Antibacterial activity of alkyl gallates is a combination of direct targeting of FtsZ and permeabilization of bacterial membranes

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Front Microbiol. 2015 Apr 29;6:390

Experiments presented in Fig 1, Fig 6, Fig 7 and Fig S1 were performed by A. de Sousa Borges.

Król et al. has been previously published in the thesis of Ewa Król (2016).
ABSTRACT

Alkyl gallates are compounds with reported antibacterial activity. One of the modes of action is binding of the alkyl gallates to the bacterial membrane and interference with membrane integrity. However, alkyl gallates also cause cell elongation and disruption of cell division in the important plant pathogen *Xanthomonas citri* subsp. *citri*, suggesting that cell division proteins may be targeted by alkyl gallates. Here, we use *Bacillus subtilis* and purified *B. subtilis* FtsZ to demonstrate that FtsZ is a direct target of alkyl gallates. Alkyl gallates disrupt the FtsZ-ring *in vivo*, and cause cell elongation. *In vitro*, alkyl gallates bind with high affinity to FtsZ, causing it to cluster and lose its capacity to polymerize. The activities of a homologous series of alkyl gallates with alkyl side chain lengths ranging from five to eight carbons (C₅-C₈) were compared and heptyl gallate was found to be the most potent FtsZ inhibitor. Next to the direct effect on FtsZ, alkyl gallates also target *B. subtilis* membrane integrity – however the observed anti-FtsZ activity is not a secondary effect of the disruption of membrane integrity. We propose that both modes of action, membrane disruption and anti-FtsZ activity, contribute to the antibacterial activity of the alkyl gallates. We propose that heptyl gallate is a promising hit for the further development of antibacterial compounds that specifically target FtsZ.
INTRODUCTION

The use of plants as sources of antimicrobial agents has a long history (1). One group of plant compounds known for their antimicrobial activities are alkyl gallates, esters of gallic acid, a main product of tannin hydrolysis. Alkyl gallates have been shown to exhibit a broad spectrum of antibacterial activities against both Gram-positive and Gram-negative bacteria, including foodborne Salmonella (2), Methicillin Resistant Staphylococcus aureus (MRSA) (3, 4), Bacillus subtilis (5), the plant pathogen Xanthomonas citri subsp. citri (6), and various others (2, 3, 5). Alkyl gallates with varying alkyl side chain lengths (C1-C14), have been studied as antibacterial agents alone or as modulators of the activities of β-lactams against MRSA (2-7), a common cause of bloodstream infections in hospitals and healthcare facilities worldwide. The hydrolysis of alkyl gallates produces gallic acid and the corresponding alcohols (or alkanols), which both are common components in many plants.

Although the alkyl gallates have a head-and-tail structure similar to alkanols, suggesting that their antibacterial mode of action may be as surface-active agents affecting membrane integrity (3, 8), Kubo et al. proposed that their antimicrobial activity is unlikely to be due to their surfactant property (2, 3, 5, 7). Recently, we showed that alkyl gallates are active against X. citri subsp citri (Xac), an important plant pathogen that is the causative agent of citrus canker, one of the most damaging infections in citriculture. Pentyl, hexyl, heptyl and octyl gallate treatment resulted in elongated Xac cells and disruption of the cell division machinery in this bacterium (6). Octyl gallate has been reported to exhibit bactericidal activity only against dividing and exponentially growing cells of B. subtilis but did not affect the viability of cells in the stationary phase (5). Taken together, these results indicate that alkyl gallates may affect functions associated with cell division in Gram-positive and Gram-negative bacteria (5, 6).

Cell division is a relatively novel target for antibacterial drugs (9-11). Division is an essential process, which starts with the polymerization of the highly conserved cytoplasmic protein FtsZ in the middle of the cell leading to the formation of the so-called Z-ring (12, 13). After assembly of the Z-ring, several other proteins are recruited to mid-cell, resulting in a complex called the divisome, which carries out cell division at the correct time and place in the cell. Formation of the divisome depends on the assembly of FtsZ. FtsZ belongs to the tubulin family of cytoskeletal GTPases. The binding of GTP to FtsZ promotes the assembly of FtsZ monomers
into long filaments \textit{in vitro} \cite{10}. FtsZ is conserved among bacteria and is essential for cell viability, making it a potential target for new antibiotic discovery \cite{10,11}. Several natural, synthetic and semi-synthetic compounds were identified as inhibitors of FtsZ from Gram-positive and Gram-negative bacteria \cite{11,14-19}.

To establish whether alkyl gallates indeed target bacterial cell division, we characterized the mode of action of alkyl gallates with a side chain length ranging from five to eight carbons (Table 1) in more detail, using \textit{B. subtilis} as a model. We show that \textit{B. subtilis} FtsZ is a target for these esters and that some of these compounds bind FtsZ with high affinity, resulting in protein cluster formation and disruption of FtsZ structures \textit{in vitro} and \textit{in vivo}. Additionally, the alkyl gallates interfere with the stability of the cell membrane. FtsZ binding and inhibition and membrane integrity are differently affected based on the alkyl chain length. Our results indicate that alkyl gallate with a C\textsubscript{7} side chain is the best hit for the further development of a FtsZ specific antibacterial with minimal effects on overall membrane integrity.

**MATERIALS AND METHODS**

**General**

DNA manipulations including molecular cloning in \textit{Escherichia coli} DH5\textsubscript{α}, PCR, DNA sequencing, restriction, ligation, and transformation were performed using

![Image](image_url)

\textbf{Table 1.} Structures of the alkyl gallates and their activity against \textit{B. subtilis} 168

<table>
<thead>
<tr>
<th>C\textsubscript{2}H\textsubscript{2}O\textsubscript{5}\textsubscript{-R}</th>
<th>R</th>
<th>MIC\textsubscript{90}\textsuperscript{a} (µg/ml)</th>
<th>MIC\textsubscript{50}\textsuperscript{a} (µg/ml)</th>
<th>MIC\textsubscript{24h}\textsuperscript{b} (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>Compound 8</td>
<td>pentyl gallate</td>
<td>(CH\textsubscript{2})\textsubscript{4}CH\textsubscript{3}</td>
<td>235</td>
<td>141</td>
</tr>
<tr>
<td>Compound 9</td>
<td>hexyl gallate</td>
<td>(CH\textsubscript{2})\textsubscript{5}CH\textsubscript{3}</td>
<td>90</td>
<td>65</td>
</tr>
<tr>
<td>Compound 10</td>
<td>heptyl gallate</td>
<td>(CH\textsubscript{2})\textsubscript{6}CH\textsubscript{3}</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Compound 11</td>
<td>octyl gallate</td>
<td>(CH\textsubscript{2})\textsubscript{7}CH\textsubscript{3}</td>
<td>105</td>
<td>75</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined by the REMA assay
\textsuperscript{b} Determined as minimum concentration that shows no growth after 24 hours in a 2-fold dilution series.
standard methods (20). Restriction enzymes, T4 DNA Ligase and Phusion DNA polymerase were used as specified by the supplier (Fermentas). Both E. coli and B. subtilis were grown at 37°C on solid medium (LB Lennox plus agar 1.5% w/v) (21), and liquid medium (LB Lennox). When appropriate, ampicillin and spectinomycin were added to final concentrations of 100 and 50 µg/ml, respectively. Starch (Sigma Aldrich) was used at 0.1%. Primers are listed in Table 2. Plasmids and strains are listed in Table 3. B. subtilis 168 genomic DNA was isolated using the Wizard genomic DNA kit (Promega) according to the suppliers’ instructions.

