SepF, a novel FtsZ-interacting protein required for a late step in cell division

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Summary

Cell division in nearly all bacteria is initiated by polymerization of the conserved tubulin-like protein FtsZ into a ring-like structure at midcell. This Z-ring functions as a scaffold for a group of conserved proteins that execute the synthesis of the division septum (the divisome). Here we describe the identification of a new cell division protein in Bacillus subtilis. This protein is conserved in Gram positive bacteria, and because it has a role in septum development, we termed it SepF. sepF mutants are viable but have a cell division defect, in which septa are formed slowly and with a severely abnormal morphology. Yeast two-hybrid analysis showed that SepF can interact with itself and with FtsZ. Accordingly, fluorescence microscopy showed that SepF accumulates at the site of cell division, and this localization depends on the presence of FtsZ. Combination of mutations in sepF and ezrA, encoding another Z-ring interacting protein, had a synthetic lethal division effect. We conclude that SepF is a new member of the Gram positive divisome, required for proper execution of septum synthesis.

Introduction

Cell division in nearly all bacteria is initiated by the polymerization of the conserved tubulin-like protein FtsZ into a ring-like structure at midcell. This so-called Z-ring functions as a scaffold for the proteins that execute the synthesis of the division septum. The assembly of this complex and dynamic protein structure, also referred to as the divisome, has been thoroughly investigated in the model organisms Escherichia coli and Bacillus subtilis (reviewed in Errington et al., 2003). Most of the components of the divisome are conserved; however, there are distinct differences between the mechanisms of divisome assembly in B. subtilis and E. coli. It is likely that this reflects structural differences between the cell-envelopes of Gram positive and Gram negative bacteria. We will focus here on cell division in B. subtilis.

The divisome complex spans the cytoplasmic membrane. At the core of the cytoplasmic site of the divisome lies the Z-ring, assembly of which is supported by at least two other cytosolic proteins. Most important is the conserved actin-like division protein FtsA. This protein is essential and interacts with the C-terminus of FtsZ (Erickson, 2001). It has recently been shown that the conserved C-terminus of FtsA contains an amphipathic helix that is essential for targeting of the protein to the membrane. This led to the suggestion that FtsA serves as the principal membrane anchor for the Z-ring (Pichoff and Lutkenhaus, 2005). In addition, there is a small 85-amino-acid cytoplasmic protein, ZapA, which promotes polymerization of FtsZ. However, a deletion of zapA showed no apparent phenotype in B. subtilis or E. coli (Gueiros-Filho and Losick, 2002; Johnson et al., 2004). EzrA is another conserved protein that binds to the Z-ring (Levin et al., 1999). This large protein has a single N-terminal transmembrane domain, but this domain is not required for interaction with FtsZ (Haeusser et al., 2004). The role of EzrA in the activity of the divisome is not entirely clear. An ezrA mutant is viable, yet it tends to assemble multiple Z-rings both at polar and midcell positions. It is assumed that this protein is a negative regulator of Z-ring formation (Levin et al., 1999).

At least two of the conserved B. subtilis divisome proteins are involved in peptidoglycan synthesis: the penicillin-binding protein Pbp2B and the integral membrane protein FtsW (R.A. Daniel and J. Errington, unpublished; Daniel et al., 2000; Aarsman et al., 2005). The 10 membrane spanning domains of FtsW suggest a possible role in transport, and it has been speculated that FtsW translocates the lipid-linked precursor for the septal peptidoglycan matrix (Holtje, 1998). The periplasmic part of the divisome comprises, aside from Pbp2B, three other conserved cell division proteins: FtsL, DivIB and DivIC. Like Pbp2B, these proteins are attached to the membrane by a single transmembrane domain. Not much is known about the function of these three proteins. They seem to
be intrinsically unstable and biochemical data suggest that they form heteromultimers that ensure their stability (Sievers and Errington, 2000; Robson et al., 2002). Therefore, it has been proposed that these proteins may fulfill a regulatory role in divisome assembly and/or disassembly.

