Localization of FtsZ in *Helicobacter pylori* and consequences on cell division

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ABSTRACT

Of the various kinds of cell division, the most common mode is binary fission, the division of a cell into two morphologically identical daughter cells. However in the case of asymmetric cell division, *C. crescentus* produces two morphological and functional distinct cell types. Here, we have studied cell cycle progression of the human pathogen *Helicobacter pylori* using a functional GFP-fusion of FtsZ protein and membrane staining. In small cells, representing newly divided cells, FtsZ localizes to a single cell pole. During the cell cycle, spiral intermediates are formed, until a FtsZ-ring is positioned with little precision, such that central as well as acentral rings can be observed. Daughter cells showed considerably different sizes, suggesting that *H. pylori* divides asymmetrically. FRAP analyses demonstrate that the *H. pylori* FtsZ-ring is about as dynamic as that of *E. coli* but that polar assemblies show less turnover. Strikingly our results demonstrate that *H. pylori* cell division follows a different route from that in *E. coli* and *B. subtilis*. It is also different from that in *C. crescentus*, where cytokinesis regulation proteins like MipZ play a role. Therefore this report provides the first cell biological analysis of FtsZ dynamics in the human pathogen *H. pylori* and even in ε-proteobacteria to our knowledge. In addition, analysis of the filament architecture of *H. pylori* and *E. coli* FtsZ filaments in the heterologous system of *D. melanogaster* S2 Schneider cells revealed that both have different filamentation properties *in vivo* suggesting a unique intrinsic characteristic of each protein.
INTRODUCTION

*Helicobacter pylori* is a Gram negative, highly motile, microaerophilic, spiral-shaped organism, which belongs to the class of the epsilon proteobacteria. The natural habitat of this pathogen is the human gastric mucosa and infection of humans results in persistent gastritis, which can develop into peptic ulcer disease and adenocarcinoma (1, 2). Today at least half of the world's human population is infected (3). Although extensive research has been conducted to *H. pylori*, remarkably little is known about the molecular basis of cell division in this important human pathogen. The comparison of the complete genome sequences of two *H. pylori* strains revealed that fourteen homologs of *Escherichia coli* cell division and chromosome segregation genes have been recognized (4) and it was suggested that the basic mechanisms of replication and cell division are similar to those of *E. coli*. These are genes such as *ftsZ*, *ftsA* and the ring inhibitor genes *minC*, *minD* and *minE*. However, some orthologues of cell division proteins like ZipA and all periplasmic connector proteins are missing in *H. pylori*. These differences may be attributed to the smaller genome size of *H. pylori* as well as the lifestyle, i.e. *H. pylori* is adapted to its unique niche in gastric mucus with fixed temperature and slow doubling time whereas *E. coli* is a free living organism with fast proliferation under different temperatures (5).

In most organisms, cell division occurs after placement of a septum through the midpoint of the dividing cell and equal distribution of the cellular components into the two daughter cells (6). Division site determination is accomplished by FtsZ ring formation at the future septum. The Z ring is usually positioned at mid-cell early during the division process (7, 8) and serves as a scaffold for the assembly of the other cell division proteins. FtsZ assembly is tightly regulated, and a diverse repertoire of accessory proteins contributes to the formation of a functional division machinery that is responsive to cell cycle status. In rod-shaped bacteria like *E. coli* or *B. subtilis*, FtsZ localizes either diffusely in the cell, in a helical pattern underneath the cell membrane, or as FtsZ-ring at the beginning of the division process.
The positioning of the Z-ring is dependent on the so called Min and nucleoid occlusion systems (9), which prevent the assembly of Z-rings at the cell poles and over chromosomal DNA. In particular, in E. coli, Min proteins oscillate from pole to pole resulting in the formation of a zone of FtsZ inhibition at the cell poles. Protection of the replicated nucleoid DNA near the mid-cell from bisection by the Z-ring is ensured by Noc in B. subtilis and by SlmA in E. coli (10). Both the Z-ring and the helical localization of FtsZ are highly dynamic with a high turnover rate (11, 12). In contrast to E. coli and B. subtilis, FtsZ of C. crescentus is clustered at a single cell pole before it is induced to assemble at mid-cell, a process that is regulated by MipZ (13).

In this study, we investigated cell cycle progression of the human pathogen H. pylori by monitoring FtsZ. To this end, we used our previously developed system (14) that permits in vivo localization of individual proteins in H. pylori. Our results indicate that H. pylori FtsZ-ring is positioned with very little precision resulting in daughter cells showing considerably different sizes. FtsZ-ring formation and disassembly is also different from the process in E. coli and B. subtilis. Thus this report provides the first cell biological analysis of FtsZ dynamics in the human pathogen Helicobacter pylori and even in ε-proteobacteria to our knowledge. Furthermore our results demonstrated that H. pylori and E. coli FtsZ filaments have different filamentation properties in vivo in the heterologous system of D. melanogaster S2 Schneider cells suggesting a unique intrinsic characteristic of each protein despite the common function.
MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains are listed in table 1. H. pylori strains were routinely cultivated on Dent blood agar in a microaerobic atmosphere as described earlier (15). Growth experiments were performed in Brucella broth with 5% fetal calf serum (BBF). Growth rate was assessed by optical density (OD₆₀₀). E. coli strains were grown aerobically at 37°C in Luria-Bertani medium. When appropriate, growth media were supplemented with 50 µg/ml ampicillin (Ap) or 20 µg/ml chloramphenicol (Cm).

