigated the ability of MinD<sub>cd</sub> to interfere with the Min system of <i>B.subtilis</i> by introducing <i>yfp-minD<sub>Cd</sub></i> fusion under the control of an IPTG-inducible promoter (P<sub>hyperspank</sub>) into an amyE locus, creating strain IB1415. We assumed that YFP-MinD<sub>cd</sub> could functionally substitute for the native MinD<sub>cd</sub>, as both GFP-MinD<sub>bs</sub> and GFP-MinD<sub>ec</sub> are functional in their respective native organisms (Pavlendová et al. 2010; Raskin and de Boer 1999a). Cell length measurements were performed without IPTG and with two different IPTG concentrations, 0.1 mmol/L and 0.5 mmol/L. In the absence of inducer, the cell length of the strain harboring <i>yfp-minD<sub>Cd</sub></i> (IB1415) was unchanged relative to the parental MO1099 strain (2.4 μm; only 0.8% of cells are longer than 4 μm) and no minicells were observed, suggesting that even though P<sub>hyperspank</sub> is known to be leaky (Vavrová et al. 2010), the MinD<sub>cd</sub> amounts resulting from this leakiness are not sufficient to induce cell elongation. The addition of an inducer, regardless on the concentration, triggered elongation, with the average cell length reaching 4 μm and 40% of cells becoming longer than this (Table S3). Additionally, we determined the cell length of a strain harboring MinE<sub>cd</sub> to verify the effect of MinE<sub>cd</sub> alone on cell division. To prepare a <i>B.subtilis</i> strain producing MinE<sub>cd</sub> (IB1410), we placed the corresponding gene under the control of a xylose-inducible promoter (P<sub>xyl</sub>) into a thrC locus. We observed no notable change in average cell length (2.3–2.5 μm) regardless of the presence of inducer at either concentration (Table S3). This is the same behavior we observed for MinE<sub>ec</sub> in a previous study (Pavlendová et al. 2010). In <i>E.coli</i>, MinE<sub>ec</sub> overexpression is characterized by the production of minicells (de Boer et al. 1989), but neither MinE<sub>cd</sub> nor MinE<sub>ec</sub> seemed to induce their formation when introduced into <i>B.subtilis</i> cells.

Finally, we assessed the effects of simultaneous MinD<sub>cd</sub>E<sub>cd</sub> expression on the length of <i>B.subtilis</i> cells. A strain harboring both MinD<sub>cd</sub> and MinE<sub>cd</sub> (<i>yfp-minD<sub>Cd</sub></i> minE<sub>cd</sub>; IB1417) was prepared by transformation using chromosomal DNA as described in the Experimental procedures and Table 1. In the absence of inducers, IB1417 cells retained the same length as the parental wild-type strain MO1099 (2.5 μm; Table S3). Both induction conditions lead to comparable elongation, with average cell length that exceeded 3.7 μm and 33% of cells were longer than 4 μm (Table S3). Apparently, increasing the inducer concentration, and thus the amounts of MinD<sub>cd</sub> and MinE<sub>cd</sub>, does not further increase cell length. Taken together, these results show that MinC<sub>cd</sub> and MinD<sub>cd</sub> but not MinE<sub>cd</sub> elongate cells and induce minicell formation when overexpressed in <i>B.subtilis</i>. Elongation was slightly less distinct when both MinD<sub>cd</sub> and MinE<sub>cd</sub> were coexpressed at low inducer concentrations.

**Complementation of the <i>B.subtilis</i> Min system with <i>C.difficile</i> Min proteins**

It is known that the absence of MinC, MinD, or both in <i>B.subtilis</i> causes a slight cell elongation and the formation of minicells (Levin et al. 1992, 1998). There are several studies showing that the Min proteins of one organism can complement the function of the Min system of a different organism (Szeto et al. 2001; Tavva et al. 2006). For example, a functional exchange of Min systems between gram-negative and gram-positive bacteria showed that the expression of a heterologous <i>E.coli</i> MinD<sub>ec</sub> protein was able to partially rescue the ΔminD<sub>bs</sub> phenotype of <i>B.subtilis</i>; however, the same could not be said for <i>E.coli</i> MinC<sub>ec</sub>, which failed to improve the ΔminC<sub>bs</sub> phenotype (Pavlendová et al. 2010).

