BldC delays entry into development to produce a sustained period of vegetative growth in *Streptomyces venezuelae*

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Abstract

Streptomyces are filamentous bacteria that differentiate by producing spore-bearing reproductive structures called aerial hyphae. The transition from vegetative to reproductive growth is controlled by the bld (bald) loci, and mutations in bld genes prevent the formation of aerial hyphae, either by blocking entry into development (mutations in activators) or by inducing precocious sporulation in the vegetative mycelium (mutations in repressors). One of the bld genes, bldC, encodes a 68-residue protein with a winged Helix-Turn-Helix (wHTH) DNA-binding motif. Here we exploit the benefits of the new model species, Streptomyces venezuelae, which sporulates in liquid culture, to study the biological role of BldC. Using electron microscopy and time-lapse imaging, we show that bldC mutants are bald because they initiate development prematurely, bypassing the formation of aerial hyphae. This correlates with premature expression of BldC target genes, showing that BldC acts as a repressor to sustain vegetative growth and delay entry into development.
Introduction

The complex Streptomyces life cycle involves two distinct filamentous cell forms: the growing or vegetative hyphae and the reproductive or aerial hyphae, which differentiate into long chains of spores (1-5). Genetic studies identified the regulatory loci that control entry into development, which are called bld (bald) genes because null mutations in these loci prevent the formation of aerial hyphae. However, baldness can arise for two different reasons. The larger class of bld mutants, which define positive regulators, fail to initiate development, forming colonies of undifferentiated vegetative mycelium. In contrast, a smaller but growing class of bld mutants, which define negative regulators, enter development prematurely, inducing sporulation in the vegetative mycelium and bypassing the formation of aerial hyphae. Thus, macroscopically these two classes of mutants look similar, forming smooth colonies that lack the ‘hairy’ appearance of the wild type, but microscopically it is apparent that they arise for diametrically opposed reasons (4, 6-8).

bldC is known to encode a short, 68 residue protein related to the DNA-binding domain of MerR-family proteins (9), but there has been less insight into its biological role and impact on Streptomyces development. In part, this is because previous studies have focussed on the classical model species, S. coelicolor, which sporulates only on solid medium. Here we exploit the benefits of the new model species, Streptomyces venezuelae, which sporulates in liquid culture (10), to study the biological role of BldC. We show that bldC mutants are bald because they enter development prematurely, bypassing the formation of aerial hyphae. This correlates with premature expression of BldC target genes, showing that BldC acts as a repressor to sustain vegetative growth and delay entry into development.
Results

Deletion of bldC causes premature initiation of development

We constructed an S. venezuelae bldC mutant by replacing the bldC coding region with an apramycin resistance (apr) cassette. The resulting mutant was bald, unable to produce the reproductive aerial hyphae that give mature wild-type Streptomyces colonies their characteristic fuzzy appearance (Fig 1.). However, scanning electron microscopy (SEM) of mature colonies of the bldC mutant showed that most of the biomass consisted of spores, rather than undifferentiated vegetative hyphae (Fig. 2). Comparison of the growth of the wild type and the bldC mutant on plates over time showed that after 1 day they looked similar (vegetative growth only) but after 2 days the wild type had produced aerial hyphae while the bldC mutant was still restricted to vegetative growth. After 3 days, the aerial hyphae of the wild-type had differentiated into spores, and most of the biomass of the bldC mutant had also differentiated into spores, bypassing aerial mycelium formation. The bldC mutant also seemed to produce higher levels of extracellular matrix than the wild type (Fig. 2). The bldC mutant phenotype was fully complemented by introducing a single copy of the bldC gene under the control of its native promoter, expressed in trans from the ΦBT1 integration site (Figs. 1 and 2).