The divisomes of *B. subtilis* and *E. coli* appear to be comparable in composition (Errington et al., 2003). However, the *E. coli* divisome comprises several more components, such as FtsK, FtsE, FtsQ, FtsN and AmiC (Aarsman et al., 2005). This complexity could be a consequence of the fact that in *E. coli* cytokinesis requires constriction of outer membrane, as well as inner membrane, and peptidoglycan layers. An intriguing difference between *B. subtilis* and *E. coli* is the way their divisomes assemble. In *E. coli*, the recruitment of the different components seems to occur in an ordered manner, whereby the depletion of one cell division protein leads to the absence of the downstream proteins in the interaction pathway [the order being: FtsZ (FtsA, ZipA, ZapA), FtsK, FtsQ (FtsL, FtsB), FtsW, FtsI, FtsN and AmiC] (Aarsman et al., 2005; Goehring et al., 2005). This linearity of recruitment is not observed in *B. subtilis*. The assembly of the *B. subtilis* divisome seems to depend on the presence of all the essential division proteins, and depletion of FtsA, DivIC, FtsL or Pbp2B, abolishes the positioning of the other cell division proteins at midcell. Only the polymerization of the Z-ring does not require the other division proteins (Errington et al., 2003). Although much is known about the assembly of the divisome, the actual mechanism of constriction remains unclear. The homology of FtsZ and FtsA with tubulin and actin, respectively, makes it plausible that these proteins lead the constriction of the divisome. However, whether they are sufficient to drive this process, or whether the synthesis of the peptidoglycan provides the ultimate force for constriction, remains to be established (Bramhill, 1997; Errington et al., 2003).

In this paper we describe the identification of a new FtsZ-interacting cell division protein, which we designated SepF. Combination of a *sepf* and *ezrA* mutation results in a synthetic-lethal division defect, suggesting that one or both of these proteins is necessary for proper functioning of the Z-ring. However, *sepf* single mutants affect only the later stages of septum constriction indicating that SepF is important throughout the process of division. While this work was in progress, we learned that Ogasawara and coworkers had obtained similar results implicating SepF in cell division of *B. subtilis*.

**Results**

**Deletion of the ylmB-H region has a mild cell division defect**

In rod-shaped bacteria the conserved protein couple MinC/MinD prevents aberrant Z-ring assembly close to the poles of the cell. Mutations in these proteins result in polar division and anucleate minicells. MinC is the actual inhibitor of FtsZ assembly but requires the membrane-bound ATPase MinD for its activity (Hu et al., 1999; Marston and Errington, 1999). In *B. subtilis* the activity of this protein couple is confined to the cell poles due to interactions with the polar localized protein DivIVA (Edwards and Errington, 1997). How DivIVA accumulates at the poles is unknown. In many Gram positive bacteria the conserved *ylm* genes are found upstream of *divIVA* (Fig. S1). Several of the *ylm* genes have been disrupted previously (Kobayashi et al., 2003) (see also, http://genome.jouy.inra.fr/cgi-bin/micado/index.cgi). However, none of the mutants were reported to have a phenotype. The genes *ylmE* and *ylmH* were not tested, and to assess a potential role of the *ylm* genes in DivIVA localization, we replaced the complete *ylmb-H* region with an antibiotic resistance marker (hereafter referred to as *ylm* mutant, Fig. 1). Light microscopic observations of the resulting strain (*B. subtilis* 3317) seem to indicate a normal wild-type phenotype, although the cells appeared longer. To analyse this in more detail, we measured cell length of an *ylm* mutant and wild-type culture. As shown in Fig. 1C and D, the difference was modest during exponential growth, with an average length of mutant cells approximately 20% longer than wild-type cells (4.6 and 5.5 µm for wild-type and *ylm* mutant respectively). In the stationary phase this difference increased to about 40% (2.8 and 4.0 µm for wild-type and *ylm* mutant respectively). It was clear that the *ylm* genes were not required for DivIVA localization because a normal polar and septal fluorescent pattern was observed when the *ylm* deletion was introduced into a strain carrying a *divIVA-gfp* fusion (Fig. 1F). However, there was a clear difference in spacing of green fluorescent protein (GFP) bands, which reflects the increase in cell length of the mutant.