Here, we investigated whether the MinC<sub>cd</sub> and MinD<sub>cd</sub> proteins of the <i>C.difficile</i> Min system could restore defects caused by deleting their homologues in <i>B.subtilis</i>, and
Figure 1. Cell length histograms. Left column: effects of overexpression of \emph{C. difficile} Min proteins in wild-type background. Expression was induced using 0.1 mM IPTG and/or 0.02\% xylose, except for strain expressing MinC\textsubscript{Cd} (IB1549), in which 0.3\% xylose was used. Right column: complementation of MinBs proteins absence by MinC\textsubscript{Cd} proteins. Shown are induction conditions exhibiting the most notable complementation, that is 0.1 mM IPTG and/or 0.02\% xylose except for strain \(\Delta\text{minDc}\) MinDE\textsubscript{Cd} (IB1418), in which higher concentrations were used (0.5 mM IPTG and 0.3\% xylose). Parental strains are in gray. Summary of all measurements can be found in Table S3.
whether coexpressing MinD_{Cd} and MinE_{Cd} together could restore defects caused by the absence of MinD_{Bs}. If MinC_{Cd} or MinD_{Cd} do complement MinC_{Bs} and MinD_{Bs}, the cells should become shorter and minicell formation should decrease. Previously utilized constructs with respective C. difficile genes under the control of inducible promoters were introduced into various B. subtilis mutant backgrounds. These strains and their complete genotypes are listed in Table 1. The average cell lengths of the resulting strains were measured in the presence of varying inducer concentrations and compared with those of their parental mutant strains. All of the following measurements are summarized in Table S3 and are illustrated in histogram in Figure 1. The length of parental strains B. subtilis ΔminD_{Bs} (IB1056) and ΔminC_{Bs} (IB1141) were both determined to be 4 μm on average, with 45% of cells being longer than 4 μm.

To investigate the ability of MinC_{Cd} to complement the absence of MinC_{Cd}, we created a strain producing MinC_{Cd} from a xylose-inducible promoter (P_{xyl}) in a ΔminC_{Bs} background (IB1550). The experiments were performed without xylose induction and with two different xylose concentrations (0.02% and 0.3%). Complementation effect was already observed in the absence of inducer, when leaky expression of MinC_{Cd} was sufficient to shorten the cells from 4.1 μm to 3.4 μm on average. Induced expression improved the phenotype even further, causing the percentage of cells longer than 4 μm drop from 45% to 12% (Table S3). Minicells were present in all samples of ΔminC_{Bs} minC_{Cd}.

Introducing a YFP-MinD_{Cd} into a ΔminD_{Bs} background (IB1056) yielded strain ΔminD_{Bs} yfp-MinD_{Cd} (IB1416). The experiments were carried out without IPTG and with two different IPTG concentrations, 0.1 mmol/L IPTG and 0.5 mmol/L IPTG. Measurements of strain IB1416 grown without inducer produced cells with an average length similar to that of the originating ΔminD_{Bs} strain (Table S3). The low levels of MinD_{Cd} due to leaky expression therefore appeared to have no visible effect on cell length. Moderate expression of MinD_{Cd} (induction with 0.1 mmol/L IPTG) seemed to have a slight effect on cell division, as cell length decreased to 3.6 μm and the proportion of cells longer than 4 μm went down to 28%. Increasing the concentration of inducer to 0.5 mmol/L IPTG, however, lead to cell elongation (average of 4 μm and 43% of cells longer than 4 μm), just as seen during overexpression on a wild-type background (IB1415) (Table S3). Minicells, which are a phenotype of both ΔminD_{Bs} mutation and also, as we have shown here, MinD_{Cd} overexpression, were observed in all samples, but their frequency was not evaluated.

We also investigated changes in cell length when MinD_{Cd} is expressed together with MinE_{Cd} in a strain lacking MinD_{Bs} (ΔminD_{Bs} minD_{Cd} minE_{Cd}; IB1418). In this strain, only the induced expression of MinD_{Cd} decreased both cell length and the proportion of cells longer than 4–3.5 μm and 29%, respectively, when using a lower induction level (0.1 mmol/L IPTG, 0.02% xylose), and to 3.3 μm and 21% when using a higher induction level (0.5 mmol/L IPTG, 0.3% xylose) (Table S3).