**Plasmid and strain construction**

To generate vector pDJ108, a PCR product containing the coding sequence of *eyfp* (enhanced Yellow Fluorescent Protein) was amplified from vector pMK13, using primer AB7/AB55 (Table 2). The PCR product was sequenced and subsequently digested with *BamHI/NheI*. *ftsZ* was amplified from the genomic DNA of *B. subtilis* 168 using primer AB10/AB56 (Table 2). The PCR product was sequenced and subsequently digested with *EcoRI/BamHI*, and ligated with the digested *eyfp* PCR product and *EcoRI/NheI* digested pDOW01 resulting in plasmid pDJ108, which was verified by sequencing. Subsequently, *B. subtilis* 168 was transformed with pDJ108, and correct integration at the *amyE* locus was verified on starch plates (23). A positive colony was selected, expression and localization of the fusion protein FtsZ-eYFP was verified by microscopy, resulting in strain 4055.

**Table 2. Primers used for cloning**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’−3’)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>A87</td>
<td>GCAGCGCTAGCATTACTTGTACAGCTCGTC CATGCGCAAG</td>
<td>reverse primer for <em>eyfp</em> with <em>NheI</em> site</td>
</tr>
<tr>
<td>A810</td>
<td>GCCGCAAGAATTCAATGTGGAGTTGAAAC</td>
<td>forward primer for <em>ftsZ</em> with <em>EcoRI</em> site</td>
</tr>
<tr>
<td>A855</td>
<td>TAGCATGGATCCGGCCGGCGCGCTCCGGGT GGTGGTGGTCAGCAGCAG ACTTACGAGG</td>
<td>forward primer for <em>eyfp</em> with flexible linker sequence and <em>BamHI</em> site</td>
</tr>
<tr>
<td>A856</td>
<td>TAGCATGGATCCGGCGCTTTATACGGTTTC</td>
<td>reverse primer for <em>ftsZ</em> with <em>BamHI</em> site</td>
</tr>
</tbody>
</table>

Restriction sites used to clone are in bold. Initiation or termination codons are underlined.
Alkyl Gallates (Compounds 8–10)

Alkyl gallates with side chains varying from five to eight carbons [pentyl (compound 8), hexyl (compound 9), heptyl (compound 10) and octyl (compound 11) gallates] were synthetized as described (6). Compound numbers were chosen to keep in line with our previous report (6).

MIC assays

REMA - The antibacterial activity of alkyl gallates was tested by the standard resazurin microtiter assay (REMA) plate method with some modifications (6, 24). Briefly, an overnight culture of *B. subtilis* was grown in 5 ml LB medium. The overnight culture was diluted with LB medium and distributed into a 96-well microtiter plate to a final volume per well of 100 μl (10^5 CFU/well). The alkyl gallates were serially diluted with LB (1000 μg/ml to 15 μg/ml), and 100 μl dilutions were added to the 96-well microtiter plate. Kanamycin (50 μg/ml) was used as control antibiotic. The plate was subsequently incubated at 37°C for 4 hours. After 4 hours, 15 μl of a 0.01% (w/v) freshly prepared resazurin solution in H2O was added to each well and the plate was incubated for 1 hour. Subsequently, the fluorescence in each well was measured in a Synergy Mix.
Microplate Reader (BioTek), with excitation and emission wavelengths at 530 nm and 590 nm, respectively. All the experiments were done in triplicate and MIC$_{50}$ and MIC$_{90}$ values were calculated using a nonlinear regression approach (6).

**Dilution** – Cells from an exponentially growing *B. subtilis* culture were inoculated at OD$_{600}$ of 0.2 in LB medium, at 37°C, with alkyl gallates in 2-fold dilution series. After 24 hours, the lowest alkyl gallate concentration at which no visible growth occurred was scored as MIC$_{24h}$.

**General microscopy analysis**

Cells were resuspended in small volumes of CH medium (25) or Phosphate Buffered Saline (PBS, 58 mM Na$_2$HPO$_4$; 17 mM NaH$_2$PO$_4$; 68 mM NaCl, pH 7.3), and mounted on an agarose pad (1% w/v in PBS). Cells were imaged using a Nikon Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with a Hamamatsu Orca Flash4.0 camera. Image analysis was performed using the software packages ImageJ (http://rsb.info.nih.gov/ij/) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA).

**In vivo Z-ring analysis**

An overnight culture of *B. subtilis* 4055 grown in LB with spectinomycin at 37°C was diluted 1:100 into fresh LB with 0.02 mM IPTG to express *ftsZ-eyfp*. When the culture reached an OD$_{600}$ of 0.4, 1 ml culture was centrifuged (1 min, 22,000 x g), resuspended in 100 µl LB, and incubated at 37°C in the presence of either dimethyl sulfoxide (DMSO, 2% v/v), nisin (1.5 µg/ml), carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 0.2 mM) or alkyl gallates at MIC$_{50}$ concentrations (see Table 3). After either 2 min or 15 min of incubation, the cells were collected (1 min., 22,000 x g), resuspended in 50 µl of PBS and mounted on agarose pads for microscopy analysis.

**Brightfield microscopy**

An overnight culture of *B. subtilis* 168 grown in LB at 37°C was diluted 1:100 into fresh LB until early exponential phase, OD$_{600}$ of 0.3. 3 ml samples of the culture were taken and incubated at 37°C in the presence of 2 µg/ml 3-((6-chlorothiazolo[5,4-b]pyridin-2-yl)methoxy)-2,6-difluorobenzamide (PC190723, Merck), and alkyl gallates at various concentrations. After 1h, 2h or 3h of continued growth in the presence of the compounds, a sample of 1 ml
was collected (1 min, 22,000 x g) and cells were fixed by the addition of 1 ml of 8% formaldehyde and incubation at 23°C for 1 hour. Subsequent to fixation, cells were washed twice in PBS and resuspended in 10 to 30 µl of PBS before mounting on agarose pads for microscopy analysis.

