A Novel System of Cytoskeletal Elements in the Human Pathogen *Helicobacter pylori*

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Abstract

Pathogenicity of the human pathogen *Helicobacter pylori* relies upon its capacity to adapt to a hostile environment and to escape from the host response. Therefore, cell shape, motility, and pH homeostasis of these bacteria are specifically adapted to the gastric mucus. We have found that the helical shape of *H. pylori* depends on coiled coil rich proteins (Ccrr), which form extended filamentous structures *in vitro* and *in vivo*, and are differentially required for the maintenance of cell morphology. We have developed an *in vivo* localization system for this pathogen. Consistent with a cytoskeleton-like structure, Ccrr proteins localized in a regular punctuate and static pattern within *H. pylori* cells. Ccrr genes show a high degree of sequence variation, which could be the reason for the morphological diversity between *H. pylori* strains. In contrast to other bacteria, the actin-like MreB protein is dispensable for viability in *H. pylori*, and does not affect cell shape, but cell length and chromosome segregation. In addition, mreB mutant cells displayed significantly reduced urease activity, and thus compromise a major pathogenicity factor of *H. pylori*. Our findings reveal that Ccrr proteins, but not MreB, affect cell morphology, while both cytoskeletal components affect the development of pathogenicity factors and/or cell cycle progression.

Introduction

*Helicobacter pylori* is a Gram negative, highly motile, microaerophilic, spiral-shaped organism, which colonizes the stomachs of at least half of the world’s population [1]. Infection of humans results in persistent gastritis, which can develop into peptic ulcer disease and adenocarcinoma [2,3]. Motility is a key factor in the adaptation of infection, allowing for the penetration of the mucus and enabling the bacteria to colonize and to persist in the gastric lumen [4]. Both spiral shape and flagella contribute to the motility of this human pathogen. Whereas flagella of *H. pylori* have been studied intensively, our knowledge of the maintenance and establishment of spiral structure in *H. pylori* and in fact for any bacterium is marginal. In addition, nothing is known about any cytoskeletal protein in this pathogen.

Maintenance of cell morphology is highly important or essential for functioning and survival of most eukaryotic and prokaryotic cells. For many eukaryotic cells, it is also vital to be able to change the shape of the cell, and/or to be able to move via flexible extension/retraction of the cell membrane. Cytoskeletal elements actin and intermediate filaments are key elements of the eukaryotic cytoskeleton that controls cell morphology and cell rigidity. Due to its rapid polymerisation/depolymerization properties, actin is the driving force for motility involving membrane rearrangements, and is also involved in trafficking of vesicles and in cell division [5]. IF proteins, on the other hand, are characterized by extended coiled coil regions. The proteins are believed to be highly elongated and assembled into sheet structures based on extensive interactions between coiled coils [6]. IF like proteins provide mechanical strength to e.g. skin or blood vessel cells, and are involved in positioning of cellular organelles [7].

For most rod shaped bacteria analysed so far, the loss of genes affecting cell shape is lethal. *Escherichia coli* or *Bacillus subtilis* cells are unable to grow as round cells, into which they turn when gene products of *rodA*, *mreB*, or *mreC* are depleted. While RodA and MreC are membrane proteins, whose function is still unclear, MreB is an actin like protein that forms filaments *in vitro*, dependent on ATP [8]. *In vivo*, MreB forms filamentous helical structures underneath the cell membrane [9,10]. In *B. subtilis* and in *Caulobacter crescentus*, these filaments are highly dynamic, and appear to move along the membrane with dynamics similar to those of eukaryotic actin [11,12]. Movement of filaments is most likely based on ratchet-like extension of filaments at one end, and depolymerization (and thus shrinkage) at the other end. *E. coli* MreB and an MreB ortholog, Mbl, in *B. subtilis*, have been shown to interact with MreC [13,14], which in turn appears to interact with enzymes that synthesize the extension of the murein sacculus [15]. Because the incorporation of new cell wall material occurs in a helical pattern [16], it has been proposed that the helical organization of MreB filaments in the cytosol may direct the helical localization of cell wall synthetic proteins within the periplasm/outside the cell. A disputed question is the effect MreB exerts on the segregation of duplicated chromosomes. Interfering with MreB levels or polymerization activity has been shown to
Author Summary