Using an established microfluidic system and methodology (10), we conducted fluorescence time-lapse microscopy to further study the developmental defects associated with deletion of bldC. As in previous studies (6,10), we introduced an FtsZ-YPet translational fusion into the wild type, mutant and complemented mutant strains, enabling us to monitor each of the two distinct modes of cell division that occur in Streptomyces. In Fig. 3, the scattered single Z-rings mark the position of vegetative cross-walls, which do not constrict or give rise to cell-cell separation, but simply divide the vegetative hyphae into long, box-like compartments (e.g. Figs. 3A + C, panel 2). In contrast, during reproductive growth, long ladders of regularly spaced
Z-rings are synchronously deposited along sporogenic hyphae. These Z-rings mark the sites of sporulation septa, which do constrict, ultimately leading to the formation of chains of spores (e.g. Figs. 3A + C, panels 3 +4). Time-lapse imaging of strains harbouring the FtsZ-YPet fusion showed that the duration of vegetative growth was shorter in the bldC mutant compared to the wild type and the complemented mutant (Fig.3 and Movies S1 A/B, S2 A/B and S3 A/B). Noticeably, following germination, hyphal outgrowth in the bldC mutant was associated with an immediate increase in FtsZ-YPet expression, leading to the precocious formation of ladders of Z-rings (Fig. 3B and Movie S2A/B). However, although ladders of Z-rings were observed as early as 4 hours in the bldC mutant, mature spores were not observed in the corresponding DIC images until 21 hours, the same time mature spores were also seen in the wild type (Figs. 3A and B). Wild-type patterns of FtsZ expression and sporulation were restored in the complemented mutant (Fig. 3C and Movie S3A/B). From these data, we concluded that the overall role of BldC is to sustain vegetative growth and delay entry into development.

BldC levels are highest early in development

Using an anti-BldC polyclonal antibody, we monitored BldC levels in S. venezuelae during sporulation in liquid culture. Western blotting showed that BldC is abundant throughout the life cycle, but that BldC levels are highest early on, during vegetative growth (Figure 4).

BldC represses transcription of its target genes whiI and smeA

ChIP-chip studies in S. coelicolor showed that BldC is a transcription factor and that it has a large regulon of ~280 genes (11). These targets include many genes encoding key transcriptional regulators of the Streptomyces developmental cascade (e.g. bldM, whiB, whiD, whiH, whiI, sigF and bldC itself), as well as others encoding proteins involved in chromosome condensation and segregation during sporulation (e.g. hupS, smeA-sffA). Schumacher et al. (11)
characterised the interaction of *S. coelicolor* BldC with the promoters of two of its targets, *whiI* and the *smeA-ssfA* operon. *whiI* encodes an orphan response-regulator that is essential for the later stages of sporulation, when it forms a functional heterodimer with a second orphan response-regulator, BldM, enabling WhiI to bind to DNA and regulate the expression of ~40 late sporulation genes (12). The *smeA-ssfA* operon encodes a small membrane protein (SmeA) that recruits a DNA translocase (SffA) to sporulation septa (13). Deletion of *smeA-ssfA* results in a defect in spore chromosome segregation and has pleiotropic effects on spore maturation (13).

BldC directly regulates the *whiI* and *smeA* promoters in *S. coelicolor* (11) and ChIP-chip analysis confirmed that they are also BldC targets in *S. venezuelae* (Table S1, Fig. 5). To assess the regulatory influence of BldC on the *whiI* and *smeA* promoters, we performed qRT-PCR using RNA prepared from both wild-type *S. venezuelae* and the *bldC* mutant at time points throughout differentiation in liquid culture (Fig. 6). In the wild type, expression of both BldC targets comes on in the 14-h time point. In contrast, in the *bldC* mutant, expression of both *whiI* and *smeA* is on in the 8- and 10-h time points. We conclude that BldC functions to repress the transcription of these target genes during early vegetative growth.

**Discussion**

Canonical *bld* mutations block entry into development and so the resulting colonies do not form aerial hyphae and spores. Such mutations typically define positive regulators such as the response regulator BldM (12) or the sigma factor BldN (14). In contrast, we have shown that *S. venezuelae bldC* mutants are bald because they enter development prematurely, bypassing the formation of aerial hyphae, and that this correlates with premature expression of BldC target genes like *whiI* and *smeA*. Thus, BldC functions as a repressor to sustain vegetative growth and...
delay entry into development. As such, BldC joins a growing class of Bld regulators known to
function as a developmental “brake”.

BldD was the first Bld regulator of this alternative class to be clearly recognized. BldD sits at
the top of the developmental cascade and represses a large regulon of ~170 sporulation genes
during vegetative growth. BldD activity is controlled by the second messenger c-di-GMP,
which mediates dimerization of two BldD protomers to generate a functional repressor. In this
way, c-di-GMP signals through BldD to repress expression of the BldD regulon, extending
vegetative growth and inhibiting entry into development (4, 8, 15). Because it is a BldD-(c-di-
GMP) complex that represses the BldD regulon and not BldD alone, engineering the
degradation of c-di-GMP in vivo also causes a precocious hypersporulation phenotype like that
of a bldD null mutant (8).