DNA techniques and mutagenesis of H. pylori

Restriction and modifying enzymes (New England Biolabs, USA) were used according to the manufacturer's instructions. Cloning was performed in E. coli according to standard protocols. Plasmids were isolated with a QIAprep Spin Miniprep Kit from Qiagen (Qiagen 27104). For the generation of a C-terminal GFP-fusion of FtsZ, the ftsZ gene was amplified by PCR using primer pair FtsZup/FtsZdw (Table 1) and the resulting 511 bp fragment was cloned at Apal and EcoRI restriction sites on pSG1164 vector (16). Integration of the fluorescent tag vector pSG1164-FtsZGFP into the H. pylori chromosome was achieved via single crossover using electroporation according to standard procedures (17). H. pylori 1061-FtsZGFP clones carrying the FtsZ-GFP fusion were selected on Dent blood agar with 20 mg/l chloramphenicol. The correct placement of the integration was verified by PCR.

To create C-terminal fusions of E. coli and H. pylori FtsZ with YFP (eYFP) for transfection of the S2 cells, the coding sequence of FtsZ was amplified by PCR, using primer pair EcftsZ_up/EcftsZ_down_NS and HpftsZ_up/HpftsZ_down_NS respectively. Additionally, E. coli and H. pylori FtsZ were amplified by PCR using primer pair EcftsZ_up/EcftsZ_down_S and HpftsZ_up/HpftsZ_down_S respectively. Plasmid pFD1 was constructed by combining the multiple cloning site as well as the coding sequence of the fluorophore of the plasmid pSG1164 (16) with the plasmid pRMHa3 (18) using KpnI and...
SpeI. Subsequently amplicates were cloned into the vector pFD1 using ApaI and ClaI giving rise to the plasmids pFD323 (EcFtsZ-YFP), pFD320 (HpFtsZ-YFP), pFD321 (EcFtsZ) and pFD319 (HpFtsZ) respectively.

**Immunoblotting and determination of GFP stability**

Cells were grown until log phase and then treated with puromycin dihydrochloride (50mg/ml) for 10 minutes to one hour. After protein determination equal amounts of the samples were lysed in SDS-PAGE loading buffer by boiling for 5 min. SDS-PAGE and transfer of protein to polyvinylidene fluoride (PVDF) membrane was performed with standard procedures. Samples were probed with α-GFP primary antibody. The same amount of wild type protein extract and a verified GFP-fusion protein served as negative and positive control respectively.

**Cell culture of Schneider cells and transient transfection**

*D. melanogaster* S2 Schneider cells were grown in Schneider’s *Drosophila* medium (Lonza Group Ltd.) supplemented with 5-10% fetal calf serum (FCS) at 25°C without addition of CO₂. Cells were passaged every 2 to 3 days to maintain optimal growth. S2 cells were transfected using FuGENE® 6 Transfection Reagent (Roche). The S2 cells were spread in a 6-well plate at 1 x 10⁶ cells per well in 3 ml medium with 5% FCS. Supercoiled plasmids (0.3 µg of each plasmid) were complexed with lipid (10 µl FuGENE® reagent) in 200 µl serum-free medium. The complex was incubated at room temperature for 15 min, filled up with serum-free medium to 1 ml and was added to cells from which the growth medium had been removed (cells were washed once with serum-free medium). After 18 hrs, the supernatant was removed and replaced by 3 ml of medium containing 5% FCS. After further incubation for 24 hrs, the production of the proteins was induced by adding CuSO₄ to a final concentration of 1 mM.

**Immunofluorescence**

Immunofluorescence of *H. pylori* cells was performed as described earlier (19) with the following modifications: anti *C. glutamicum* FtsZ antibody (1:100 dilution in 1 x PBS, 100
µg/ml BSA) was used as primary antibody, which was detected by the secondary antibody goat anti guinea pig coupled to FITC (Invitrogen) (1:100 dilution in 1x PBS, 100 µg/ml BSA). For immunofluorescence of the S2 Schneider cells, 100 µl of transfected S2-cells were transferred onto a poly-L-lysine treated glass slide and left to settle for 15 min. For fixation the slide was placed for 5 min in cooled methanol (-20°C) and afterwards 30 s in cooled acetone (-20°C). After rehydrating with 1 x PBS for 5 minutes at RT, 100 µl image-iT Fx signal enhancer (Invitrogen) were applied and incubated over night at 4°C. For visualisation of FtsZ, anti *C. glutamicum* FtsZ antibodies (1:100 dilution in 1 x PBS, origin: guinea pig) were added and incubated for 90 min at RT. Unbound antibodies were removed by three times washing with 1x PBS (5 min, RT) and FtsZ signals were visualized by the secondary antibody goat anti guinea pig coupled to FITC (Invitrogen) (1:100 dilution in 1 x PBS, 1 h, RT). After washing for 5 min at RT, cells were mounted with fluorescent mounting medium (Dako).