The human pathogen Helicobacter pylori lives in the hostile environment of the human stomach. H. pylori possesses a spiral shape and high motility that enable the bacterium to swim through the stomach lumen and to come into close contact with epithelial cells. High urease activity in the bacterium counterbalances the low pH within the stomach, in order to persist within the viscous mucus layer. In this work, we analysed the molecular basis of the spiral structure of H. pylori. We demonstrate that the helical cell shape depends on so-called coiled coil rich proteins (Ccrrp), which form extended filamentous structures in vitro and in vivo, and are differentially required for the maintenance of proper cell morphology. In most bacteria analysed so far, the actin-like protein MreB affects cell morphology. Contrarily, H. pylori MreB is not involved in the maintenance of cell shape, but affects the progression of the cell cycle. Mutant cells were highly elongated, characteristic for a delay in cell division, and contained non-segregated chromosomes. The persistence of H. pylori in the hostile environment of the human stomach depends on the activity of urease. Interestingly, mreB mutant cells displayed significantly reduced urease activity, revealing a novel connection between the cytoskeletal element and an enzyme, and thus with pathogenicity. These experiments show that H. pylori has a novel type of system setting up helical cell shape, which has not yet been described for any bacterium. Our work will allow studying H. pylori cell cycle and pathogenicity at a new visual level.

strongly impair chromosome segregation in several organisms [10,17], but arguments against a direct involvement of MreB in segregation have also been put forward [18].

The question of how bacterial cells can obtain a curved shape has only been investigated in the vibrio-shaped bacterium C. crescentus. Ccs encodes for a coiled coil protein, crescentin, which has high similarity to IF proteins. Crescentin forms filamentous structures in vitro without the addition of any nucleotides. Deletion of ccs leads to the generation of straight cells, and thus to loss of cell curvature, while the culture doubling time or any other obvious physiological aspect of the cell is not affected [19]. Crescentin localizes as a defined ribbon structure along the short side of the cells, suggesting that it forms a filamentous structure in vivo [19]. Recent evidence suggests that crescentin exerts its effect on cell curvature through mechanical control of cell growth [20].

In this work, we set out to analyse cytoskeletal elements in the human pathogen Helicobacter pylori. We have systematically inactivated genes encoding coiled coil-rich proteins, and for mreB. Surprisingly, deletion of mreB is not lethal, but affects a variety of cellular parameters, such as chromosome segregation, but not cell shape. Deletions of Ccrrp (coiled coil rich proteins) genes have different effects on cell shape in different strains, from loss of helical shape to complete loss of a regular morphology. We have also established a system for the visualization of proteins in H. pylori, and show that Ccrrp proteins have a specific pattern of localization, consistent with their function in cell shape maintenance.

Results

Genetic organization of genes predicted to encode for coiled coil rich proteins potentially serving as cell shape determinants

To gain insight into the question of how H. pylori gains its helical cell shape, we searched for elements similar to known cytoskeletal or cell morphological elements. Chromosomes of all H. pylori strains analysed contain a gene with high similarity to mreB, followed by a mreC gene. Like in E. coli and B. subtilis, the MreC gene product is predicted to contain a single membrane span, and coiled coil regions. No mreD gene could be found in the genomes, but a mreA like gene, and several phb genes (not shown). Interestingly, all strains contain two genes that have already been suggested to encode for IF-like proteins (HP0059 and HP1143 in strain 26695) [19], which are predicted to contain several extended heptad repeat regions, but also a so-called stutter, where coiled coil 4 is clearly discontinued for few amino acids [21]. However, HP0059 is almost entirely composed of heptad repeat regions, and lacks the characteristic N- and C-terminal domains of IF proteins, which are predicted to be globular. According to their predicted secondary structure we suggest to term this class of proteins as “coiled coil rich proteins” (Ccrrp). We designate the H. pylori HP0059 or HP1143 gene products as Ccrrp59 or Ccrrp1143, respectively.