Membrane permeability assay

An overnight culture of *B. subtilis* 168 grown in LB was diluted 1:100 into fresh LB and grown at 37°C until exponential phase, OD$_{600}$ of 0.5. A sample of 1 ml was collected and cells were resuspended in 50 µl of CH medium (25). Membrane integrity was assessed using the commercial assay Live/Dead BacLight bacterial viability kit (Invitrogen) for microscopy, according to the manufacturer's instructions. The dyes propidium iodide (20 mM) and SYTO 9 (3.34 mM) were combined in equal amounts and 0.15 µl of the dye mixture was used to stain the DNA of cells resuspended in 50 µl of CH medium. Cells were incubated for 15 min. at 23°C in the presence of DMSO (2%), Nisin (2.5µg/ml), CCCP (0.2 mM) and alkyl gallates at MIC$_{50}$ and MIC$_{90}$ (see Table 3). After incubation, cells were mounted on agarose pads for microscopy analysis and the green and red fluorescence were imaged. In total, two independent experiments were performed. Per experiment, more than 250 cells for each condition were scored for green (intact membrane) or red (permeabilized membrane) fluorescence. The values were converted into percentages and the mean and standard deviation was plotted on a graph using Excel.

Protein expression and purification

*B. subtilis* FtsZ was expressed and purified using the ammonium sulfate precipitation method as described before (26, 27). His-EzrA$_{cyt}$ was expressed as described (22). For purification, pellet from one liter of culture was resuspended in 20 ml of Buffer A (50 mM tris(hydroxymethyl)aminomethane [Tris]/HCl pH=7.5, 250 mM NaCl, 10 mM imidazole) supplemented with a EDTA (ethylenediaminetetraacetic acid)-free protease inhibitor tablet (Roche). The cells were disrupted at 18 kpsi (Constant systems OneShot disruptor, LA biosystems) and the insoluble fraction was removed by centrifugation at 7,000 x g for 20 minutes at 4°C. Supernatant was applied onto Ni-NTA Agarose resin (Qiagen). The resin was washed with Buffer A containing 25 mM imidazole and His-EzrA$_{cyt}$ protein was eluted with the same buffer containing 300 mM imidazole. Samples
were dialyzed and stored at -80°C in a buffer containing 20 mM Tris/HCl, pH=7.5, 250 mM NaCl, 10% glycerol.

**GTPase assay**

The FtsZ GTP hydrolysis rate was determined using the malachite green phosphate assay described in (26) with the following modifications. Two fold concentrated stocks of alkyl gallates with FtsZ were prepared in polymerization buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [Hepes]/NaOH, pH=7.5; 300 mM KCl; 0.02% Triton X-100) and incubated for 5 minutes at 30°C. After that, 2 mM GTP dissolved in 50 mM Hepes/NaOH, pH=7.5; 300 mM KCl was added. The final concentrations of GTP and Triton X-100 in the sample were 1 mM and 0.01%, respectively.

**Binding of alkyl gallates to FtsZ**

Binding of alkyl gallates to FtsZ was assessed by monitoring the increase of alkyl gallate fluorescence upon binding to protein, analogous to (8). Alkyl gallates at constant concentration were incubated with increasing amounts of FtsZ (0.6-14.4 µM). After addition of protein, the solution was incubated for 3 minutes at room temperature to allow equilibration of binding. Fluorescence was excited at 271 nm and emission spectra (320-450 nm) were acquired in a QuantaMaster™ spectrofluorometer controlled by the FelixGX program (Photon Technology International, Inc.). The fluorescence spectra of corresponding blanks resulting from titration of FtsZ into buffer alone were recorded and subtracted from the respective data sets. All measurements were done in polymerization buffer: 50 mM Hepes/NaOH pH=7.5, 50 mM KCl in the absence of GTP. The change in fluorescence of alkyl gallates upon binding to FtsZ was used to determine the dissociation constant (K_d) of the interaction between the compounds and FtsZ as described in (28). The dissociation constant was determined using MATLAB (The MathWorks, Inc., Massachusetts, U.S.A.).

**FtsZ sedimentation assays**

FtsZ (10 µM) was mixed with the alkyl gallates or an equal volume of DMSO as control at 50 or 100 µg/ml in the polymerization buffer (50 mM Hepes/ NaOH, pH=7.5, 50 mM KCl) supplemented with 10 mM MgCl₂. After incubation for 5 minutes at 30°C, an equal volume of GTP, GDP or polymerization buffer was
added (final concentration of nucleotides 2 mM). The samples were incubated for another 20 minutes and centrifuged at 186,000 x g or at 350,000 x g when indicated for 10 minutes at 25°C. Pellet and supernatant fractions were analyzed by sodium dodecyl sulfate - poly-acrylamide gel electrophoresis (SDS-PAGE) as described (26). FtsZ interacting protein His-EzrA<sub>cyt</sub> and bovine serum albumin (BSA) (both at 10 µM) were used as controls.

**Electron Microscopy**

Electron microscopy samples were prepared essentially as described for the sedimentation assays. FtsZ (10 µM) was mixed with alkyl gallates at 50 µg/ml in polymerization buffer (50 mM Hepes/ NaOH, pH=7.5, 50 mM KCl, 10 mM MgCl<sub>2</sub>), incubated for 5 minutes at 30°C with shaking (300 rpm), after which GTP was added to a final concentration of 2 mM. The samples were incubated at 30°C for another 20 minutes and the polymerization mixture was applied to an electron microscopy grid as described in (26). To test the effect of alkyl gallates on pre-polymerized FtsZ, the protein was incubated at 30°C in polymerization buffer (25 mM piperazine-N,N′-bis(2-ethanesulfonic acid) [PIPES]/NaOH pH=6.8, 300 mM KCl, 10 mM MgCl<sub>2</sub>) and polymerized for 2 minutes with 2 mM GTP – this results in maximal polymerization as determined by light scattering (26). After 2 minutes, alkyl gallates (50 µg/ml) were added and the samples were incubated for another 6 minutes. Samples were collected at two times, immediately after alkyl gallates were added and after 6 minutes of incubation with alkyl gallates, and grids were prepared as described in (26). The grids were examined in a Philips CM120 electron microscope equipped with a LaB<sub>6</sub> filament operating at 120 kV. Images were recorded with a Gatan 4000 SP 4 K slow-scan CCD camera at 36,400× magnification.