**Scanning electron microscopy**

Infected monolayers grown on cover slips were fixed with 3% glutaraldehyde in PBS at room temperature. After several washing steps with PBS, samples were dehydrated with a gradient series of ethanol (30, 50, 60, 70, 80, 90 and 100%) at room temperature for 12 hours for each step. Samples were then subjected to critical-point drying with liquid CO₂ (CPD030, Bal-Tek). Dried samples were covered with gold film by sputter coating for 80 s (SCD 050, Bak-Tek). Examinations were performed in a field emission scanning electron microscope (FEI Quanta 250 FEG) using an Everhart Thornley detector and an acceleration voltage of 5 kV.

**Fluorescence microscopy**

Fluorescence microscopy was performed on a Zeiss Axioobserver Z1 microscope using a 100 x objective with A = 1.45. Cells were mounted on agarose gel pads containing brucella liquid medium on object slides. Images were acquired with a digital Cascade EM-CCD camera (Photometrix); signal intensities and cell length were measured using the *Metamorph 6.3*
software (Universal Imaging Corp., USA). Membranes were stained with FM4-64 (final concentration 1 nM). Filters used were: GFP – ex460–495, dc505, em510–550, FM4–64 ex480–550, dc570, em590. For monitoring cell division under the microscope we used a heating stage at 32°C and a CO₂ atmosphere of 5% producing a microaerophilic milieu (Tokai Hit).

**Fluorescence after photobleaching (FRAP)**

Cells were imaged and FRAP experiments were performed on a Zeiss Axioobserver Z1 microscope equipped with a 50 mW solid phase laser. The laser was focussed to 1 µm through a lense system and the cell in question was positioned into the stationary 405 nm laser beam. Fluorescence of the selected region of interest was bleached for 0.1 s. All images and FRAP measurements were taken at room temperature. Prebleach image and postbleach images were acquired with 488 nm laser excitation. Postbleach image series were taken automatically every 0.1 s using the Metamorph program and statistically analysed using ImageJ (NIH) in combination with EXCEL (Microsoft). Calculations were done according to Schulmeister et al. (20). Briefly, fluorescence intensity of the region of interest (ROI) was measured automatically in image sequences, using a custom-written ImageJ plug-in. Gradual bleaching of the image was compensated during scanning by normalizing the fluorescence of the ROI to the integral fluorescence of the entire cell in the same image. To facilitate comparison of multiple experiments with different bleaching depth and different cluster intensity, the relative fluorescence intensity of the ROI in the image sequence was normalized again to the relative ROI intensity before bleaching. Data were subsequently processed by using EXCEL.
RESULTS

FtsZ localization in *H. pylori*

To analyse cell cycle progression in *H. pylori*, we generated a C-terminal GFP-fusion of FtsZ that is expressed as the sole source of the protein in strain 1061. Analysis of the growth characteristics revealed that the fusion was fully functional at 32°C, and partially functional at 37°C, because cells displayed a filamentous phenotype at higher temperature (Suppl. Fig. 1A). We further confirmed fusion protein abundance and fusion protein stability by Western blot analysis of cell extracts with antibodies to GFP (Fig. 1A). To this end, *H. pylori* cells were treated with puromycin to inhibit protein biosynthesis. This treatment revealed that the FtsZ-GFP fusion is very stable, because the fusion was still visible even after 60 minutes of incubation. (Fig. 1A). In addition this experiment confirmed that GFP was not cleaved off.

Subsequently, we analysed 1061-FtsZ-GFP cells grown in liquid culture until log phase using epifluorescence microscopy. In this phase *H. pylori* has a tight spiral shape, whereas coccoid cells start to appear at the earliest in late log phase (21). Single mid-cell bands of FtsZ were visible in large cells (> 2.2 µm) verifying that the GFP tagging system can produce functional fusions in live cells (Fig 1C, white triangles). Surprisingly, FtsZ bands were positioned off centre in about 50% of the cells (Fig. 1C, red triangles, 85 cells analysed) suggesting an asymmetric cell division. As spiral or mildly curved bacteria might introduce visual artifacts (e.g. as the entire bacterial body might not be in the same focal plane) we further confirmed our finding using 1061-FtsZ-GFP *H. pylori* cells which displayed a straighter phenotype. To this end, we performed Z stacks of the asymmetric localized Z ring demonstrating that the localization is indeed not a visual artifact (Fig. 1F). In this context it has to be noted, that straight wt cells are always found to a small extend in *H. pylori* cell cultures.