**Disruption of the ylm locus has a synthetic lethal effect when combined with ezrA**

It seemed possible that the mild mutant phenotype produced by the *ylm* deletion might be exacerbated when combined with other mutations that impair cell division. To test whether the *ylm* mutant had increased sensitivity to FtsZ levels, the *ylm* deletion was introduced into cells in which *ftsZ* expression is under control of the IPTG-inducible Pspac promoter. Although low IPTG concentrations resulted in elongated cells, the presence of an *ylm* deletion did not substantially aggravate this phenotype. Various other mutations in genes that regulate FtsZ polymerization, such as *minC* and *zapA*, were combined with an *ylm* deletion, but the effects on cell length were again marginal (data not shown). In contrast, the number of transformants was much lower than expected when the *ylm* mutant was transformed with chromosomal DNA from
an *ezrA* mutant. The few colonies that emerged appeared to have lost the kanamycin-resistance marker, and therefore had been co-transformed with the wild-type *ylm* locus from the donor strain. This finding supported the view that one or more of the *ylm* genes plays a role in cell division.

Deletion of the *ylm* locus leads to impaired septa

In view of the possible deficiency in cell division resulting from deletion of the *ylm* locus, the ultrastructure of division septa was analysed using electron microscopy (EM). It appeared that in *ylm* mutant cells septa showed strong deformations (Fig. 2). This was most apparent in the early stages of septum synthesis, as shown in Fig. 2D and E. Division seemed to proceed normally when septation has been completed, although distortions in matured septa were still observed (Fig. 2F). These distortions did not seem to lead to deformed cell poles (data not shown).

The *ylmD-H* genes comprise an operon

Inspection of the DNA sequence of the conserved *ylm* region revealed only a single large space likely to accom-
modulate a promoter: a 165 bp non-coding region between ylmC and ylmD. To test whether this region contains a promoter, and simultaneously, to examine whether ylmB and/or ylmC contributed to the cell division defect, two deletion mutants were constructed: strains 4054 and 4055. In strain 4055, ylmB, and ylmC including the 165 bp non-coding region, were replaced with the IPTG-inducible Pspac promoter. Strain 4054 was similar except that the 165 bp potential promoter region remained in position upstream of ylmD. When an ezrA mutation was introduced into these strains, the resulting transformants grew normally in the presence of IPTG. However, transformants from strain 4055 became elongated and eventually lysed when IPTG was omitted, whereas those of 4054 retained normal cell length and viability (data not shown). These results showed that loss of ylmB and ylmC does not give a division phenotype, and strongly suggested that the gene or genes responsible for the division effect lay downstream of the 165 bp region and required transcription from that promoter (ylmD promoter).

To test which of the genes downstream of the 165 bp region were transcribed from the putative promoter, RNA was extracted from wild-type B. subtilis, and strain 4055 (grown in the absence of IPTG). As shown in Fig. 3, probes directed against ylmD, ylmG and ylmH gave a clear signal when RNA from wild-type cells was used, but almost no signal when RNA from the mutant was used. This suggests that there is a single polycistronic mRNA, and that the promoter for this mRNA is absent from strain 4055. In contrast, a probe against the downstream-located gene divIVA hybridized only to a faster migrating band that was present in both strains. The latter supported previous findings that divIVA has its own promoter (Edwards and Errington, 1997).

ylmF (sepF) is responsible for the division defect
To determine which of the ylm genes was responsible for

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**Fig. 3.** Northern analysis of the ylmD-divIVA region. Total RNA from wild-type (wt), and a B. subtilis mutant (mut) that contains the Pspac promoter immediately upstream of the ylmD open reading frame (B. subtilis 4055), was hybridized with radioactively labelled probes directed against ylmD, ylmG, ylmH and divIVA respectively. B. subtilis 4055 was grown in the absence of IPTG.

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**SepF interacts with itself and FtsZ**
In most Gram positive bacteria sepF lies in a conserved gene cluster together with ylmE, ylmG and ylmH homologues (see Fig. S1). This suggested that their gene products might be functionally related. To assess whether SepF, YlmE, -G and -H physically interacted, the corresponding open reading frames were expressed in yeast as fusions to the GAL4 activation domain (AD), and to the GAL4 binding domain (BD). Interactions were tested by a yeast two-hybrid mating assay, as described in *Experimental procedures*. The result of a typical mating experiment is shown in Fig. 4A. The self-activation of the interaction reporter genes by the BD-YlmH fusion prevented any interpretation for this protein. In contrast, BD-SepF interacted specifically with AD-SepF, suggesting that the protein can dimerize or oligomerize. Various division proteins were then tested for interaction with SepF, YlmE and YlmG, including: DivIB, DivIC, DivIVA, EzrA, FtsA, FtsL, FtsZ, Pbp2B and ZapA. Only a single interaction emerged from this extensive screening experiment: a clear interaction between SepF and FtsZ (Fig. 4B). This result provided strong support for the notion that SepF is part of the divisome complex.