**RESULTS**

Alkyl gallates with carbon side chain length C<sub>5</sub>-C<sub>8</sub> inhibit *B. subtilis* growth

Recently, we showed that pentyl (compound 8), hexyl (compound 9), heptyl (compound 10) and octyl (compound 11) gallates disrupt cell division in Xac. Delocalization of GFP-ZapA from the cell division site suggested that
the FtsZ-ring is a target of these compounds (6). To further study the mode of action of these compounds we tried to overexpress and purify Xac FtsZ, but failed to do so (data not shown). Therefore, we chose to use B. subtilis FtsZ for a more detailed characterization of the mechanism of action of alkyl gallates. First, we tested the antibacterial activity of compounds 8-11 against B. subtilis 168, using the resazurin microtiter assay (REMA). This assay determines the concentrations at which compounds block bacterial metabolic activity over a period of 4 hours. Using this assay, these compounds were found to block cell activity with MIC_{90} concentrations ranging from 50 µg/ml to 235 µg/ml (Table 1). In the REMA assay, compound 10 (C7) was the most potent (Table 1). In an earlier report, Kubo and co-authors found similar MIC values for compounds 9 and 10, and a lower MIC for compound 11 against B. subtilis strain ATCC 9372 (12,5 µg/ml) using the macrodilution method (5). Since compound 10 was the most potent against B. subtilis and alkyl gallates with longer and shorter side chains than compounds 8-11 did not disrupt septum formation in Xac (6), we focused on compounds 8-11 in our study.

Alkyl gallates disrupt Z-ring formation in vivo

To study whether FtsZ ring formation is the target of the alkyl gallates, we used a B. subtilis strain (4055) that expresses an additional copy of ftsZ fused to the fluorescent protein eYFP from the ectopic amyE locus. In this strain, the FtsZ-eYFP fusion protein localized to the division site at mid-cell (Fig. 1) and mid-cell localization was not affected by DMSO, which was used as a solvent for the compounds. Incubation with alkyl-gallates at MIC_{50} interfered with the formation or stability of the Z-ring. After 2 minutes of incubation, the FtsZ-eYFP fluorescence pattern was mostly found spread throughout the cell, even though some Z-rings could still be detected (most noticeably after incubation with compound 9 and compound 11). After 15 minutes of incubation the effect was more pronounced, with fluorescence throughout the cytosol, sometimes with occasional fluorescent spots, but hardly any Z-rings. The disruption of the Z-ring seemed to be fast, although it became more evident with an increase of incubation time. The occasional observance of rings was consistent with the fact that the cells were incubated with compounds at MIC_{50}, meaning that it was expected that not every cell was affected. To confirm that the disappearance of the FtsZ rings is not caused by a generic loss of membrane integrity, the effect
Figure 1. Alkyl gallates disrupt the Z-ring. B. subtilis cells expressing ftsZ-eyfp were incubated with DMSO (2%) or alkyl gallate compounds at MIC\_50 for 2 min. (left columns) or 15 min. (right columns). Brightfield and fluorescence microscopy images are shown. Incubation with alkyl gallates led to the disappearance of Z-rings and increase of fluorescence in the cytoplasm. Scale bar (same for all): 5 µm.

of membrane potential dissipation and membrane pore formation on FtsZ rings was determined using carbonyl cyanide m-chlorophenylhydrazone (CCCP) and Nisin (29). After incubation of the strain 4055 with CCCP and nisin, various Z-rings localized at the mid-cell (Fig. S1), although rings are less bright after CCCP treatment as reported before (29). The presence of Z-rings after CCCP
and nisin treatments indicate that the disappearance of the FtsZ-rings caused by the alkyl gallates is not caused by a generic loss of membrane integrity.

Alkyl gallates inhibit GTPase activity of FtsZ

The disruption of the Z-ring in cells treated with alkyl gallates suggested that FtsZ is a direct target for these compounds. To test this we made use of the polymerization-associated GTP hydrolysis activity of FtsZ. GTP hydrolysis is often used to screen for FtsZ inhibitors from a compound library (14). The GTPase activity of FtsZ in the presence of 50 µg/ml of the alkyl gallates was reduced nearly 6-fold compared to the control sample. However, residual GTPase activity was still detected in all of the samples, indicating that FtsZ was not completely inactive (Fig. 2). All of the compounds showed similar levels of inhibition of the GTPase activity of FtsZ. The GTP hydrolysis assay was performed in the presence of Triton X-100 as it has been reported that some compounds identified in high-throughput screens form small aggregates that inhibit FtsZ activity non-specifically. This aspecific inhibition is abolished by the inclusion of Triton X-100 in the assay (14). The experiment was also performed in the absence of Triton X-100 and similar results were obtained (not shown). Combined, this indicates that the effect of the alkyl gallates on FtsZ hydrolysis was specific and not caused by aggregation of the compounds.

FtsZ is a direct target for alkyl gallates

The binding of alkyl gallates to FtsZ was monitored using the intrinsic fluorescence of the compounds. FtsZ was titrated into a quartz cuvette containing

![Figure 2. Alkyl gallates inhibit FtsZ GTPase activity. FtsZ GTPase activity was determined as described in Materials and Methods. Compound concentrations were 50 µg/ml, 1.5% DMSO (compound vehicle) was used as a control. Three independent experiments were performed. Average and standard deviation are shown.](image)
a compound at fixed concentration (see Materials and Methods) and fluorescence emission spectra of the alkyl gallates were recorded with the excitation wavelength set at 271 nm as described in (8). Compounds 10 and 11 showed strong binding to FtsZ: the fluorescence emission maximum shifted from 389 nm to 366 nm and the maximum signal intensity increased upon FtsZ addition (Fig. 3A, B). The fluorescence of alkyl gallates was plotted as a function of FtsZ concentration and the dissociation constant was determined using 1:1 binding formalism described in (28). The best fits (R²=0.99) were obtained for a fixed concentration of compounds at 3.3 µM. The estimated K_d values obtained were 0.08 ± 0.03 µM for compound 10 and 0.84 ± 0.22 µM for compound 11 (Fig. 3C, D).

Tryptophan and tyrosine fluorescence are also excited at 271 nm. However, FtsZ does not contain tryptophan and the emission maximum of tyrosine occurs at 303 nm and does not change according to solvent polarity. The emission

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**Figure 3.** Binding of compounds 10 and 11 to FtsZ monitored by fluorescence spectroscopy. Fluorescence emission spectra of compound 10 (A) and 11 (B) acquired in the presence of 0, 1.2, 2.4, 3.6, 4.8 µM FtsZ (from bottom to top). (C, D) Binding curves of compounds 10 (C) and 11 (D) to FtsZ. The change in fluorescence intensity at 366 nm was plotted against FtsZ concentration (from 0 to 9.6 µM for compound 10 and from 0 to 12 µM for compound 11).
maximum of alkyl gallates occurs at 389 nm. We observed that tyrosine emission does not change upon binding of FtsZ to compound 10 and 11. However, addition of compound 8 and 9 significantly quenched tyrosine fluorescence of FtsZ. During analysis, the fluorescence spectra obtained for the compounds in the presence of FtsZ were corrected for protein fluorescence by subtracting the spectra of samples containing only FtsZ. The change in the FtsZ emission spectra due to tyrosine quenching made proper analysis of binding of compound 8 and 9 to FtsZ very difficult (Fig. S2A, B). The resulting shift in emission maximum for compounds 8 and 9 was severely reduced compared to compounds 10 and 11. For compound 8, an estimated $K_d$ of $3.1\pm2.0 \mu M$ could be calculated, with a worse fit ($R^2=0.83$) than was obtained for compounds 10 and 11 (Fig. S2C). For compound 9, an estimated $K_d$ could not be calculated. Combined, these results show that compounds 10 and 11 specifically bind FtsZ, with compound 10 having the highest affinity. The quenching observed with compounds 8 and 9 indicates interaction of the compounds with FtsZ but the low shifts in emission maximum and the difficulties in $K_d$ estimation indicate that the binding of compounds 8 and 9 to FtsZ may be aspecific.