To support the idea of asymmetric cell division, we measured the distance of the division septum to the two old cell poles in dividing cells (Fig. 1D, Table 2). The measurement of cells...
(n= 60) demonstrated that on average one daughter cell had 80.8% of the length of the other daughter (please compare Table 2 for hard data), in support of an asymmetric cell division. To specify this finding we split the cells into two groups according to their Z-ring position: Cells with symmetric cell division were defined to have a mid-cell position of the Z-ring which varies less than 10% of the middle position, whereas the asymmetric localization was defined as more than 10% variation of the Z-ring from the cell centre. Thus, the average size of the small part in percentage of the larger part was 90.7% and 70.3% in the case of symmetric or asymmetric cell division, respectively. This shows that positioning of the Z-ring during cell division is more relaxed in *H. pylori* than in *E. coli* or in *B. subtilis*, where the central positioning of the Z-rings varies less than 5% (22). Strikingly, this is even true in the case of the symmetric positioned *H. pylori* Z-ring. Thus, cell division in *H. pylori* occurred in an asymmetric manner more similar to *C. crescentus*.

Interestingly, small cells (up to 2.3 µm) predominantly contained clear FtsZ-GFP foci at a single cell pole (Fig. 1B, green triangles). These small cells constitute young cells just after complete cell division because these cells are predominantly found in the exponential growth phase. By contrast, coccoid cells displayed a complete delocalization of FtsZ (data not shown). In this context it is of note that, because of polar FtsZ-GFP foci, viable cells were clearly distinguishable from cells which started dying and therefore became coccoid. Thus, this observation suggests that polar FtsZ-GFP foci relocate from the new pole to mid-cell region during the course of the cell cycle.

In order to confirm these different localizations independently of the GFP-fusion, we performed immunofluorescence analysis in strain 1061, using an anti-FtsZ-antibody against *Corynebacterium glutamicum* FtsZ. This antibody was used because BLAST search using the Comprehensive Microbial Resource (CMR) website (http://cmr.jcvi.org) revealed that *C. glutamicum* FtsZ is more closely related to *H. pylori* FtsZ than *E. coli* FtsZ (58.9% similarity and 38% identity compared to 55.3% similarity and 33.1% identity). Indeed,
immunofluorescence analysis verified symmetric and asymmetric positions of the Z-ring as well as polar foci (Fig. 1C). As a further control we performed immunofluorescence analysis with aztreonam treated \textit{H. pylori} wt cell. Unevenly spaced Z rings in the filamentous cells could be seen (Suppl. Fig. 1B) supporting the asymmetric positioning of the Z-ring.

Thus, our results show that there are two main patterns of localization of \textit{H. pylori} FtsZ: at one single cell pole and in the Z-ring, which can be positioned symmetrically as well as asymmetrically. This finding is strikingly dissimilar to the situation in \textit{E. coli} and \textit{B. subtilis} and cell cycle progression is more reminiscent of that in \textit{C. crescentus}. However, \textit{H. pylori} does not contain homologs of cytokinesis regulation proteins that are abundant in \textit{C. crescentus}, e.g. MipZ and TipN.

\textbf{Asymmetrically positioned Z-ring results in two daughter cells with different size}

Next, we wanted to analyse if the asymmetric localization of the Z-ring results in two daughter cells with different size or if our finding constitutes only a snap-shot followed by increased growth of the smaller cell. Therefore, we measured the cell length of newly divided wild type cells in \textit{H. pylori} strains 1061, 26695 and KE88-3887 using a fluorescent membrane stain. Newly divided cells were defined as two cells which were separated with membrane (which could be easily seen with the membrane stain) but which were still attached to each other. Our measurements demonstrate that the asymmetric position of the Z-ring indeed resulted in two daughter cells of different size (Fig. 1E, please consult Table 3). All three wild type strains displayed the same relaxed type of cell division. In 50\% of the dividing wild type cells the length of the cells were 95 +/- 0.5\% of each other whereas the other 50\% wild type cells had one daughter which was only 80 (+/- 1\%) in length of the other (with about 80 cells analysed for each strain).

In addition, we analysed the division site placement by observing the cell constriction using Scanning Electron Microscopy (SEM) of \textit{H. pylori} cells. AGS cells (human gastric
adenocarcinoma epithelial cell line) were infected with strain KE88-3887. SEM pictures clearly corroborated with the findings that the little precision of the Z-ring positioning causes asymmetric (Fig. 1G, red triangles) as well as symmetric (Fig. 1G, white triangles) cell constriction. Intriguingly, these results demonstrate that this mode of cell division also occurred during *H. pylori* infection. To rule out that growth conditions affect the precision of cell division we grew *E. coli* K12 wt cell both under aerobic and microaerophilic conditions and measured the cell length of newly divided cells according the procedure in *H. pylori*. Cell length was identical with an aberrance of less than 5% in both conditions (data not shown).

These experiments substantiate that the observed asymmetrically localized Z-ring indeed generated daughter cells that differ in size. Furthermore, a possible wild type strain dependent effect could be ruled out.