More recently, bldO was identified as a second member of this emerging class of bld mutant
(6-7). In contrast to BldD and BldC, which both control large regulons, BldO functions to
repress a single developmental gene, whiB. The precocious hypersporulation phenotype of the
bldO mutant arises from premature expression of whiB, and in line with this, constitutive
expression of whiB alone is sufficient to induce precocious hypersporulation in wild-type S.
venezuelae (6). WhiA and WhiB act together to co-control the same set of promoters to initiate
developmental cell division in Streptomyces (16-17). WhiA is constitutively present
throughout the life cycle, but it only binds to its target promoters at the onset of sporulation
(16). This is because WhiA and WhiB function cooperatively and in vivo DNA binding by
WhiA depends on WhiB, and vice versa (17). As a consequence, the regulation of whiB
expression is key in controlling the switch between hyphal growth and sporulation. This critical
role for WhiB is reflected in the extensive developmental regulation to which whiB
transcription is subject, being directly repressed by BldC (11), BldD (18) and BldO (6), and
directly activated by BldM (12).
Materials and Methods

Construction and complementation of an *S. venezuelae* bldC null mutant. Using ‘Redirect’ PCR targeting (19-20), bldC mutants were generated in which the coding region was replaced with a single apramycin resistance (*apr*) cassette. A cosmid library that covers > 98% of the *S. venezuelae* genome (M.J. Bibb and M.J. Buttner, unpublished) is fully documented at http://strepdb.streptomyces.org.uk/. Cosmid 4O24 was introduced into *E. coli* BW25113 containing pIJ790 and the bldC gene (*sven3846*) was replaced with the *apr*-oriT cassette amplified from pIJ773 using the primer pairs bldCdis_F and bldCdis_R. The resulting disrupted cosmids were confirmed by restriction digestion and by PCR analysis using the flanking primers bldCcon_F and bldCcon_R, and introduced into *S. venezuelae* by conjugation (Keiser *et al.*, 2000). Null mutant derivatives, generated by double crossing over, were identified by their apramycin-resistant, kanamycin-sensitive and morphological phenotypes, and their chromosomal structures were confirmed by PCR analysis using the flanking primers bldCcon_F and bldCcon_R. A representative bldC null mutant was designated SV25. For complementation, bldC was amplified with the primers bldCcomp_F and bldCcomp_R, generating an 846bp fragment carrying the coding sequence and the bldC promoter, and cloned into HindIII-KpnI/Asp718 cut pIJ10770 to create pIJ10618. The plasmid was introduced into the bldC mutant by conjugation and fully complemented all aspects of the mutant phenotype.

Time-lapse imaging of *S. venezuelae*. Fluorescent time-lapse imaging was conducted essentially as described previously (6,11). Before imaging, fresh *S. venezuelae* spores for each of the strains imaged were first prepared by inoculating 30 ml cultures of MYM with 10 µl of the appropriate spore stock or 20 µl of the appropriate mycelial culture. Cells were cultured at 30 ºC and 250 rpm until fully differentiated (16-24 h for hypersporulating strains, otherwise 36-40 h). 1 ml of each culture was spun briefly to pellet mycelium, the supernatant spores were
diluted 1:50 in fresh MYM, and 50 µl was transferred to the cell loading well of a prepared B04A microfluidic plate (Merck-Millipore). The remaining culture was filter-sterilised to obtain spent MYM that was free of spores and mycelial fragments. The ONIX manifold was then sealed to the B04A plate before transferring to the environmental chamber, pre-incubated at 30 ºC. Spores were loaded onto the B04A plate, at 4 psi for 15 seconds using the ONIX microfluidic perfusion system. Fresh MYM medium was set to flow at 2 psi during the first 3 hours during germination, before the 2-psi flow of spent MYM medium for the remainder of the experiment. The system was left to equilibrate for 1 h prior to imaging.