Sedimentation of FtsZ is enhanced in the presence of alkyl gallates

Additionally, we used a sedimentation assay to study the effect of alkyl gallates on the assembly of FtsZ in vitro. FtsZ was mixed with the alkyl gallates (at 50 µg/ml) or 1.5% DMSO and polymerization was started by addition of GTP or GDP (control) to the sample. We noticed that in the presence of compounds 8, 10 and 11, FtsZ was recovered in the pellet fraction above background levels independent on the presence and type of nucleotide used (Fig. 4A). This result suggested that the compounds induce protein clustering or aggregation. As both the FtsZ interacting protein His-EzrA$_{ct}$ (Fig. 4A) and BSA (not shown) did not sediment in the presence of the compounds, the observed FtsZ sedimentation is not the result of aspecific protein aggregation. Compound 10 has the strongest effect on FtsZ sedimentation (all FtsZ protein was present in the pellet fraction), whereas approximately 50% of FtsZ protein was recovered in the pellet fraction after treatment with compounds 8 or 11. No sedimentation was detected when FtsZ was incubated with compound 9 (Fig. 4A). However, upon higher sedimentation speed (350 000 x $g$) or at higher compound concentration (100 µg/ml), FtsZ
was recovered above background levels also with compound 9 and 100% of FtsZ was present in the pellet fraction after treatment with compounds 8, 10 and 11 (Fig. S3). The strength of the effects of the various alkyl gallates on FtsZ sedimentation are in line with the estimated $K_d$'s for the different compounds.
The structures formed by FtsZ treated with alkyl gallates were visualized by electron microscopy (EM). As expected, FtsZ formed clusters after incubation with all the alkyl gallates (Fig. 4B).

Alkyl gallates promote clustering of FtsZ and bundling of FtsZ polymers

Alkyl gallates caused the clustering of FtsZ and only a small amount of short polymers was detectable in the sample when GTP was added. When FtsZ was incubated with the respective compounds at MIC<sub>90</sub> values, only big protein clusters and aggregates were observed (Fig. S4). We assume that FtsZ was not able to polymerize because the clustering of FtsZ prevents the correct association of FtsZ molecules required for polymerization. To establish whether the alkyl gallates disrupt existing polymers, we performed an experiment in which polymerization of FtsZ was initiated before the addition of compounds to the sample. FtsZ was polymerized in a PIPES/KCl buffer that ensures optimal polymerization as determined by light scattering (26), and the compounds or DMSO were added to polymerized FtsZ. Samples were collected immediately after the addition of compounds and after an additional 6 minutes of incubation. Compounds 8, 10 and 11 bound to the polymers of FtsZ and induced the formation of irregular bundles (Fig. 5). The amount of bundles that were observed in samples treated with compounds 10 and 11 was much higher than for compound 8 – although it has to be noted that this method is not quantitative. In the samples with compound 9, bundles were almost not visible (Fig. 5). We could only detect a few small bundle-like structures under the conditions used. The presence of a high number of tubules in the samples with compounds 10 and 11 suggests that the compounds can easily bind to the polymeric form of FtsZ, whereas binding of compound 8 and 9 to polymers of FtsZ is less strong – again, this is in line with the overall stronger FtsZ-binding of compounds 10 and 11. Although the alkyl gallates induce the formation of clusters of FtsZ monomers that as a result no longer form polymers, the compounds do not disrupt existing FtsZ polymers.

Membrane integrity and cell viability are affected by alkyl gallates

Cells in which the FtsZ activity is compromised, either through mutation or by the addition of FtsZ targeting compounds, display cell elongation and filamentation
caused by delayed or defective division (30). Even though the alkyl gallates had clear and immediate effects on FtsZ rings (Fig. 1), elongated cells could only occasionally be observed upon longer incubation at MIC$_{50}$, whereas cells grown in the presence of the known FtsZ-targeting compound PC190723 (31) were clearly filamentous (Fig. 6 A). After 1 hour of incubation with the compounds 9, 10, and 11 at MIC$_{50}$, we noticed that some cells had already lysed, and lysis was more noticeable when the incubation time was extended to 2 hours. At

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**Figure 5.** Structures of FtsZ formed in the presence and absence of alkyl gallates. 10 µM FtsZ was polymerized with 2 mM GTP for 2 minutes. After polymers were formed, alkyl gallates or DMSO (1.5% w/v) were added and immediately sample 0 was collected (0 min, A, C, E, G, I). After 6 minutes of incubation, another sample was collected (6 min, B: DMSO, D: compound 8, F: compound 9, H: compound 10, J: compound 11). Scale bar: 100 nm.
MIC<sub>50</sub> compounds 9, 10, and 11 caused cell death at an equivalent or higher proportion (data not shown). Incubation with compound 8 did not cause noticeable lysis – at least not to the extent as the other compounds - however, the large majority of the non-lysed cells did not appear elongated. These results indicate that the alkyl gallates can cause cell death via another mechanism than FtsZ inhibition. These alternative mechanisms may correspond to the ones reported by the REMA assay for antibacterial activity, as elongation still requires metabolic activity. Therefore, we investigated the effects of the alkyl gallates at lower concentrations than the MIC<sub>50</sub> determined in the REMA assay. Lowering the concentrations of the compounds revealed that compounds 9, 10 and 11 are capable of causing cell elongation, as expected for FtsZ inhibitors, whereas compound 8 had no effect on cell length (Fig. 6B). The minimal inhibitory activity (MIC<sub>24H</sub>) of the compounds was established by a 2-fold dilution series in which prolonged incubation revealed that compound 11 can prevent cell growth at a lower concentration than the one determined by the REMA assay (25 µg/ml Table 1). This is closer to the value reported in the literature for this compound (5). Compounds 8 and 9 and 10 show similar inhibition of growth after 24 hours as previously determined by the REMA assay. Taken together these results indicate that alkyl gallates with a high affinity for FtsZ in vitro can induce cell death by directly targeting FtsZ. Additionally it is observed that at higher concentrations of the alkyl gallates an alternative mechanism is responsible for the quick cell death that takes place without cell elongation (Fig. 6B).