In conclusion, our results suggest that MinC_{Cd} is able to complement for the absence of MinC_{Bs}. MinD_{Cd} alone can only partially substitute for a missing MinD_{Bs}, but when coexpressed with MinE_{Cd} considerably enhanced complementation is observed.

### Localization of C. difficile MinD and MinE in B. subtilis

As observation using fluorescent proteins is not yet commonly feasible in the anaerobic C. difficile, we explored the localization of its Min proteins in a heterologous B. subtilis system, which has previously proven to be a suitable environment for the study of Min proteins. We introduced a YFP-MinD_{Cd} fusion into B. subtilis cells under the control of an IPTG-inducible promoter at the amyE locus. This fusion was introduced into wild-type, ΔminD_{Bs} and ΔminI_{Bs} mutant backgrounds creating strains IB1415, IB1417, and IB1553, respectively. As expected from the similarity of MinD_{Cd} to MinD_{Bs}, MinD_{Cd} localized to the cell membrane and often formed foci (Figs. 2A–C). The localization pattern between the wild-type and mutant strains did not differ and we often observed short helical-like structures resembling those seen previously with B. subtilis MinD (Barák et al. 2008). In many instances, we observed localization to the sites of vegetative and asymmetric septa as well as the polar sites (Figs. 2A and B).

The localization of MinE_{Cd} was examined using a MinE_{Cd}-GFP fusion placed under the control of a xylose-inducible promoter in a wild-type background (IB1552). The observed signal was dispersed throughout the cytoplasm (not shown), which is similar to the localization of E. coli MinF_{Ec}-GFP in B. subtilis observed previously (Pavlendová et al. 2010).

### Oscillation of C. difficile MinDE proteins in B. subtilis

We have previously shown that the oscillation of the E. coli Min system can be reproduced in B. subtilis (Jamroškovicić et al. 2012). Because manipulation of an anaerobic pathogenic bacteria poses a number of complications, we decided to explore the behavior of the C. difficile Min proteins in B. subtilis cells. We introduced MinE_{Cd} under the control of a xylose-inducible promoter at the thrC locus, into strains already harboring a YFP-MinD_{Cd} fusion at the amyE locus. This gave rise to strains

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carrying the \textit{C. difficile} genes MinD$_{Cd}$ and MinE$_{Cd}$ in wild-type, Δ\textit{minD\textsubscript{Bs}} and Δ\textit{minD\textsubscript{Bs}} Δ\textit{minJ\textsubscript{Bs}} mutant backgrounds (strains IB1417, IB1418 and IB1546). When these proteins were coexpressed using 0.1 mmol/L IPTG and 0.02% xylose on the wild-type background (IB1417), we observed oscillation of YFP-MinD$_{Cd}$ from one pole to the other in a small number of cells (roughly 3% of 250 cells). This is in contrast with the behavior of the corresponding \textit{E. coli} proteins in \textit{B. subtilis}, which showed no oscillation at all in the wild-type. This effect was probably due to an interaction between MinD$_{Bs}$ and MinD$_{Ec}$ (Jamroškovic et al. 2012).

In \textit{E. coli}, Min oscillation cycle period is 20–50 sec (Raskin and de Boer 1999a; Touhami et al. 2006); the oscillation of the \textit{E. coli} proteins in \textit{B. subtilis} is somewhat slower, with a period of 1.5–3 min (recorded against a Δ\textit{minD\textsubscript{Bs}} Δ\textit{divIVA\textsubscript{Bs}} background; IB1242), and increasing the temperature to 30°C or 37°C does not affect the oscillation speed (Jamroškovic et al. 2012). Strain Δ\textit{minD\textsubscript{Bs}} Δ\textit{divIVA\textsubscript{Bs}} yfp-\textit{minD\textsubscript{Ec}} min\textit{E\textsubscript{Ec}} (IB1242) was used as a control strain in this study, to ensure our conditions are properly set, as it showed the most extensive oscillation of \textit{E. coli} Min proteins in \textit{B. subtilis}.