**H. pylori** flagella do not act as polar marker during cell division

We also analysed the localization of the monopolar flagella of newly divided cells in order to determine whether there is a preferred positioning of flagella during cell division. *H. pylori* possess 2-8 polar flagella, which are covered by a membranous sheath (23). Thus, these flagella are visible by the use of a fluorescent membrane stain. However, it must be pointed out that the flagella are not observed in all cells because they can become sheared during application of cells onto the agarose-containing microscope slides or may be present in a different focal plane from that of the cell body. Analysis of *H. pylori* wt cells during the exponential growth phase revealed that the polar flagella were visible at both ends of newly divided cells irrespective of cell length (Fig. 1H, white triangles). In addition, we found some large cells, which already possessed two polar bundles of flagella, indicating that the formation of flagella occurs prior the placement of a septum (Fig. 1H, yellow triangles). It was not possible to define the precise time point of flagella formation during cell cycle progression caused by the fastidious growth requirements of this bacterium (see below).
Fluorescence recovery after photo bleaching (FRAP) of the Z-ring and the polar FtsZ foci

In *E. coli* and *B. subtilis*, the Z-ring shows high turnover inside live cells, with fluorescence after photobleaching (FRAP) half times of 6-9 s (24). In order to study the *in vivo* dynamics of the Z-ring in *H. pylori* we used FRAP to examine the dynamics of the *H. pylori* FtsZ polymers. To facilitate data analysis, the fluorescence intensity in the region of interest (ROI) was normalized to the fluorescence of the entire cell at each time point; these ratio values were subsequently renormalized to the prebleach ratio. One representative FRAP time series is shown in Fig 2A (left side). We bleached half of a Z-ring and calculated a half time of recovery of about 10 s (n=5) (Fig. 2A right side), which is similar to those measured in *E. coli* and *B. subtilis*. Nevertheless, this result is interesting as far as *H. pylori* has a considerably slower cell cycle with a generation time of about at least 3 hours (25) in comparison to a maximal doubling time of twenty minutes in *E. coli*. Therefore our results indicate that the duration of the cell cycle is independent of the turnover rate of the Z-ring.

Next we performed FRAP experiments of FtsZ at its polar location. We bleached a small area close to a cell pole and monitored the recovery of fluorescence. Figure 2B (left side) shows an example of a FRAP experiment. Interestingly, we calculated a half time of recovery of about 18 s (n=5) (Fig. 2B right side). Therefore we assume that these foci are at least ordered structures. Also, these structures were distinct from the filaments in the Z-ring as the half time was almost twice as long.

Monitoring of FtsZ during cell cycle progression in *H. pylori*

In order to visualize cell cycle progression in *H. pylori* we monitored cell division under the microscope using a heating stage at 32°C and a CO₂ atmosphere of 5% producing a microaerophilic milieu. Due to the fastidious requirements of *H. pylori* to his environment, monitoring was not possible over a complete cell cycle in this human pathogen; however, we
were able to perform time lapse microscopy, in which we could follow FtsZ-GFP (Fig. 3) over different time periods. Thus, it was possible to observe FtsZ-GFP moving from the polar localization (Fig. 3, white triangles) to the localization where the Z-ring was build (Fig. 3, yellow triangles). This confirms that the polar foci of FtsZ-GFP are functional and that the polar accumulation of FtsZ is indeed part of the cell cycle progression in *H. pylori*.

Interestingly, this movement seemed to follow a spiral pattern (Fig. 3, white asterisks), which appears to be similar to results obtained in *E. coli*, *B. subtilis* and *Streptomyces coelicolor*, in which FtsZ also localizes in dynamic helical pattern associated with repositioning of Z-ring (26). These helical FtsZ patterns in *H. pylori* could be visualized as a clear distinct pattern (Fig. 3) because there was very little background fluorescence within the cell. In the numerous time lapse experiments taken, the FtsZ spirals condensed at central as well as at peripheral positions in a random manner.

FtsZ filaments of *H. pylori* and of *E. coli* differ in the heterologous system of S2 Schneider cells.

Previously, we have shown that the heterologous system of *D. melanogaster* S2 Schneider cells (derived from macrophages) is convenient to study filamentation properties of different cytoskeleton elements in vivo (14). In order to characterize filament architecture and filament dynamics, we transfected S2 cells with plasmids (listed in table 1) containing *H. pylori* or *E. coli* FtsZ-YFP in combination with the respective wild-type FtsZ. Co-induction of wild-type and tagged proteins was used to avoid tag-artefacts. For *E. coli* straight and rarely branched filamentous structures could be observed soon after induction of transcription (Fig. 4A lower panel). Further induction resulted in long filaments that were ordered in parallel and exclusively found underneath the membrane, which was shown by imaging of different Z-planes within cells (Fig. 4A upper panel, left side). Interestingly, some of these filaments reached a length of more than 20 µm causing striking cell extrusions (Fig. 4A upper panel,
right side). The localization underneath the membrane, which enables the highest possible straightness of these filaments as well as the caused protrusions, support the idea of a remarkable stiffness of *E. coli* FtsZ filaments in S2 Schneider cells. Qualitative FRAP experiments demonstrated that fluorescence recovery occurred within one minute indicating functional filaments with subunit turnover (Fig. 4A lower panel).