**SepF localizes to division sites dependent on FtsZ but not later components of the divisome**
To further test the interaction of SepF with FtsZ, we examined the localization of the protein *in vivo* by fusing it to GFP. As shown in Fig. 5A, cells expressing a sepF-gfp fusion showed a pattern of regular transverse bands (arrowheads) and dots (arrows) corresponding to septa in the early and late stages of division. Unfortunately, the
The ylm locus in B. subtilis and related bacteria

In many bacteria the genes of the main players in cell division and cell wall synthesis are located together in a locus. Unfortunately, the exact localization in Bacillus subtilis (B. subtilis) is not well understood. One of the key proteins involved in cell division is SepF, a novel FtsZ-interacting protein. To study the role of SepF in cell division, a SepF-GFP fusion was introduced into a strain in which the division proteins FtsL and Pbp2B could be simultaneously depleted. This led to a rapid block in assembly of the divisome, but Z-rings still assembled, as visualized with an ftsZ-gfp fusion (Fig. 6A). Some of the Z-rings appeared abnormal, like double structures or short helices (arrowheads). Such abnormal structures were also observed in ezrA single mutants, but they appeared more often when SepF was depleted, as well. Nevertheless, the conclusion must be that SepF is not essential for the formation of Z-rings in an ezrA mutant. To test whether SepF affects a later step in division, the experiment was repeated with a gfp-pbp2B fusion. As shown in Fig. 6E and F, bands of GFP-Pbp2B (arrows) were still visible after depletion of SepF, albeit with a reduced intensity. The persistence of the GFP-Pbp2B banding pattern suggests that SepF is not required for assembly of the divisome under these conditions.

The GFP experiments suggested that the sepF ezrA arrest in division occurs at a very late step. EM was used to examine the ultrastructure of the arrested cells. In the presence of IPTG division septa had a fairly normal appearance, indicating that deletion of ezrA does not significantly affect the formation of the division septum (Fig. 6L–N). In the absence of IPTG, normal division septa were essentially absent. In some cells the early stages of constriction were visible (Fig. 6L–N). It is likely that these are the remains of septa that had been initiated just before SepF became limiting.

**Discussion**

**The ylm locus in B. subtilis and related bacteria**

In many bacteria the genes of the main players in cell division and cell wall synthesis are located together in a
cluster that is well conserved, the DCW cluster (Massidda et al., 1998). In many Gram positive bacteria the ylm locus is located next to the DCW cluster (Fig. S1). An indication that the ylm genes might have a role in cell division came from a deletion study in Streptococcus pneumoniae (Fadda et al. 2003). In this organism ftsA, ftsZ, the ylm genes, and divIVA form an operon. Deletion of divIVA resulted in a strong morphological effect with incomplete septa, strongly deformed cells and occasional anucleate cells. Insertions in the individual ylm genes resulted in thin septa, and a slightly altered cell shape. Unfortunately, interpretation of these data was difficult due to potential polar effects on the expression of ylm genes and divIVA.

Most Gram negative bacteria contain homologues of ylmE and ylmG, but they lack genes that show similarities to sepF or ylmH. Interestingly, cyanobacteria harbour cell division proteins that are typical of both Gram positive and Gram negative bacteria. In the genomes of several sequenced cyanobacteria, homologues of both divIVA and minE are present, and sepF, ylmE, ylmG and ylmH are all conserved (Miyagishima et al., 2005). However, these genes are not clustered at a single locus in the genome. Despite their outer membrane, molecular phylogenetic studies now group the cyanobacteria separately from the other Gram negatives (Woese et al., 1990; Miyagishima et al., 2005). It was recently shown that in the cyanobacterium Synechococcus elongatus, an interruption of sepF resulted in a doubling in cell length (Miyagishima et al., 2005). The relatively mild effect of sepF disruption on cell length in B. subtilis may explain why this gene had not been picked up in previous mutant searches. Deletion of sepF resulted in aberrant septum synthesis according to the EM images of Fig. 2. These structural deformations could delay maturation of the division septum, which may explain why sepF mutations result in longer cells.