Imaging was conducted using a Zeiss Axio Observer.Z1 widefield microscope equipped with a sCMOS camera (Hamamatsu Orca FLASH 4), a metal-halide lamp (HXP 120V), a hardware autofocus (Definitive Focus), a 96-well stage insert, an environmental chamber, a 100x 1.46 NA Oil DIC objective and the Zeiss 46 HE shift free (excitation 500/25 nm, emission 535/30 nm) filter set. DIC images were captured with a 150 ms exposure time, YFP images were captured with a 100 ms exposure time. Images were taken every 30 min. In all experiments, multiple x/y positions were imaged for each strain and in each experiment. Representative images were transferred to the Fiji software package (http://fiji.sc/Fiji), manipulated and converted into the movie files presented here, as described previously (Schlimpert et al., 2016).

**Chromatin immunoprecipitation-microarray (ChIP-chip) analysis.** To carry out the ChIP-chip experiments, cultures of *S. venezuelae* and the congenic *bldC* null mutant strain SV25 were grown for 12 h in MYM liquid medium. Formaldehyde was added to cultures at a final concentration of 1% (v/v) and incubation was continued for 30 min. Glycine was then added to a final concentration of 125 mM to stop the cross-linking. Cultures were left at room temperature (RT) for 5 min before the mycelium was harvested and washed twice in PBS buffer.
pH 7.4. Each mycelial pellet was resuspended in 0.5 ml lysis buffer (10 mM Tris HCl pH 8.0, 50 mM NaCl) containing 15 mg/ml lysozyme and protease inhibitor (Roche Applied Science) and incubated at 25 °C for 1 h. An equal volume of IP buffer (100 mM Tris HCl pH 8, 250 mM NaCl, 0.5% Triton-X-100, 0.1% SDS) containing protease inhibitor was added and samples were chilled on ice. Samples were sonicated for 7 cycles of 15 s each at 10 microns to shear the chromosomal DNA into fragments ranging from 300-1000 bp in size. Samples were centrifuged twice at 13,000 rpm at 4 °C for 15 min to clear the cell extract, after which 10 µl of cell extract was set aside for total DNA extraction. The remainder was incubated with 10% (v/v) protein A-sepharose (Sigma) for 1 h on a rotating wheel to remove non-specifically binding proteins. Samples were then centrifuged for 15 min at 4°C and 13,000 rpm to remove the beads. Supernatants were incubated with 10% (v/v) anti-BldC antibody (9) overnight at 4 °C with rotation. Subsequently, 10% (v/v) protein A-sepharose was added to precipitate BldC and incubation was continued for 4 h. Samples were centrifuged at 3500 rpm for 5 min and the pellets were washed four times with 0.5x IP buffer. Each pellet was incubated overnight at 65 °C in 150 µl IP elution buffer (50 mM Tris HCl pH 7.6, 10 mM EDTA, 1% SDS) to reverse cross-links, and 10 µl of the total cell extract control was treated in the same way. Samples were centrifuged at 13,000 rpm for 5 min to remove the beads. Each pellet was re-extracted with 50 µl TE buffer (10 mM Tris HCl pH 7.4, 1 mM EDTA) and the supernatant incubated with 0.2 mg/ml Proteinase K (Roche) for 2 h at 55°C. The resulting samples were extracted with phenol-chloroform and further purified using QiaQuick columns, eluting in 50 µl EB buffer (Qiagen). DNA labelling, hybridization to DNA microarrays and data processing were carried out as described previously (10).