The oscillation of the \textit{C. difficile} proteins observed against a wild-type \textit{B. subtilis} background (IB1417) was even slower than the \textit{E. coli} ones, at a pace of about 3–5 min per cycle at room temperature. The oscillation period in strains depleted of MinD$_{Bs}$ or both MinD$_{Bs}$ and MinJ$_{Bs}$ did not change and remained at 3–5 min per cycle. However, the absence of these components seemed to increase the proportion of cells in which oscillation was observed. In a strain lacking MinD$_{Bs}$ (IB1418, Fig. 2D, Video S1), the effect was similar to the wild-type strain (4% of 50 cells), but when both MinD$_{Bs}$ and MinJ$_{Bs}$ were absent (IB1546), the oscillation was observed in up to 50% of the cells (Video S2). Regardless of the strain observed, the oscillation often stopped after 10 min and the YFP signal became dispersed throughout the cell. The speed and extent of oscillation for various organisms and heterologous systems is summarized in Table 2.

To determine if \textit{C. difficile} MinE could drive oscillation of MinD of \textit{B. subtilis}, we prepared a strain carrying a combination of GFP-tagged MinD$_{Bs}$ and MinE$_{Cd}$ in the \textit{B. subtilis} Δ\textit{minD\textsubscript{Bs}} Δ\textit{minJ\textsubscript{Bs}} background which showed the most efficient oscillation of MinD$_{Cd}$  minE$_{Cd}$. Fluorescence microscopy of this strain (Δ\textit{minD\textsubscript{Bs}} Δ\textit{minJ\textsubscript{Bs}} gfp-\textit{minD\textsubscript{Bs}}...
minE_{Cd} (IB1562) revealed that no oscillation or movement of foci could be observed after induction with 0.04% xylose (not shown). The GFP signal was distributed as random foci throughout the cell and along the helical structures which are characteristic of MinD_{Bs} (Barák et al. 2008).

Swap of Min system oscillating components between C. difficile and E. coli

To investigate the interchangeability of the Min proteins from the gram-positive C. difficile and the gram-negative E. coli and their ability to oscillate together, we prepared B. subtilis strains ΔminD_{Bs} ΔdivIVA_{Bs} YFP-minD_{Ec} minE_{Ec} (IB1412) and ΔminD_{Bs} ΔdivIVA_{Bs} yfp-minD_{Ec} minE_{Cd} (IB1413). After inducing the expression of YFP-MinD_{Ec} and MinE_{Cd} we observed oscillation of YFP-MinD_{Ec} with a period of 3–5 min, similar to that observed before for strain expressing MinDE originating from C. difficile (IB1417). This oscillation was only observed in a small portion of the ΔminD_{Bs} cells (IB1412) and improved in cells with a ΔminD_{Bs} ΔdivIVA_{Bs} background (IB1413; not quantified statistically). These results show that there is some compatibility between the oscillating systems of these two evolutionarily distant gram-positive and gram-negative species.

Oscillating Min system of C. difficile interferes with B. subtilis sporulation

In our previous study, we showed that the oscillating E. coli Min system blocks sporulation at the asymmetric septum formation step (Jamroškovič et al. 2012). An intriguing question is therefore whether spore-forming C. difficile also possesses an oscillating Min system that interferes with its sporulation. We assessed the sporulation efficiency of various B. subtilis strains in the presence of inducers (0.5 mmol/L IPTG, 0.5% xylose). The sporulation efficiency of both the wild-type and a ΔminD_{Bs} strain harboring the oscillating C. difficile MinDE proteins (IB1417 and IB1418) dropped to 32% and 45%, respectively (Fig. 3). A dramatic decrease in sporulation efficiency, down to 0.03%, was observed in the strain which lacked both MinD_{Bs} and MinE_{Bs} (IB1546). This was also the strain with the most effective oscillation. This drop in sporulation efficiency seems to be related to the proportion of cells in which oscillation is observed.