**A late role for SepF in cell division**

In this study we have shown that SepF is an important cell division protein. The conserved genomic localization of sepF upstream of divIVA might suggest a functional relationship. However, deleting sepF does not result in a minicell phenotype, and the typical targeting of DivIVA to division sites and cell poles was unaffected in such mutant. In addition, deletions of divIVA and minC/D did not interfere with the localization of SepF (data not shown). This suggests that SepF functions in a different pathway from DivIVA. The information that can be extracted from the primary sequence of the ylm operon is rather limited. Only ylmE shows sequence homology that could point towards a specific activity in the cell division process, because this protein has homology with pyridoxal phosphate-dependent enzymes. The cofactor pyridoxal phosphate is involved in a wide variety of catalytic reactions, including the conversion of L-alanine to d-alanine by alanine racemases, which is one of the first steps in the synthesis of peptidoglycan (Shaw et al., 1997). Nevertheless, the yeast two-hybrid study did not reveal an interaction between SepF and YlmE, YlmG or YlmH. In addition, none of the ylmE, ylmG or ylmH mutants showed a filamentous phenotype in an ezrA background that would resemble a sepF ezrA double knockout. So far, our data do not provide support for the assumption that SepF and the other Ylm proteins function in the same pathway, as the conserved operon organization would suggest.
To facilitate the discussion on the function of SepF, we dissect the process of septum formation into three sequential steps: (i) Z-ring formation, (ii) divisome assembly and (iii) septum synthesis (Fig. 7). The first step, the formation of the Z-ring, begins with the polymerization of FtsZ monomers into protofilaments that subsequently assemble into a Z-ring. FRAP studies (fluorescence recovery after photobleaching) have shown that the Z-ring is a dynamic structure with a turnover of subunits within seconds (Stricker et al., 2002; Anderson et al., 2004). This remodelling of the Z-ring appears to depend on the hydrolysis of GTP by FtsZ polymers (Stricker et al., 2002; Anderson et al., 2004). Several proteins control the assembly of the Z-ring in B. subtilis such as MinC and ZapA (Fig. 7). In vitro experiments with MinC of E. coli have shown that this protein interacts with FtsZ and inhibits polymerization (Hu et al., 1999). EzrA is also a negative regulator of Z-ring formation, and recent biochemical experiments showed that purified EzrA inhibits the polymerization of FtsZ into protofilaments (Haeussser et al., 2004). ZapA has a positive effect on Z-ring formation and in vitro studies showed that this protein strongly stimulates the bundling of protofilaments (Gueiros-Filho and Losick, 2002). Interestingly, an ezrA zapA double knockout is highly filamentous (Gueiros-Filho and Losick, 2002). The reason for this is not clear, but this phenotype could be used to argue that SepF might have a comparable function with that of ZapA. However, we do not think that this is the case. A zapA mutant is sensitive to reduced FtsZ concentrations, and this was not observed for the ylm mutant. Also the introduction of minC or zapA deletions into the ylm mutant did not affect cell length more than the mutations did individually. Finally, in the absence of both SepF and EzrA, Z-rings are still formed. Therefore, we assume that SepF is not involved in Z-ring formation, but is active in a later step of septum synthesis. Interestingly, SepF requires only the Z-ring for its localization, which is so far unique for a late cell division protein in B. subtilis.

After the Z-ring has formed the divisome assembles. In E. coli there is a considerable time delay between these two steps: 15–20 min, depending on the growth rate (Aarsman et al., 2005). Whether this is also the case for B. subtilis is not known. As described in Introduction, assembly of the divisome in this organism is a cooperative process and requires the presence of a number of essential division proteins (Fig. 7). SepF is not essential for this step of the process, because the ylm mutant grew normally. Even when both SepF and EzrA are absent, and

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septation is blocked, the divisome still assembles, as the fluorescent GFP-Pbp2B bands in Fig. 6E indicated. Thus, it is unlikely that the main function of SepF is to stimulate the assembly of the divisome complex. However, the EM images of Fig. 2 suggest that the synthesis of the division septum occurs in an irregular manner when SepF is absent. Such aberrant septa have not been described for B. subtilis cell division mutants. Although mutants that affect Z-ring formation can result in misplaced septa, they do not show such strong deformations and thickening of division septa. For example, Fig. 6I–K illustrates that septa have a wild-type appearance in an ezrA background, whereas it is clear from Fig. 2 that maturation is strongly affected in a sepF mutant septum. Therefore, we favour a model in which SepF participates in the third step of the cell division process: the constriction of the divisome and/or synthesis of the septum wall.