Because of the genetic (and morphological) variability of Helicobacter pylori, we generated constructs in several different strains, to obtain information on the general validity of gene deletions or localization patterns of fusion proteins. We focussed our work on the reference strain 26695 (moderately motile), on KE88-3887, a hyper-motile variant of strain 26695, and on the clinical isolates G27 and 1061, all of which are relatively well amenable for genetic analysis.

Deletion of HP0059 abolishes helical cell shape in several strains, while deletion of HP1143 affects cell morphology in strain 1061

It should be noted that H. pylori strains have somewhat different morphologies. Strain 26695 is highly helical (Fig. 1A) (similar to KE88-3887, Fig. 2D), with an average of length of 3.0 +/- 0.5 μm (n = 72) and can be up to 4.0 μm in length, while cells of strain 1061 are much shorter with an average of length of 2.3 +/- 0.5 μm (n = 100) and their helical shape is less pronounced (compare Fig. 2A). Other strains of H. pylori also have varying degrees of cell curvature.

In order to study possible functions of genes predicted to encode for cell shape determinants, we inactivated genes HP0059 and HP1143 in H. pylori strains 26695, KE88-3887, G27 and 1061. To ensure expression of the downstream genes, all genes were disrupted by insertion of a cat gene driven by its own promoter but lacking a terminator. Growth analysis of all mutants revealed that inactivation of none of these genes showed any effect on the growth rate of H. pylori.

Interestingly, the inactivation of gene HP0059 resulted in the formation of 100% straight rods in strains 26695, KE88-3887 and G27 (Fig. 1B, Fig. 2E, and data not shown for G27), or 85% straight cells in strain 1061 (Fig. 2B), revealing a complete loss of the spiral shape in the absence of the HP0059 gene product. To rule out a possible downstream effect on gene HP0060, HP0060 was disrupted by introducing a pcat cassette. No change in growth or cell curvature could be detected compared with wild type cells (data not shown) showing that the loss of helical cell shape is due to the inactivation of gene HP0059. The same observation was made with strains 1061, KE88-3887 and G27 (data not shown). Inactivation of HP1143 had a mild effect on cell shape. Whereas 10 to 15% of 26695 wild type cells were straight (Fig. 1A), about 60% of HP1143 mutant cells were straight (Fig. 1G, or 52% for strain KE88-3887, Fig. 2F). These experiments show that the loss of genes HP0059 or of HP1143 affects cell curvature to different extents.
The deletion of HP1143 had an even more dramatic effect on cell shape in 1061 cells, about 70% of the cells were round, oval or irregularly shaped, while the remaining cells were straight or bulgy rod shaped (Fig. 2C). Single non-aggregated cells were basically undetectable. Thus, deletions of genes HP0059 and HP1143 have different effects on cell shape in different strain backgrounds. Absence of HP0059 generates loss of cell curvature in all strains tested, while lack of HP1143 results in a complete loss of cell shape in strain 1061.

*H. pylori* undergoes a transition from helical cells to coccoid cells upon prolonged starvation. We analysed whether *ccr* mutant cells influence this morphological transition, whose mechanism is still poorly understood. Like wild type cells, all HP0059 or HP1143 mutant cells were coccoid 7 days after inoculation (i.e. 5 days into stationary phase), showing that the inactivation of *Ccr* encoding genes does not influence the helical to coccoid transition.