Contrarily, *H. pylori* FtsZ filaments were found to have two distinct patterns. Either FtsZ-YFP filaments were curled (Fig. 4B upper panel, left side) or straight (Fig. 4B upper panel, right side). Interestingly, there were no cells with mixed types of filaments. Furthermore, even straight filaments differed from *E. coli* filaments as they were completely detached from the membrane and never caused cell extrusions. These filaments were also seen by immunofluorescence using the anti-FtsZ-antibody against *Corynebacterium glutamicum* FtsZ supporting the specificity of this serum (Fig. 4C). Qualitative FRAP experiments confirmed the subunit turnover of both kinds of filaments (Fig. 4B lower panel and data not shown).

Surprisingly, it was possible in some cases to follow filament polymerisation via time lapse microscopy (Fig. 4D). Therefore it was possible to calculate overall filamentation speed by measuring filament extension over time (n=5). The average of polymerization/depolymerization was 0.35 ± 0.04 μm/min with a maximum of 0.4 μm/min.

Thus, our results demonstrated that *H. pylori* and *E. coli* FtsZ filaments have different filamentation properties in vivo in the heterologous system of *D. melanogaster* S2 Schneider cells, suggesting unique intrinsic characteristics of each protein despite their common function.
This report provides the first cell biological analysis of FtsZ dynamics in the human pathogen *Helicobacter pylori* and even in ε-proteobacteria, a group of organisms that has hardly been studied at the cell biological level so far. Indeed, recent research discerned that biological strategies employed by model organisms, which have contributed greatly to our knowledge of basic biology and pathogenesis, do not always represent those of other bacterial species as these model species represent only a small fraction of the known bacterial diversity (27).

By using an *ftsZ-gfp* fusion expressed from the original gene locus we demonstrate that approximately 50% of the *H. pylori* cells show clearly asymmetrically localized FtsZ rings. However, even in cells with an apparent mid-cell Z-ring there was 10% variation of Z-ring positioning, which is much larger than the at most 5% variation observed in *E. coli* or *B. subtilis* (22). Furthermore, measurement of the cell length of newly divided wild type cells revealed, that the asymmetrically positioned Z-ring indeed resulted in daughter cells of different lengths. We confirmed our findings by observing the cell constriction of *H. pylori* cells by the use of Scanning Electron Microscopy (SEM) during infection of a human gastric adenocarcinoma epithelial cell line. Moreover, these results demonstrate that asymmetric cell constriction also occurred during *H. pylori* infection in this model system. Thus, in half of the cases *H. pylori* FtsZ ring localizes asymmetrically, giving rise to two daughter cells of different size, which leads to the suggestion of an asymmetric cell division. However, we cannot exclude the possibility of the asymmetry arising from differential growth of the daughter cell compartments. These resulting daughter cells are of different size but seem to have an apparent identical function. On the other hand, there could be a not yet identified difference in cell fate of these cells which will be subject of further studies.

In contrast to *E. coli*, which has flagella in a peritrichous arrangement, *H. pylori* has to define the localization of the formation of the new flagella. However, *H. pylori* does not contain a TipN homologue, which regulates cell polarity and therefore the placement of the
flagellum in *C. crescentus*. In any case, it is clear that both, large and small, *H. pylori* daughter cells are indistinguishable in terms of obtaining the new flagella machinery, because flagella were always observed at both cell poles, even shortly before cell division. These findings suggest that asymmetry may be a stochastic process, although it is possible that asymmetric factors exist which have not yet been identified.

In addition, small cells often displayed polar FtsZ-GFP foci. Time-lapse-analyses following the movement of FtsZ from its polar position to mid-cell suggest that FtsZ relocates from the new pole to the mid-cell region, forming spiral-like intermediates, during the course of the cell cycle. This behaviour is reminiscent of the dynamics of FtsZ in *C. crescentus*. However, *H. pylori* does not contain MipZ, which regulates FtsZ localization in *C. crescentus* (13), but instead has MinC, MinD and MinE, which prevent the formation of a Z-ring close to the cell poles in *E. coli* and *B. subtilis* (28). These observations show that cell division in *H. pylori* follows a different route from that in *E. coli* and *B. subtilis*, and is also dissimilar to that of *C. crescentus*.