As several described FtsZ inhibitors also affect cell membranes (32), we decided to study the effect of the alkyl gallates on cell membrane integrity in vivo. The Live/Dead BacLight kit, which combines the green membrane permeable fluorescent DNA dye, SYTO 9, and the red membrane impermeable fluorescent DNA dye, propidium iodide, was used to assess membrane integrity. Cells were incubated with both dyes and alkyl gallates. Subsequently, cells were imaged and red or orange cells were classified as cells with affected membrane integrity, whereas green cells were classified as cells with intact membranes. Nisin, which is known to make pores in the membrane, and CCCP, which disrupts the membrane potential but does not make pores (29), were used as controls. All alkyl gallates were found to be able to create membrane pores in vivo, to different extents (Fig. 7). Compound 8, at both MIC<sub>50</sub> and MIC<sub>90</sub> concentrations, permeabilized all cells, which is in line with the observed cell death without elongation –
Figure 6. Alkyl gallates cause cell elongation and lysis. (A) controls: *B. subtilis* cells were incubated with nothing (168) or with PC190723 at 2 µg/ml for 1h, 2h or 3h. (B) *B. subtilis* cells were incubated with alkyl gallates at MIC$_{50}$ for 1h (1st column) or 2h (2nd column), or
although it has to be noted that cells incubated with compound 8 at MIC\textsubscript{50} did not noticeably lyse (Fig. 6). Compounds 9, 10, and 11 showed a concentration-dependent membrane permeabilization – but importantly, incubation at MIC\textsubscript{50} concentrations never resulted in more than 50% permeabilized cells, indicating that the alkyl gallates function by targeting membrane integrity, FtsZ function, and possibly other mechanisms.

**DISCUSSION**

The use of alkyl gallates as anti-bacterial agents has been proposed in various studies in which the pharmacological activity of these compounds was described (2-5, 7). As semi-synthetic compounds that are derived from gallic acid, a plant metabolite, these compounds could be interesting and environmental-friendly...
alternatives for the control of bacterial infections of agricultural crops. To this end, we previously characterized the activity of alkyl gallates against the important citrus pathogen *Xanthomonas citri* subsp *citri* (Xac) (6). Our initial studies indicated that the alkyl gallates disrupt cell division in Xac, possibly by targeting the FtsZ-ring, which is different from the observed mechanism of membrane binding that is also described for these compounds (8). Here, we investigated the mode of action of four alkyl gallates in more detail.

As we neither had access to a Xac strain expressing fluorescent FtsZ, nor were able to purify Xac FtsZ, we turned to *B. subtilis*, which is also killed by alkyl gallates (5). Here, we provide both *in vivo* and *in vitro* evidence that FtsZ is a target for the alkyl gallates. The addition of alkyl gallates to cells expressing a fluorescent variant of FtsZ causes immediate disruption of Z-rings (Fig. 1), and cultures exposed to compounds 9, 10 and 11 display the classical elongation phenotype of cells affected in cell division (Fig. 6). Purified FtsZ is blocked from polymerizing in the presence of alkyl gallates (Fig. 4 and Fig. S4), and some of the alkyl gallates bind FtsZ with high affinity, most notable heptyl gallate with an estimated Kd of 80 nM (Fig. 3). It should be noted that the effect of the alkyl gallates on *B. subtilis* indicated that FtsZ is not the sole target— the classical phenotype of cell elongation by division inhibition was observed for compounds 9, 10 and 11 (Fig. 6), but all compounds also affected membrane integrity as observed with the permeability assay (Fig. 7). Therefore, alkyl gallates probably promote cell death by a combination of mechanisms: FtsZ inhibition, membrane permeabilization and, possibly, another activity.

Two recent studies pointed out some issues with antibacterials that have been identified as ‘FtsZ-inhibitors’. The first study, by Anderson *et al.* (14), showed that many compounds identified as FtsZ inhibitors in high-throughput screening assays based on FtsZ-mediated GTP hydrolysis, in fact form small aggregates that block GTP hydrolysis. The addition of Triton X-100 to these assays prevents aggregate formation and reveals normal GTP hydrolysis levels in the presence of “false-positive” compounds. The effect of alkyl gallates on GTP hydrolysis is the same, irrespective of whether Triton X-100 is present or not, indicating that alkyl-gallates are not false-positive GTP hydrolysis inhibitors (Fig. 2). Also, our other *in vitro* assays show that – at least some – of the alkyl gallates bind FtsZ with high affinity and that these compounds cluster FtsZ and prevent polymerization (Fig. 3, 4). Cluster formation is not caused by aspecific protein aggregation as shown by
control experiments with His-EzrA<sub>cyt</sub> and BSA. Combined, the in vitro work clearly shows that FtsZ is inhibited by the alkyl gallates. The second study, from Foss et al. (32) indicated that many compounds, identified as FtsZ inhibitors, target the membrane. Alkyl gallates have already been identified as membrane binding agents (8) – therefore we examined the effect of the alkyl gallates on membrane integrity. Intriguingly, compound 8, with the shortest alkyl chain length, had the most disruptive effect on membranes as monitored by the influx of propidium iodide into cells (Fig. 7). The other compounds also affected membrane integrity albeit to different extents. We observed that compounds 9-11 permeabilize less than 50% of the cells at concentrations where 50% of the cells are metabolically inactive (MIC<sub>50</sub>) as determined by the REMA assay. At MIC<sub>50</sub>, cell elongation cannot be observed and many cells in the culture lyse. At lower concentrations of the compounds, elongation can clearly be observed, and it is evident, from the dilution series (Table 1), that concentrations that cause elongation are sufficient to inhibit cell growth. We conclude that, especially for compounds 10 and 11, FtsZ inhibition occurs at lower concentrations than MICs determined with the REMA assay. Increasing the concentration to MIC values leads to disruption of membrane integrity – this is particularly obvious for compound 8. The effect of alkyl gallates on membrane integrity is not the cause for FtsZ ring disruption as compounds that disrupt membrane integrity or that dissipate the membrane potential do not affect the Z-ring in vivo, whereas all alkyl gallates, at MIC<sub>50</sub> and MIC<sub>90</sub>, quickly disrupt most of the Z-rings in cells. Combined, our experiments show that alkyl gallates, in addition to targeting membrane integrity (8, 31), can also directly target FtsZ. Given the antimicrobial activity of alkyl gallates against a variety of bacteria (2-6), the disruption of model membranes by alkyl gallates (8) and the disruption of cell division in both Gram-negative and Gram-positive bacteria (6, this work), we consider it very likely that both membrane disruption and FtsZ inhibition by alkyl gallates apply to many bacteria. Heptyl-gallate (compound 10) was found to bind to FtsZ with very high affinity, resulting in blocked cell division at low concentrations and FtsZ cluster formation in vitro. The length of the alkyl chains affects both the interaction with the membrane and with FtsZ. As the most promising anti-FtsZ agent, heptyl gallate can be used as a hit for the design of innovative compounds that have enhanced specificity towards FtsZ and less activity on the membrane. Several modifications on the structure of heptyl gallate are currently being made in our laboratories.
CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Morten Kjos and Ruud Detert Oude Weme (Molecular Genetics, University of Groningen, NL) for plasmids pMK13 and pDOW01.