The reasoning behind the model of Fig. 7 presents us with a problem, namely, how to explain the synergistic effect of an ezrA sepF double knockout. The precise role that EzrA fulfils in the division process is not entirely clear. Fluorescence microscopy studies have shown that EzrA primarily localizes at the site of cell division, and that this localization is FtsZ-dependent (Levin et al., 1999). This suggests that EzrA is active during synthesis of the septum, which is surprising because EzrA is an inhibitor of FtsZ polymerization. However, biochemical experiments provided an explanation for this paradox because EzrA is unable to disassemble preformed FtsZ-polymers in vitro (Haeusser et al., 2004). The polymerization of FtsZ is very sensitive to differences in cellular FtsZ concentrations, and even a twofold increase can result in doublet and polar Z-rings (Weart and Levin, 2003). A local increase in the FtsZ concentration in the vicinity of a dynamic and contracting Z-ring could therefore lead to the assembly of a doublet, which could eventually result in the formation of a minicell. Possibly, EzrA represses formation of adja-

Fig. 6. Depletion of SepF in an ezrA background. The effect on Z-ring assembly is presented in the upper panel. B. subtilis strain 4218 (ezrA, Pspac-ylmD-H, Pxy-gfp-ftsZ) was grown in the absence (A, B) or presence of 1 mM IPTG (C, D), for 2 h at 30°C. Arrowheads indicate aberrant Z-rings. The fluorescence images in the middle panel were taken from cells expressing a GFP-Pbp2B fusion (B. subtilis 4219; ezrA, Pspac-ylmD-H, Pxy-gfp-pbp2B). The strain was grown in the absence (E, F) or presence of 1 mM IPTG (G, H), for 2 h at 30°C. Some of the GFP-Pbp2B bands are pointed out by arrows. Membranes were stained with fluorescent membrane dye FM5-95 (B, D, F, H). Scale bars indicate 5 µm. The TEM images in the lower panel show some division septa of B. subtilis 4077 (ezrA, Pspac-ylmD-H) grown in the presence of 1 mM IPTG (I, J, K) or absence of IPTG (L, M, N).

Fig. 7. A model of the different stages leading to septum synthesis. The process is divided in three subsequent steps: Z-ring formation, divisome assembly, and septum synthesis accompanied by constriction of the Z-ring. Grey and black lines indicate cell wall and cytoplasmic membrane respectively. Open circles indicate FtsZ molecules, and the different proteins that constitute the divisome are depicted as open rectangles.
cent Z-rings when septum synthesis is in progress. Thus, EzrA could be most important during the period when SepF is also active. Blocking both processes may therefore be too much to bear for the division machinery.

In conclusion, SepF is a new member of the divisome of Gram positive bacteria. We postulate that it acts as a late division protein, even though it is an early recruit to the Z-ring. We now need to know whether the protein is required for divisome constriction, or plays a more direct role, perhaps in septal peptidoglycan synthesis.

**Experimental procedures**

**General methods and materials**

Molecular cloning, polymerase chain reactions (PCR) and *E. coli* transformations were carried out using standard techniques. *B. subtilis* strains were grown in Difco antibiotic medium 3 (PAB). *E. coli* was used as cloning intermediate. *B. subtilis* chromosomal DNA for transformation and PCR reactions was purified as described by Venema et al. (1965). Transformation of competent *B. subtilis* cells was performed using an optimized two-step starvation procedure based on the method of Anagnostopoulos and Spizizen (Anagnostopoulos and Spizizen, 1961; Hamoen et al., 2002). Selection for *E. coli* and *B. subtilis* transformants was carried out on nutrient agar supplemented with: 100 µg ml⁻¹ ampicillin, 5 µg ml⁻¹ kanamycin, 4 µg ml⁻¹ erythromycin, 5 µg ml⁻¹ chloramphenicol, 50 µg ml⁻¹ spectinomycin, 0.2 µg ml⁻¹ phleomycin or 5 µg ml⁻¹ tetracyclin.