Deletion of both, HP0059 and of HP1143 exacerbates the cell shape defect in strain 26695

To investigate if HP0059 and HP1143 are genetically linked, we generated a strain from the parent 26695, in which both genes are deleted. Interestingly, cells of the double mutant strain displayed a

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**Figure 1. Deletion of genes encoding for Ccrp proteins in strain 26695.** Images show fields of cells with Nomarski differential interference contrast (DIC) or with fluorescence of membrane stain. “pcat” indicates deletion of the corresponding gene, “DKO” refers to the double deletion of HP0059 and of HP1143. A) 26695, wild type strain; B) 26695-0059PCAT; C) 26695-1143PCAT; D) 26695-DKO; Black bars 2 μm. doi:10.1371/journal.ppat.1000669.g001
variety of cell shapes: while 65% of the cells were straight, 30% had an irregular curved shape, and 5% had a highly bent shape, such that the cell ends came together (Fig. 1D), which is never observed for wild type cells. For strain KE88-3887, the double deletion resulted in even more highly bent cells (suppl. Fig. S1). These findings show that the loss of both Ccrp encoding genes leads to a complete loss of regular cell shape in strains 26695 and KE88-3887, and exacerbates the phenotype of the single gene deletions. The double deletion of HP0059 and HP1143 in strain 1061 was similar to that of the HP1143 single gene deletion, in that most (>80%) double mutant cells were round, oval or irregularly shaped, while the remaining cells were straight or bulgy rod shaped (data not shown).

Ccrp59 forms filamentous structures in vitro and in vivo

We wished to obtain insight into the biochemical properties of Ccrp proteins. Towards this end, we purified an N-terminally strep-tagged version of Ccrp59 to more than 95% purity (Fig. 3A). Ccrp59 could be purified in very low quantities as a soluble protein upon mild and short time (2 h) induction of the protein in E. coli cells, but appeared in inclusion bodies after prolonged induction. On SDS-PAGE, Ccrp59 migrated as monomer but also as a band that corresponded to a dimer (Fig. 3A), which is apparent in the Western blot in Fig. 3B. When subjected to centrifugation, a major proportion of Ccrp59 appeared in the pellet fraction (Fig. 3C), suggesting that it forms large assemblies. Over time (i.e. days to weeks), the amount of Ccrp59 in the pellet fraction increased (data not shown).
Importantly, time lapse microscopy showed that Ccrp59-GFP signals were not moving through the cells, but were stationary.

Localization of Ccrp59 in H. pylori

We wished to obtain insight into the pattern of localization of cytoskeletal elements in live H. pylori cells. We adapted a system for the generation of GFP fusions for Bacillus subtilis cells to H. pylori, which allowed integration of the fusions at the original locus within the chromosome. This strategy was successful with strain 1061, and in some cases also with 26695, which does not easily take up and integrate plasmid DNA (in contrast to linear DNA).

Ccrp59 was visualized through the generation of a C-terminal GFP fusion that was integrated at the original locus, such that it was expressed under the native promoter, and was the sole source of Ccrp59 within the cells. Cells of strain 26695 expressing Ccrp59-GFP were helical like wild type cells and not straight (Fig. 6A), showing that the fusion was functional, even in the absence of wild type Ccrp59. Discrete Ccrp59-GFP foci could be detected within exponentially growing cells (Fig. 6A–E). Small cells contained 2 to 3 foci, while the number of foci increased with cell size. Foci were not of uniform fluorescence, but showed different intensities. Foci with high fluorescence intensity (indicated by grey triangles in Fig. 6A, C and D) were positioned at relatively regular intervals within the cells, with an average of 0.89 μm ± 0.2 (n = 62) between the foci, and were frequently interspersed with foci of low intensity. Imaging of different Z-planes within cells and ensuing 3D deconvolution suggested that some of the foci were connected with each other (Fig. 6C). Due to the low cell diameter of H. pylori (0.78 μm), and because of the weak fluorescence of Ccrp59-GFP (which allows capturing of only 4–5 Z-planes) it was not possible to clearly determine if the foci are arranged in a helical pattern (which some images suggest), or in which other pattern. However, the data are compatible with a helical localization of Ccrp59 filaments along the long axis of the cells (Fig. 6F).