This work was funded by the bilateral research programme “Biobased Economy” from the Netherlands Organisation for Scientific research (NWO) and the São Paulo Research Foundation (FAPESP 2013/50367-8, Brasil, to DJS and HF), a NWO Vidi grant (to DJS), a doctoral grant (SFRH/BD/78061/2011) from POPH/FSE and FCT (Fundação para a Ciência e Tecnologia) from Portugal (to ASB) and a Science without Borders sandwich grant (CNPq process: 201196/2012-3, to IS).
REFERENCES


**SUPPLEMENTAL MATERIAL**

**Figure S1.** *B. subtilis* cells expressing ftsZ-eyfp (strain 4055) without treatment or after incubation with CCCP (2 mM) or nisin (1.5 µg/ml) to disrupt membrane potential or integrity. Brightfield and fluorescence microscopy images are shown. Scale bar (same for all): 5 µm.

**Figure S2.** Binding of compound 8 and 9 to FtsZ monitored by fluorescence spectroscopy. Fluorescence emission spectra of compound 8 (A) and 9 (B) acquired in the presence of 0, 1.2, 2.4, 3.6, 4.8 µM FtsZ-compound 8 and 0, 2.4, 3.6, 4.8, 7.2 µM FtsZ-compound 9 (from bottom to top). Compound 9 was used at 4x higher concentration than compound 8. The change in fluorescence intensity of compound 8 (C) at 366 nm plotted vs increasing concentrations of FtsZ (from 0 to 14.4 µM). The plot of fluorescence intensity vs FtsZ concentration for compound 9 was impossible to obtain due to high instability of the fluorescence signal.
Figure S3. Sedimentation of FtsZ in the presence of 100 µM alkyl gallates and at high sedimentation spin. (A) 10 µM FtsZ was incubated with alkyl gallates (100 µg/ml) before GTP/GDP was added. In the sample without nucleotide (no nucl), the same volume of polymerization buffer was added to the sample as for GTP/GDP. (S) indicates supernatant and (P) pellet fractions from the experiment. As a control, 1.5% DMSO was used. Samples were spun down at 186,000 x g for 10 min. All experiments were performed in duplicate. (B) The same experiment as in (A) with alkyl gallates at 50 µg/ml. Samples were spun down at 350,000 x g for 15 min.

Figure S4. Structures formed by FtsZ with alkyl gallates at MIC₉₀ concentration. 10 µM FtsZ was incubated with alkyl gallates at MIC₉₀ concentration for 5 minutes. After that, 2 mM GTP was added and samples were incubated for another 20 minutes. As a control, 1.5% DMSO was used. Scale bar: 100 nm.
ANNEX

The sporulation experiments included in this annex are part of the following paper: Król E., de Sousa Borges A., Kopacz M. and DJ Scheffers. 2017. Metal-dependent SpoIIE oligomerization stabilizes FtsZ during asymmetric division in Bacillus subtilis. PLoS ONE 12: e0174713.

Improving the fusion construct ftsZ-eyfp

Strain 4055 (trpC2 amyE::spc PamyE::spc ftsZ-eyfp) was constructed as described in the M&Ms section of this chapter (1). In this strain, IPTG is used to express ftsZ-eyfp integrated in the amyE locus. This strain is the result of the testing of two ftsZ-eyfp fusions with different linker sequences between ftsZ and eyfp. In the first fusion construct tested, the coding sequence of FtsZ was attached to the coding sequence of eYFP via a linker that we hereby designate as linker 1. Linker 1 was previously used in the Veening laboratory with success for C-terminal eYFP fusions (2). Linker 1 consisted of 15 amino acids (SRGSGEAAAKATGS) and contained a flexible (in bold) and a rigid (underlined) section. In order to see FtsZ-eYFP rings with this construct, relatively high amounts of IPTG were required (Fig. 1E). In order to analyze if the FtsZ-linker1-eYFP fusion protein was subject to proteolytic cleavage, cells were incubated with various concentrations of IPTG so that different amounts of this fusion protein would be produced. Cell lysate was obtained from these different samples and equalized amount of proteins were loaded on a gel and blotted against FtsZ (Fig. 1, panel A). In addition, the same amount of cell lysate was also loaded on a gel in order to study the stability of the fluorescent fusion protein by detecting the eYFP fluorescence in-gel (Fig. 1, panel B).

In panel A, the band corresponding to the size of FtsZ wild-type (~40 kDa) increased together with the production of FtsZ-linker1-eYFP due to the increase in IPTG concentration. This result suggests that linker 1 was being cleaved resulting in an increase in full-length FtsZ and eYFP. This was corroborated by the presence of free fluorescent eYFP (~25 kDa, Fig. 1, panel B). The eYFP fluorescence intensity at ~25 kDa was roughly equal to the fluorescence intensity displayed by the complete fusion protein (~65 kDa), indicative of a cleavage rate of approximately 50%. Interestingly, in panel A we also noticed a higher band that was detected when blotting against FtsZ, and although we cannot determine what is the fusion protein associated with this band, we concluded it wouldn’t interfere with our results as there is no fluorescence associated with this band (Fig. 1, panel B and D).
Figure 1. Stability of fluorescent fusion proteins. Panels A and B, the cell lysates of cells producing FtsZ-linker1-eYFP in the presence of different IPTG concentrations were loaded on a gel and blotted against FtsZ (A), or imaged for in-gel GFP fluorescence (B). On the right, the kDa marker for protein size. Panels C and D, the cell lysates of cells producing FtsZ-linker1-eYFP and FtsZ-linker2-eYFP after the addition of 0.2 mM IPTG were loaded on a gel and blotted against FtsZ (C), or imaged for in-gel GFP fluorescence (D). As controls, cells grown without IPTG, to check for leaky expression, and wild-type cells (168) were loaded. Panel E, microscopy of exponential phase cells producing either FtsZ-linker1-eYFP in the presence of 0.2 mM IPTG (top) or FtsZ-linker2-eYFP in the presence of 0.2 mM or 0.02 mM IPTG (bottom). Scale bar: 2 µm.