**Strain construction**

The relevant *B. subtilis* strains are listed in Table 1. Primers used for PCR reactions are listed in Table S1. Deletion of the ylmB-H region (*B. subtilis* 3317) was accomplished by double crossover of a kanamycin marker. The upstream region was amplified by PCR using primers IIGA and ylmB2, the downstream region was amplified using primers ylmD1 and ileS2, and the kanamycin marker was amplified using primers Km3 and Km4. As template for PCR reactions, chromosomal DNA from *B. subtilis* 168 was used. The kanamycin marker was amplified from an aph3 containing plasmid. The PCR fragments were digested, ligated, and the ligation product was directly transformed to competent 168 cells. An ezrA deletion (*B. subtilis* 3362) was constructed by double crossover of a tetracycline marker. The upstream region was amplified by PCR using primers nifZ2 and ezrA1, and the downstream region was amplified using primers ezrA2 and ytrP1. The tetracycline marker was cut out of pBEST309, ligated with the upstream and downstream fragments, and the ligation product was directly transformed to competent *B. subtilis* 168 cells. *B. subtilis* 4055 was constructed as follows. The ylmB-C region plus the ylmD promoter region was replaced with the pMUTIN4 derivative pMut by double crossover. pMut is pMUTIN4 without the lacZ gene, and was derived by PCR reaction on pMUTIN4 with primers pmut1 and pmut2. The ylmB-C upstream region was amplified by primers sigE1 and ylmA2, and the downstream region was amplified by primers ylmC1 and ylmF2. The amplified products were digested, ligated and transformed to *B. subtilis* 168. In case of *B. subtilis* 4054 only the ylmB-C region was replaced by double crossover by pMut. For this, the upstream region was amplified by primers sigE1 and ylmA2, and the downstream region was amplified by primers ylmD3 and ylmF2. Insertion deletion of ylmE (*B. subtilis* 4214) and ylmH (*B. subtilis* 4215) were obtained by single crossover of pMut. An internal fragment of ylmE was amplified using primers ylmE8 and ylmE9. pMut was again amplified from pMUTIN4 using primers pmut1 and pmut2. After digestion and ligation the ligation mixture was first transformed to *E. coli*, and plasmid DNA from the correct clone was transformed to *B. subtilis* 168. The same procedure was followed for the construction of the ylmH integration except that pMut was amplified using primers pmut4 and pmut5, and an internal ylmH fragment was obtained using primers ylmH11 and ylmH14. In case of the ylmG deletion (*B. subtilis* 4094), a double crossover was required. The upstream region was amplified by primers ylmD1 and ylmF8, and the downstream region was amplified by primers ylmH15 and ileS10. pMut was again amplified from pMUTIN4 using primers pmut1 and pmut2. The amplified products were digested, ligated and transformed directly to *B. subtilis* 168. All chromosomal integrations were verified by PCR, restriction digestion, and sequencing. For the construction of a sepF-gfp fusion (*B. subtilis* 4181), we made use of the *B. subtilis* GFP-cloning vector pSG1154 (Lewis and Marston, 1999). The sepF open reading frame was amplified using primers ylmF1 and ylmF4. Cloning this fragment into pSG1154 resulted in a C-terminal GFP fusion under control of the xylose-inducible *Pxyl* promoter. After transformation to *B. subtilis* 168, the gene fusion was integrated at the amy locus by double crossover.

**Fluorescence light microscopy**

Cells from overnight cultures grown in PAB were inoculated into fresh PAB. The following *B. subtilis* strains required 1 mM IPTG for growth: 4077, 4216, 4217, 4218 and 4219. *B. subtilis* 4219 also required 0.5% xylose. To induce the sepF-gfp, gfp-ftsZ and gfp-pbp2B fusions, 0.1%, 0.2% and 0.5% xylose was used respectively. Depletion of FtsZ, FtsL, Pbp2B or SepF was achieved by growing exponentially growing cultures for 2 h in the absence of IPTG. For fluorescence microscopy, cultures were grown in PAB at 30°C, samples were taken at exponentially growth, and mounted onto microscope slides coated with a thin layer of 1.5% agarose. Images were acquired with a Zeiss Axiosvert 200M coupled to a CoolsnaphQ CCD camera, and using Metamorph imaging software (Universal Imaging). When required, cells were grown in the presence of the membrane dye FM5-95 (90 µg ml⁻¹, Molecular Probes). For cell length measurements, cells were mixed with the membrane dye Nile Red (2.5 µg ml⁻¹, Molecular Probes), prior to microscopic examinations.