Previous research using FRAP (fluorescence recovery after photobleaching) analysis revealed that the Z-ring is very dynamic and that subunits in the Z-ring are exchanging with those in the cytoplasm on a time scale of 8 to 11 s (29). As the turnover rate was very similar in both *E. coli* and *B. subtilis*, it has been suggested that it is a common feature in bacteria. Consistent with this assumption we found a half time recovery of 10 s of the Z-ring in *H. pylori* despite a considerably slower cell cycle (25) of this bacterium. In contrast to this, FRAP analysis of the Z-ring in *Mycobacterium smegmatis*, which has a slower cell cycle as well, gave an average turnover half time of 34 s, with a broad spread from 10 to 70 s (29). However, these authors already considered that the FRAP data for *M. smegmatis* were much more scattered than for *E. coli*, and that they had to exclude 20% of the measurements (30). In addition, we also performed FRAP experiments of the polar FtsZ foci in *H. pylori*. Interestingly, we calculated a twofold slower turnover half time for this ill-defined structure.
In *E. coli*, the dynamics of FtsZ outside of the Z-ring are fast, indicating freely diffusing molecules (12, 31). These findings demonstrate that the situation of FtsZ outside of the Z-ring in *H. pylori* is different from that in *E. coli* and that these foci are at least partially ordered structures. However, whether these structures were distinct from the filaments in the Z-ring or associated with a distinct set of proteins regulating their dynamics in a different manner will be subject of further studies. Time-lapse experiments confirmed that the polar foci of FtsZ-GFP are functional and that the polar accumulation of FtsZ was indeed part of the cell cycle progression in *H. pylori*.

Furthermore, we characterized and compared filament architecture and filament formation of both *H. pylori* and *E. coli* FtsZ in the heterologous system of *D. melanogaster* S2 Schneider cells (14). Whereas *E. coli* FtsZ built up long filaments that were ordered parallelly and were exclusively found underneath the cell membrane, *H. pylori* FtsZ filaments were found in the cytoplasm detached from the membrane in two distinct patterns, which were either curled or straight. One possible explanation for these two distinct filament architectures might be a concentration-dependent switch in polymerization. Hence, single curled filaments were seen in case of low fusion protein amount. By exceeding a threshold concentration, the same single filaments intercoiled and therefore gave rise to a bundle of filaments. This bundle would have appeared as a single smooth structure due to the resolution of fluorescence microscopy. The finding that, indeed, curled filaments displayed a lower fluorescence intensity, compared to the straight structures, supports our model. Previous studies have shown that *E. coli* FtsZ requires a membrane-tether to attach to the membrane (10). We therefore assume that localization underneath the membrane occurred because this position allows the highest possible straightness of FtsZ filaments being longer than the cell diameter of the round S2 cell. Thus, this localization as well as the finding that some of these filaments caused striking cell extrusions suggests a remarkable stiffness of *E. coli* FtsZ filaments in S2 Schneider cells.
To summarize, we suggest a model of cell division in *H. pylori*, in which FtsZ accumulates at one cell pole after a complete cell division and from which it starts moving to the next localization of cell division building up the Z-ring. Thereby, FtsZ ring is positioned with little precision, such that central as well as acentral rings can be observed. Daughter cells showed considerably different sizes, suggesting that *H. pylori* divides asymmetrically. Overall, our results provide evidence that the cell cycle of *H. pylori* is clearly dissimilar to the *E. coli* cell cycle, and more similar to that of *C. crescentus*, in spite of a dissimilar division machinery, which has important implications for the future research on the human pathogen.

ACKNOWLEDGMENTS

We thank Dr. Marc Bramkamp for providing the anti-*C. glutamicum* FtsZ-antibody, Maren Lingnau for technical assistance, Jihad El Andari for technical assistance concerning S2 Schneider cell culture and Prof. Peter Graumann for helping with fluorescence microscopy and writing of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (WA2574/1-1, WA2574/1-2 and FOR 929).

REFERENCES


Table 1: strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid, primer</th>
<th>Relevant characteristics</th>
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<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
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<td></td>
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<td>F, φ80lacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(k2, mK), phoA, supE44, λ, thi-1, gyrA96, relA1</td>
<td>Bethesda Research Laboratories</td>
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<td><strong>H. pylori</strong></td>
<td></td>
<td></td>
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<tr>
<td>26695</td>
<td>wt, containing the entire cag PAI</td>
<td>(32)</td>
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<tr>
<td>1061</td>
<td>Wt</td>
<td>(33)</td>
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<td>1061, ftsZ-gfp (at original locus)</td>
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<tr>
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<td>(34)</td>
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<td>(16)</td>
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<td>bla, PxyI-ftsZ-gfpmut1, cat</td>
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<td>pFD1</td>
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<td>P.L. Graumann (unpubl. results)</td>
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<td>Hp ftsZ down S</td>
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### Table 2: distance of the division septum to the two old cell poles in dividing cells

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<tr>
<th>Z-Ring position</th>
<th>Average size of the small part in percentage of the larger part</th>
<th>Total cell length [µm]</th>
<th>Distance to Z-Ring [µm]</th>
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<td></td>
<td></td>
<td>large</td>
<td>small</td>
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<tr>
<td>Out of centre</td>
<td>70.3 %</td>
<td>3.8 +/- 1.15</td>
<td>2.3 +/- 0.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1.6 +/- 0.4</td>
</tr>
<tr>
<td>In centre (+/- 10%)</td>
<td>90.7 %</td>
<td>3.3 +/- 1.0</td>
<td>1.8 +/- 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.6 +/- 0.5</td>
</tr>
<tr>
<td>Total</td>
<td>80.8 %</td>
<td>3.6 +/- 1.17</td>
<td>2.0 +/- 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.6 +/- 0.5</td>
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### Table 3: cell length of newly divided wild type cells