Given the high rate of fusion protein cleavage, a new linker, here named linker 2, was created. Linker 2 is a highly repetitive flexible linker with 15 amino acids (SGGGSGGGGSGGGG). To compare both linkers, cells producing FtsZ-linker1-eYFP or FtsZ-linker2-eYFP fusion protein were incubated with the same amount of IPTG and the respective cell lysates were equalized for protein concentration before loading on a gel. Both the FtsZ (Fig. 1, panel C) protein
and eYFP fluorescence (Fig. 1, panel D) were detected. Panel C shows the same pattern previously obtained in panel A, but in panel D it is clear that cleavage of the fusion protein with linker 2 is reduced. The fact that the cleavage of the fusion protein FtsZ-linker2-eYFP is reduced, thus resulting in a lower amount of fluorescent free eYFP, explains why 0.2 mM IPTG yields correct Z-rings, in vivo, in the case of FtsZ-linker1-eYFP but results in the clear overexpression phenotype of multiple Z-rings in the case of FtsZ-linker2-eYFP. Even though the fusion protein FtsZ-linker1-eYFP produced suitable Z-rings with 0.2 mM IPTG (Fig. 1, panel E); it was the fusion protein FtsZ-linker2-eYFP that efficiently allowed the formation of adequate Z-rings under a very low expression, using 0.02 mM of IPTG (Fig. 1, panel E), due to the decreased levels of degradation. Therefore, the strain that had ftsZ-eyfp with linker 2 integrated at amyE was renamed strain 4055 and this strain was used, with ftsZ-eyfp expression driven by 0.02 mM IPTG, for the experiments described in this annex and in chapter 3.

The construct ftsZ-eyfp upon sporulation

As described in chapter 1, B. subtilis is capable of asymmetric division, or sporulation. During sporulation, FtsZ is redeployed from midcell to both poles to form two polar Z-rings within the cell; however, only one polar Z-ring is converted into a septum that separates the mother cell from the forespore compartment (3, 4). SpoIIE is an FtsZ-interacting protein that contributes to the formation of the asymmetric septum by switching the FtsZ positioning to the poles, and after septation, SpoIIE activates the forespore-specific transcription factor σF (5-8). In spoIIE null mutants, FtsZ still localizes to both poles but there is a delay on the switch from medial to polar rings, thus reducing the frequency of polar Z-ring formation (9, 10). Although the phosphatase activity of SpoIIE has already been assumed to be manganese dependent (11), we were interested to see whether the role of SpoIIE in polar septation, which precedes its role as a phosphatase, is also manganese dependent. To this end, the strain 4055 was used to visualize FtsZ localization during sporulation, in the presence and absence of manganese.

An overnight culture of B. subtilis strain 4055 (P_{hyperspac} -ftsZ-eyfp) grown in CH medium (12) was diluted into fresh CH to an OD of 0.1. Cells were grown at 37°C until OD of 0.7. At this point, 2 samples of 5 ml were taken and cells were collected and washed 2 times with the same volume of CH with (spo+)
or without (spo-) manganese. After the washing steps, spo+ and spo- cells were resuspended in 100 µl of CH with and without manganese, respectively. Sporulation medium (13) (with or without manganese) was added up to the volume of 5 ml in the presence of 0.02 mM of IPTG to allow low level expression of ftsZ-eyfp. Cells were allowed to sporulate at 37°C by continuing the incubation. Every hour, a 500 µl sample was taken and cells were harvested and resuspended in 20-50 µl of PBS before being mounted on an agarose pad prior to microscopy. FtsZ-eYFP was visualized as described in (1) and cells were scored according to their Z-ring localization, revealing that the polar Z-ring formation is delayed in the absence of manganese (Fig. 2).

In the absence of manganese, relocation of the Z-ring from mid-cell to the cell poles is delayed (compared to samples supplemented with manganese). We noticed that in the absence of manganese, cells contain more mid-cell rings and significantly less polar rings compared to cells in sporulation medium supplemented with manganese (Fig. 2).

**Figure 2.** The absence of Mn$^{2+}$ from the sporulation medium delays asymmetric Z-ring formation. Pie-chart representation of sporulating *B. subtilis* cells in the presence (+) and absence (-) of Mn$^{2+}$. Four different types of cell (representative images in the legend) were scored: cells without any Z-ring in the cell (no ring), cells with a Z-ring in the middle of the rod (mid-cell ring), cells with two rings assembled at the cell poles (two polar rings) and cells with only one polar ring (polar ring). Non-sporulating cells are marked in blue shades, while sporulating cells are marked in orange shades. Each pie chart is the result of two independent classification experiments in which at least 200 cells were classified per condition. Actual percentages and standard deviations are included in Table 1.
However, the absence of manganese also contributes to a delay in the sporulation phosphorelay. To exclude that, in the absence of manganese, the delay in the phosphorelay is the main cause for the delay in polar septation, the previous experiment was repeated in a strain that overproduces KinA. KinA overproduction artificially charges the phosphorelay both in sporulating and exponentially growing cells (14). The protocol used in this experiment was equal to the previous one, with a minor exception that in the new strain 4120 \((P_{\text{hyperspac}-ftsz-eyfp}, P_{\text{xyl}-\text{kinA}})\) \(\text{kinA}\) was induced with 0.5% (w/v) Xylose after resuspension in sporulation medium. The overproduction of KinA resulted in faster sporulation, and thus cells had to be analyzed after 60, 120 and 180 minutes. A similar result to the previous experiment was obtained, with more cells with midcell rings and less cells with polar rings in the absence of manganese (Table 2). As polar septation is also reduced in the absence of manganese when

### Table 1. Averaged percentages and standard deviations (avg±stdv)

<table>
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<tr>
<th></th>
<th>No-ring</th>
<th>Mid-cell ring</th>
<th>Two polar rings</th>
<th>Polar ring</th>
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<tr>
<td>2h</td>
<td>29.1±1.8</td>
<td>5.6±0.6</td>
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### Table 2. Averaged percentages and standard deviations (avg±stdv)

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The absence of Mn\(^{2+}\) from the sporulation medium delays asymmetric Z-ring formation in cells with a hypercharged phosphorelay. \(B.\ subtilis\) cells were scored in the presence (plus) and absence (minus) of Mn\(^{2+}\) as described for Fig. 2. The actual percentages and standard deviations from two independent classification experiments in which at least 200 cells were classified per experiment are depicted in table 2.
the phosphorelay is artificially charged, we can conclude that the role of SpoIIE in relocating FtsZ to the cell pole is affected in the absence of manganese, and that the formation of asymmetric septa is delayed, similar to the reported spoIIE null phenotype. Combined with biochemical experiments on SpoIIE manganese binding and its positive influence on FtsZ polymer stability (15, submitted), these experiments show that manganese facilitates the formation of polar septa during B. subtilis sporulation, most likely acting through SpoIIE.

Importantly, these two sporulation experiments exemplify the versatility of the fusion construct FtsZ-eYFP.
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Bacillus subtilis cells forming a smiling face
Figure 6B, Chapter 4