**Electron microscopy**

For transmission electron microscopy (TEM), cultures were grown in PAB at 37°C to mid-exponential phase and fixed by

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the addition of gluteraldehyde to a final concentration of 2.5%. Cells were then pelleted and fixation was continued overnight at 4°C. Cell pellets were washed with 200 mM phosphate buffer, post fixed with 1% osmium tetroxide in 100 mM phosphate buffer, and incubated for 1 h at 4°C. Pellets were then washed in distilled water and stained with 2% aqueous uranyl acetate for 1 h at 4°C in the dark. The fixed cells were washed twice in water, dehydrated in acetone, and washed in propylene dioxide. Samples were imbedded in epon-araldite resin that was allowed to polymerize overnight at 65°C. Sections of 80 nm were cut using an ultracut microtome (Reichert and Jung), and examined with a Zeiss EM 912 OMEGA Electron Microscope.

Northern blotting

Total RNA was isolated from exponentially growing cells using the FastRNA Pro-kit (Qbiogene). For Northern blotting, 8 µg of RNA was denaturated using 10 µl denaturation-mix (10× FA buffer, 37% formaldehyde and formamide; 20:34:100) at 55°C for 15 min. 10× FA buffer contains 200 mM MOPS (pH 7), 50 mM Na-acetate and 10 mM EDTA. RNA was separated on formaldehyde agarose gels (0.5 g agarose, 4 ml 10× FA buffer, 720 µl 37% formaldehyde, in 40 ml), run in 1× FA buffer (100 ml 10× FA buffer and 20 ml 37% formaldehyde, in 1 l), and blotted onto a nylon membrane (Amersham) using 20× SSC blot buffer (3 M NaCl, 0.3 M Na-citrate pH 7). RNA was immobilized at 55°C for 3 h, and hybridized with radioactively labelled probes directed against ylmD, ylmG, ylmH and divIVA. Probes were synthesized using the Prime-a-gene labelling kit (Promega). Blots were hybridized at 67°C and subsequently exposed to a phosphor screen.

Yeast two-hybrid assay

The different proteins that have been tested for interaction in the yeast two-hybrid assay are listed in the Results section. The related open reading frames were PCR amplified from chromosomal DNA, and PCR products were cloned into pGBDU (bait) and pGAD (prey) vectors (James et al., 1996) using the EcoRI and BamHI restriction sites. The resulting vectors were checked by DNA sequencing, and the yeast strains PJ69–4a (bait) and PJ69–4a (prey) (James et al., 1996) were transformed by the bait and prey vectors respectively. Bait-containing cells were selected on SC-U medium (Synthetic Complete lacking uracil), and prey-containing cells were selected on SC-L medium (Synthetic Complete lacking leucine). For each construct, two independent transformants were stored for further mating experiments. Prey- and bait-containing strains were grown on fresh selective plates at 30°C for 48 h, and cells were resuspended in 5 ml YEPD liquid medium. Mating was carried out by mixing 50 µl of prey- and bait-containing cells in 96 well plates, and depositing, with a replicating tool, approximately 3 µl of the cell mixtures (prey + bait) onto YEPD plates, which were incubated at 30°C for 48 h. Cells were transferred onto SC-LU medium (Synthetic Complete lacking leucine and uracil) for diploid selection, and the plates were incubated for 2–3 days at 30°C. Diploid colonies were then transferred onto media selecting for the expression of the HIS3 and ADE2 interaction reporters (SC-LUH and SC-LUA; Synthetic Complete lacking leucine, uracil and histidine or adenine respectively). Interaction phenotypes were scored after 5 days of growth at 30°C.

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References


**Supplementary material**

The following supplementary material is available for this article online:

**Fig. S1.** Comparison of the ylm locus in different Gram positive bacteria. The conserved ftsA, ftsZ and iles genes are also indicated. The *B. subtilis* ylm locus is located approximately 8 kb downstream of ftsZ; divIVA is indicated in black, and the conserved ylm genes (ylmE, -F, -G and -H) are marked with different shades of grey.

**Table S1.** Primers used for PCR reactions.

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