Protein–protein interactions between C. difficile MinD and B. subtilis Min proteins

To improve our understanding of the behavior of the C. difficile Min proteins in B. subtilis, we looked for interactions between the C. difficile and B. subtilis proteins using a bacterial two-hybrid system (BACTH). The strength of these interactions was quantified using a β-galactosidase assay. A very strong interaction was detected between MinD_{Cd} and MinC_{Bs}, while a weaker one was found between MinD_{Ec} and MinC_{Eb} (Fig. 4). It is possible that the lower affinity of MinD_{Cd} for MinJ_{Bs} might explain why only partial complementation was observed when MinD_{Cd} was expressed against a ΔminD_{Bs} background (IB1416), as the MinD–Min interaction would clearly be a limiting factor. The MinD proteins from the two organisms seem to interact with each other strongly as well. The interaction observed between MinD_{Cd} and MinC_{Bs} confirms that the MinD_{Cd}/MinE_{Cd} system can indeed utilize the host B. subtilis MinC, as suggested by the complementation experiments in the ΔminD_{Bs} minD_{Cd} minE_{Cd} (IB1418) strain.

Discussion

Clostridium difficile is an important human pathogen, causing serious problems in hospitals and medical facilities (reviewed in Burke and Lamont 2014). Because of its strictly anaerobic life style and its demanding transformation procedures, we have still only a limited knowledge of some of its basic processes, including cell division. For example, commonly used reporter genes, such as fluorescent proteins or luciferase, require oxygen for protein folding or enzyme activity (Heim et al. 1994; Hastings and Gibson 1967). In spite of ongoing efforts, methods and reporter assays suitable for anaerobic or low-oxygen conditions are limited.

Table 2. Comparison of oscillation times and efficiency between Min systems.

| System | Organism | Genotype | Oscillation efficiency [%] | Oscillation period | Reference |
|--------|----------|----------|-----------------------------|--------------------|-----------|
| E. coli | E. coli  | –        | 100                         | 20–50 sec          | Raskin and de Boer 1999a; Touhami et al. 2006 |
| E. coli | B. subtilis | YFP-minD_{Ec} minE_{Ec} | 0 | – | Jamroškovič et al. 2012 |
| E. coli | B. subtilis | ΔminD_{Bs} ΔdivIVA_{Bs} YFP-minD_{Ec} minE_{Ec} | −100 | 1.5–3 min | Jamroškovič et al. 2012 |
| C. difficile | B. subtilis | YFP-minD_{Cd} minE_{Cd} | 3 | 3–5 min | This study |
| C. difficile | B. subtilis | ΔminD_{Bs} YFP-minD_{Cd} minE_{Cd} | 4 | 3–5 min | This study |
| C. difficile | B. subtilis | ΔminD_{Bs} ΔminE_{Bs} YFP-minD_{Cd} minE_{Cd} | 50 | 3–5 min | This study |
conditions are now only starting to emerge (Drepper et al. 2007; Edwards et al. 2015; Buckley et al. 2015; Ransom et al. 2015). Because of these problems, we decided to investigate the mechanism of action of \textit{C.\difficile} Min proteins and their effects on vegetative cell division and sporulation in a heterologous \textit{B.\subtilis} host. This, the first study focused on the \textit{C.\difficile} Min system, may help us to understand the role of its Min proteins in the asymmetric division and spore formation of this medically significant bacterium.

Our analysis of MinC_{\textit{cd}} and MinD_{\textit{cd}} in vegetatively growing cells shows that these proteins are able to affect cell division in \textit{B.\subtilis}. We found that both MinC_{\textit{cd}} and MinD_{\textit{cd}} can complement for the missing \textit{B.\subtilis} counterparts. Interestingly, the same could not be said for MinC_{\textit{ec}}, which was previously shown to fail in MinC_{\textit{bs}}.

* $\Delta{\text{spo0A}}$ used as negative control.

| Strain   | MinD_{bs} | MinJ_{bs} | MinD_{cd} | MinE_{cd} | Sporulation efficiency | Oscillation | DSM plate |
|----------|-----------|-----------|-----------|-----------|------------------------|-------------|-----------|
| MO1099   |           |           |           |           | 100%                   |             |           |
| IB1056   | -         | MinJ_{bs} |           |           | 85 ± 2%                |             |           |
| IB1417   | MinD_{bs} | MinJ_{bs} | MinD_{cd} | MinE_{cd} | 32 ± 4%                | +           |           |
| IB1418   | -         | MinJ_{bs} | MinD_{cd} | MinE_{cd} | 45 ± 4%                | +           |           |
| IB1545   | -         | -         | -         | -         | 97 ± 4%                |             |           |
| IB1546   | -         | -         | MinD_{cd} | MinE_{cd} | 0.03 ± 0.04%           | +           |           |
| IB220*   | MinD_{bs} | MinJ_{bs} |           |           | 0.01 ± 0.004%          |             |           |

* $\Delta{\text{spo0A}}$ used as negative control.