**qRT-PCR.** Mycelial pellets from MYM cultures were washed in PBS and resuspended in 900 µl lysis solution (400 µl phenol [pH4.3], 100 µl chlorophorm:isoamyl alcohol (24 : 1) and 400
µl RLT buffer [Qiagen]) with lysing matrix B (MP Biomedicals) and homogenised using a FastPrep FP120 Cell Disruptor (Thermo Savant). Two pulses of 30 s of intensity 6.0 were applied with cooling down for 1 min on ice between pulses. Supernatants were centrifuged for 15 min, full-speed on a bench-top centrifuge at 4°C and then treated according to the instructions given in the RNEasy Kit (Qiagen). The RNA samples were treated with on-column DNase I (Qiagen), followed by an additional DNase I treatment (Turbo DNA-free, Ambion) until they were free of DNA contamination (determined by PCR amplification of hrdB). RNA was quantified and equal amounts (350 ng) of total RNA from each sample was converted to cDNA using SuperScript II reverse transcriptase and random primers (Invitrogen). cDNA was then used as template in qRT-PCR performed using the SensiFAST SYBR No-ROX kit (Bioline). Three technical replicates were used for each gene. Specific qPCR primers (Table S1, final concentration of 250 nM) were used to amplify the whiI target gene (whiIqRT_F and whiIqRT_R), the smeA target gene (smeAqRT_F and smeAqRT_R) the hrdB reference gene (hrdBqRT_F and hrdBqRT_R). To normalize for differing primer efficiency, a standard curve was constructed using chromosomal DNA. Melting curve analysis was used to confirm the production of a specific single product from each primer pair. qRT-PCR was performed using a CFX96 Touch instrument using hardshell white PCR plates (BioRad), sealed with thermostable film covers (Thermo). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: 95°C, 3 min, then 45 cycles at 95°C 5 sec, 60°C 10 sec and 72°C 7 sec. Melting curves were generated at 65 to 95°C with 0.5°C increments. The BioRad CFX manager software was used to calculate starting quantity (SQ) values for smeA and whiI at each time point. These values were divided by the mean SQ value derived from the hrdB reference at the corresponding time points, generating a value for relative expression. The resulting values were normalised against the mean relative expression of the wild type at 8 hours, which was set to 1.
Western Blotting. Samples of frozen mycelium, originating from 2 ml liquid MYM samples, were resuspended in 0.4 ml ice-cold sonication buffer [20 mM Tris pH 8.0, 5 mM EDTA, 1 x EDTA-free protease inhibitors (Roche)] and sonicated (5x 15 sec on/15 sec off) at 4.5 micron amplitude. Lysates were then centrifuged at 16,000 xg for 15 min at 4°C to remove cell debris. Total protein concentration was determined using the Bradford assay (Biorad). 1 µg of total protein from each time point was loaded in triplicate into a microplate (Proteinsimple #043-165) and anti-bldC antibody (9) diluted 1:200. BldC levels, originating from the wild type strain and the bldC mutant negative control were then assayed using the automated Western blotting machine Wes (ProteinSimple, San Jose, CA), according to the manufacturer’s guidelines.

Scanning electron microscopy. Colonies were mounted on the surface of an aluminum stub with optimal cutting temperature compound (Agar Scientific Ltd, Essex, UK), plunged into liquid nitrogen slush at approximately -210°C to cryopreserve the material, and transferred to the cryostage of an Alto 2500 cryotransfer system (Gatan, Oxford, England) attached to a FEI NanoSEM 450 field emission gun scanning electron microscope (FEI Ltd, Eindhoven, The Netherlands). The surface frost was sublimated at -95°C for 3½ mins before the sample was sputter coated with platinum for 2 min at 10 mA at below -110°C. Finally, the sample was moved onto the cryostage in the main chamber of the microscope, held at approximately -130°C, and viewed at 3 kV.
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**Figure Legends**

**FIG 1.** BldC is required for the formation of aerial mycelium.

The phenotypes of wild-type *S. venezuelae*, the *bldC* mutant, the *bldC* mutant carrying the empty vector, and the complemented *bldC* mutant, photographed after four days of growth on MYM solid medium.

**FIG 2.** Deletion of *bldC* causes premature initiation of development on solid medium.

Scanning electron micrographs showing the phenotype of the *bldC* mutant, compared to wild-type after 1 days, 2 days and 3 days of growth on MYM solid medium. The phenotype of the complemented *bldC* mutant is also shown after 3 days of growth on MYM solid medium.

**FIG 3.** Deletion of *bldC* causes premature initiation of development in liquid medium.

Time-lapse images (4, 7, 12 and 21 h post-inoculation) of (A) wild-type *S. venezuelae*, (B) the *bldC* mutant and (C) the complemented *bldC* mutant, grown in liquid MYM medium in the microfluidic system. All three strains carry the same FtsZ-YPet translational fusion expressed from the native ftsZ promoter, and both the DIC (upper) and fluorescence (lower) images are shown. Scale Bar = 10µm. For the corresponding movies, please see Supporting Information Movies S1A/B, S2A/B and S3 A/B.