<table>
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<th>Strain</th>
<th>Cell division</th>
<th>Average size of the small cell in percentage of the larger cell</th>
<th>Total cell length [µm]</th>
<th>Distance to cell constriction [µm]</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>large</td>
<td>small</td>
</tr>
<tr>
<td>26695</td>
<td>symmetric</td>
<td>95.7 %</td>
<td>4.5 +/- 0.6</td>
<td>2.3 +/- 0.3 2.2 +/- 0.4</td>
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<td></td>
<td>asymmetric</td>
<td>81.0 %</td>
<td>4.6 +/- 0.9</td>
<td>2.5 +/- 0.6 2.0 +/- 0.4</td>
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<tr>
<td>KE</td>
<td>symmetric</td>
<td>94.7 %</td>
<td>4.3 +/- 0.5</td>
<td>2.2 +/- 0.3 2.0 +/- 0.3</td>
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<tr>
<td></td>
<td>asymmetric</td>
<td>80.5 %</td>
<td>4.6 +/- 0.7</td>
<td>2.5 +/- 0.4 2.0 +/- 0.3</td>
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<tr>
<td>1061</td>
<td>symmetric</td>
<td>94.6 %</td>
<td>4.6 +/- 0.9</td>
<td>2.4 +/- 0.5 2.3 +/- 0.4</td>
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<tr>
<td></td>
<td>asymmetric</td>
<td>78.5 %</td>
<td>4.0 +/- 0.9</td>
<td>2.3 +/- 0.5 2.0 +/- 0.4</td>
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Fig. 1: A) Western Blot analysis of puromycin treated *H. pylori* cells expressing FtsZ-GFP with antibodies to GFP. Incubation time of puromycin is indicated. GFP-control (GFP fusion protein of crescentin served as positive control) and 1061 wt cells (negative control) are marked. B) Immunofluorescence images with anti *C. glutamicum* FtsZ antiserum. White triangles indicate localization of the FtsZ-ring at mid cell, red triangles indicate an asymmetric localization of the FtsZ-ring, and green triangles indicate polar foci of FtsZ in small cells, asterisks indicate speckled/helical FtsZ pattern. C) Fluorescence microscopy of *H. pylori* 1061 cell expressing the FtsZ protein as FtsZ-GFP fusion. White triangles indicate localization of the FtsZ-ring at mid-cell, red triangles indicate an asymmetric localization of the FtsZ-ring, and green triangles indicated polar foci of FtsZ in small cells, asterisks indicate speckled/helical FtsZ pattern. D) Overlay of FtsZ-GFP (green) and membrane strain (red). Red triangles indicate asymmetric localization; black triangles indicate mid-cell localization. E) Membrane stains of small (and thus young) cells supporting asymmetric division. Numbers indicate average size of smaller daughter cell relative to the larger cell. F) Z-stack of a straight *H. pylori* cell displaying different sections of the asymmetric localization of the FtsZ-Ring. G) Scanning Electron Microscopy (SEM) of *H. pylori* cells during infection of AGS (human gastric adenocarcinoma epithelial cell line) cells. Asymmetric cell division is indicated by red triangles, symmetric cell division is marked with white triangles. H) Membrane stain of exponentially growing *H. pylori* wt cells. White triangles indicate localization of flagella in newly divided cells; yellow triangles indicate flagella in cells prior to septum formation. Scaling bars in all images are 2 µm unless indicated.

Fig. 2: FRAP experiment on FtsZ-GFP-expressing *H. pylori* 1061 cells. A) FRAP of the H. pylori Z-ring. B) FRAP of polar FtsZ foci. The right side shows one representative FRAP time series and the left side demonstrates the mean value of the normalized fluorescence.
intensity plotted against time of at least 5 independent experiments. Pre, before bleaching; post, after bleaching; seconds after bleaching are indicated; circles indicate the area of bleaching.

Fig. 3: Time lapse microscopy of FtsZ-GFP; white triangles indicating polar foci of FtsZ; yellow triangles indicating the Z-ring. Spiral patterns are marked with white asterisks, numbers indicate the time in minutes; FM, membrane stain FM4-64; the lower panel shows an overlay of FtsZ-GFP fusion (green) and membrane stain FM4-64 (red). Grey scaling bars 2 µm.

Fig. 4: Expression of FtsZ-YFP in combination with wild-type FtsZ in S2 Schneider cells. A) *E. coli* FtsZ B) *H. pylori* FtsZ. Upper panels demonstrate distinct morphologies; lower panels show representative FRAP experiments. C) Immunofluorescence images of *H. pylori* expressing S2 Schneider cells with anti *C. glutamicum* FtsZ antiserum D) time series follow filament polymerisation of *H. pylori* FtsZ. Scaling bars 5 µm and 2 µm (C) respectively.
Fig. 2

A

B

Pre  Post  7.8 s  15.3 s  31.8 s  41.8 s

Pre  Post  8.1 s  15.1 s  31.1 s  41.1 s