Figure 3. Sporulation efficiency of \textit{B.\subtilis} strains. Sporulation efficiency is given as the mean ± SD of at least three independent assays, each normalized against a wild-type control. Sporulating colonies develop brown color, while nonsporulating light are brown to translucent, as seen in the $\Delta{\text{spo0A}}$ negative control (IB220).

Figure 4. Protein–protein interactions between MinD_{cd} and the \textit{B.\subtilis} Min proteins compared alongside interactions among the \textit{B.\subtilis} Min proteins as detected by bacterial two-hybrid system BACTH. Interactions were quantified using a $\beta$-galactosidase assay and are expressed in Miller units as mean values ± SD of at least three independent measurements. The color intensity corresponds to the strength of the interaction; red boxes highlight strong positive interactions between heterologous proteins. Negative controls were all below 80 MU.
The observed localization of MinD\textsubscript{Cd} along helical structures suggests that this protein recognizes the anionic phospholipids organized in a helical manner in the \textit{B.subtilis} membrane. The C-terminal region of MinD from various organisms, including \textit{E.coli} and \textit{B.subtilis}, contains a consensus amphipathic helical region that anchors it to the membrane (Szeto et al. 2002). This consensus sequence can also be found in \textit{C.difficile} MinD (Fig. 5B) and an amphipathic helix was also predicted at the C-terminus by AmphipaSeek (not shown; Sapay et al. 2006). This helix would then be responsible for the membrane localization of MinD\textsubscript{Cd} in \textit{B.subtilis}, which is similar to that observed for MinD\textsubscript{E} in the same organism.

\textit{Bacillus subtilis} and \textit{C.difficile} have very different membrane compositions, In fact, \textit{Clostridium} species display distinct variations in their major polar lipid contents and \textit{C.difficile} has an exceptionally variable membrane lipid composition, even for different isolates of the same strain (Korachi et al. 2002). Phosphatidylglycerol (PG) and cardiolipin (CL) have been identified, but interestingly, phosphatidylethanolamine is completely absent and other lipids have been proposed to balance the negative charge of PG and CL (Drucker et al. 1996; Korachi et al. 2002; Guan et al. 2014). Phosphatidylglycerol and CL represent 24\% and 4\% of the membrane phospholipids in \textit{E.coli} (Kusters et al. 1991), 40\% and 20\% in \textit{B.subtilis} (López et al. 1998), and 30\% and 16\% in \textit{C.difficile} (Guan et al. 2014).

The ability to oscillate is an intrinsic characteristic of the Min proteins and emerges whenever some minimal criteria are met (Loose et al. 2008). Thus, a different membrane composition does not pose any obstacle for oscillation, as long as enough negatively charged lipids are present, although the resulting charge density does affect oscillation parameters such as wavelength and velocity (Vecchiarelli et al. 2014; Zieske and Schwille 2014). It has been suggested that these differences arise from the differences between the mechanisms of membrane binding by MinD\textsubscript{Ec} and MinE\textsubscript{Ec} (Vecchiarelli et al. 2014). Previous successful reconstitutions of oscillation in heterologous systems (Ramirez-Arcos et al. 2002; Jamrošković et al. 2012) suggest that, in the complex environment of cell, the most limiting factor is the interaction between the heterologous Min proteins within the host organism. In our case, oscillation markedly improved when the Min\textsubscript{Cd} system was introduced into a \textit{\textDelta}\textit{minD\textsubscript{B} minF\textsubscript{E}} background (IB1546), which allowed oscillation of the heterologous Min system.