**FIG 4.** Automated Western blot analysis of BldC.

Equal amounts (1 µg) of total protein were loaded for each sample and BldC was detected with polyclonal antibody (9) using the quantitative ‘Wes’ capillary electrophoresis and blotting system (ProteinSimple – San Jose, CA). The *S. venezuelae bldC* mutant was used as a negative control. Both the wild-type and *bldC* mutant were grown in MYM liquid medium. (A) quantitation of BldO levels (area under each peak; arbitrary units). (B) virtual Western blot.
All experimental samples were analysed in triplicate and the mean value and its Standard Error are shown for each sample. Each time-point is indicated in hours, along with its relation to the developmental stage (V = vegetative growth; F = fragmentation; S = sporulation), as determined by microscopy. Cultures used to analyse BldC levels were identical to those used to prepare RNA prior to qRT-PCR analysis (Fig. 6).

**FIG 5.** BldC regulates the expression of many genes in *S. venezuelae*.

(A) Genome-wide distribution of BldC binding sites identified by ChIP-chip analysis using anti-BldC polyclonal antibody, conducted during vegetative growth (12 hr) in the wild type. DNA obtained from immunoprecipitation of BldC was labelled with Cy3 and hybridized to DNA microarrays together with a total DNA control labelled with Cy5. Data are plotted as Cy3/Cy5 ratios (y-axis), as a function of chromosome location (x-axis).

(B) ChIP-chip data for *whiI* and *smeA* in wild-type *S. venezuelae* and the *S. venezuelae ΔbldC* mutant (blue and red dots, respectively). Plots span approximately 8 kb of DNA sequence. Genes running left to right are shown in green, and genes running right to left are shown in red. The black arrow indicates the gene subject to BldC regulation.

**FIG 6.** BldC functions to repress transcription. *whiI* (A) and *smeA* (B) mRNA abundance determined by qRT-PCR in the wild type (white bars) and the bldC mutant (black bars) throughout development. Strains were grown in MYM liquid medium. Expression values were calculated relative to the accumulation of the constitutively expressed *hrdB* reference mRNA and normalised to the wild-type value at 8 h. Cultures used to prepare RNA prior to qRT-PCR analysis were identical to those used to analyse BldC levels (Fig. 4).
FIG 1

ΔbldC::apr
attB
ΔbldC::apr
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ΔbldC::apr
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Supplemental Material

Table S1. ChIP-chip data set for *S. venezuelae* BldC. Each row represents an enriched probe (probeID) with the mid-position (midpos) of each probe on the *S. coelicolor* genome recorded. Enrichment ratios are expressed as the log-fold change (logFC). Probes are listed in order of significance (adjusted p value - adj.P.Val). For each probe, the nearest gene to the left and right is identified (left/rightLocusTag), its distance to the midpos of the probe (left/rightDistance), whether the gene is on the forward (1) or reverse (-1) strand (inStrand) and the predicted function (left/rightProduct) based on annotation in strepdbname (http://strepdb.streptomycetes.org.uk). If the midpos of a probe falls within a gene, its gene identifier (inLocusTag), distance to the probe (inDistance), whether the gene is on the forward (1) or reverse (-1) strand (inStrand) and predicted function (inProduct) is also listed.

Table S2. Strains, Plasmids and Oligonucleotide primers used in this study

Movie S1. Time-lapse microscopy of the wild type strain carrying the FtsZ-YPet fusion. DIC (A) and YFP-channel (B) movies are at 5 frames per second. The time following the first image is indicated at the bottom left. Images were taken every 30 minutes (DIC 150 ms; YFP 100 ms). Movies were assembled in the Fiji software package (http://fiji.sc/Fiji).

Movie S2. Time-lapse microscopy of the *bldC* mutant carrying the FtsZ-YPet fusion. DIC (A) and YFP-channel (B) movies are at 5 frames per second. The time following the first image is indicated at the bottom left. Images were taken every 30 minutes (DIC 150 ms; YFP 100 ms). Movies were assembled in the Fiji software package (http://fiji.sc/Fiji).
**Movie S3.** Time-lapse microscopy of the complemented strain carrying the FtsZ-YPet fusion.

DIC (A) and YFP-channel (B) movies are at 5 frames per second. The time following the first image is indicated at the bottom left. Images were taken every 30 minutes (DIC 150 ms; YFP 100 ms). Movies were assembled in the Fiji software package ([http://fiji.sc/Fiji](http://fiji.sc/Fiji)).