All \textit{B.subtilis} strains expressing an oscillating Min\textsubscript{Cd} system exhibited disturbed sporulation. The severity of the sporulation defect seems to be correlated with improved oscillation efficiency. Two important questions remain: first, what is the underlying cause of this failed
sporulation, and second, does it affect the sporulation of *C. difficile* at all? It is possible that the differences in sporulation and sporulation regulation between *B. subtilis* and *C. difficile* cause oscillation to be inhibitory in the heterologous organism, but not in the native one. Another possibility is that an oscillating MinCd system only inhibits polar division during particular parts of the cell life cycle, such as vegetative growth, and is shut down or modulated during sporulation. Yet, a third possibility is that this system does, in fact, cause *C. difficile* to sporulate less efficiently than *B. subtilis*, but provides additional advantage for its different lifestyle and environmental niche.

Although complex transcriptional data for *C. difficile* are still lacking, we can still make some inferences from the work of Saujet et al. (2013). They found that the *ftsZ, minC, miniD, miniE*, and *divIVA* genes were all positively controlled by SigH, the key regulator of the transition phase in *C. difficile*, which is of comparable importance to Spo0A. SigH is also involved in the expression of *ftsZ, minC*, and *miniD* in *B. subtilis* as well (Britton et al. 2002). These results suggest that the MinCDE proteins are present in the early stages of sporulation in *C. difficile*. The genome of *C. difficile* also harbors a DivIVA homolog (Table. S4), which in *B. subtilis* has a role in sporulation, but the question of whether it serves as a polar tether for the Min system as it does in *B. subtilis* remains open, as we were not able to identify a MinJ homologue. It is still possible that some other protein fills the role of MinJ in connecting the MinCD system to DivIVA.

The Clostridia are a diverse group of bacteria, and, despite their common historical designation as gram-positive, a number of them have been found to have a membrane organization more characteristic of gram-negative bacteria (and were thus moved into a separate class, *Negativicutes*), together with the ability to form endospores (Yutin and Galperin 2013). *Acetoneum longum* is a distant relative of *Clostridium* spp. and a lesser known member of *Negativicutes*. A study of the sporulation and germination of this organism revealed a remarkable inversion of the inner membrane of the mother cell, to become the outer membrane of the germinating cell (Tocheva et al. 2011). This brings us to the question of evolution of gram-positive and gram-negative bacteria, an exciting topic on which many opposing theories exist. The work of Tocheva et al. 2011 suggests how the outer membrane of gram-negative bacteria might have evolved, and more broadly, how gram-negatives could have arisen from gram-positives. *A. longum* could therefore represent a missing link between the two groups. Our analysis of some Clostridia and Negativicutes members’ genomes shows that many possess Min proteins from both systems (Table. S4), suggesting that the two systems might have evolved in a gram-positive bacterium. Whether and how these systems could coexist in clostridia remains to be resolved by future studies. Until convenient methods for directly studying clostridia are developed, *B. subtilis* could serve as host system for these studies.

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**Conflict of Interest**

Authors declare no conflict of interest.

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**Supporting Information**

Additional supporting information may be found in the online version of this article:

**Figure S1:** Multiple sequence alignment of Min proteins.
**Video S1:** Oscillation of YFP-tagged MinD$_{Cd}$ in the presence of MinE$_{Cd}$ recorded in *B.subtilis* ΔminD$_{Bs}$ minD$_{Cd}$ minE$_{Cd}$ (IB1418). Scale bar represents 1 μm.

**Video S2:** Oscillation of YFP-tagged MinD$_{Cd}$ in the presence of MinE$_{Cd}$ recorded in *B.subtilis* ΔminD$_{Bs}$ ΔminJ$_{Bs}$ minD$_{Cd}$ minE$_{Cd}$ (IB1546). Scale bar represents 5 μm.

**Table S1:** Plasmids used in this study and their construction.
**Table S2:** Primers used in this study.
**Table S3:** Cell length measurements.
**Table S4:** The sequence similarity/identity of Min proteins of selected members of Clostridia and Negativicutes compared with their counterparts in *E.coli* and *B.subtilis*. Similarity and identity values are derived from a BLAST query (Altschul et al. 1997). For MinC and MinD, the sequence of the *B.subtilis* proteins was used as reference, since these queries gave lower E-values and higher query cover and identities than the *E.coli* sequences (not shown). The *E.coli* sequence was used as a reference for MinE, and *B.subtilis* sequences for a search of MinJ and DivIVA homologs. All listed organisms are endospore-formers except *E.coli*. 