Ccrp59-GFP also localized in a very similar arrangement in strain 1061 (Fig. 6D), suggesting that the observed localization reflects the true positioning of Ccrp59 in several if not all H. pylori strains.

Importantly, time lapse microscopy showed that Ccrp59-GFP signals were not moving through the cells, but were stationary.
We did not observe any movement of Ccrp59-GFP foci in any of the 120 cells analysed. Thus, Ccrp59-GFP foci are not freely diffusing elements, supporting the idea that they may constitute cytoskeletal elements that are statically localized along the length of the cells.

We have not been able to generate a functional Ccrp1143-GFP fusion. We created a strain derived from 1061 in which a complete Ccrp1143-GFP fusion was integrated into the original locus by single crossover, such that the fusion as well as the wild type gene HP1143 were present within the *H. pylori* chromosome. Between 40 to 60% of these cells showed abnormal cell shape (Fig. 6G), and contained one to two distinct Ccrp1143-GFP foci at random places within the cell (data not shown), suggesting that the Ccrp1143-GFP fusion is dominant negative. These data reinforce the idea that a loss of function of Ccrp1143 leads to aberrant cell morphology.

**Ccrp encoding genes are heterogeneous in size and sequence between different *H. pylori* strains**

As mentioned above, *H. pylori* strains can have different degrees of helical cell curvature and different cell lengths. Occasionally, laboratory strains lose cell curvature altogether and become rod shaped. To investigate if differences in genes encoding for Ccrp proteins may be the basis for this phenomenon, we amplified the gene region of HP0058 up to the beginning of gene HP0060 from different strains and sequenced the PCR products. Interestingly the whole region differs in length from about 1500 bp in strain
26695, 1600 bp in strain J99, about 1000 bp in strain 1061 up to only 550 bp in strain SS1 (mouse adapted). This is in agreement with a previous study that showed that HP0059 is among the most divergent genes in *H. pylori* [22]. Analysing HP0059 (encoding Ccrp59) itself, the size of 855 bp, 984 bp, 750 bp or 500 bp in strains 26695, J99, HPAG1 and 1061, respectively, was deter-

Table 1. Properties of IF and Ccrp proteins.

|                        | Eukaryotic IF-protein | Ccrp59     | Ccrp1143   |
|------------------------|-----------------------|------------|------------|
| Filamentous structures in vitro | +                     | +          | +          |
| Self assembly without cofactors | +                     | +          | +          |
| Filament diameter | 10 nm                 | ~10 nm     | ~10 nm     |
| Bundles of filaments in vitro | No                    | +          | No         |
| pH sensitivity | +                     | n.d.       | +          |
| Structural sequence organization | central coiled coil domain flanked by amino- and carboxy-terminal domains of variable size and structure | central coiled coil part but lacks non-coiled coil N- and C termini | long coiled coil region at its N terminus, which bears some features of IF proteins, but apparently lacks a stutter towards the end of the coiled coil region. |
| Recombinant protein | insoluble, need to be refolded | partially soluble | soluble |

Figure 5. Expression of Ccrp59-YFP in S2 Schneider cells. A) 2 hours after induction of transcription, B) Co-expression of Ccrp59 and of Ccrp59-YFP 2 hours after induction of expression. White triangles indicate branched structures, grey triangle straight filament. The three panels show different Z section of the S2 cell (going from up to down). C) S2 cell 4 hours after induction of Ccrp59 and of Ccrp59-YFP. Right panel is identical to the left panel except for increased fluorescence intensity. Grey bars 2 μm.

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mined. Gene jhp0050 (is similar to HP0059) in strain J99 is 663 bp long. Because strain KE88-3887 is a hyper-motile variant of strain 26695 both strains contain the same HP0059 sequence.

Deletion of \textit{mreB} affects cell division and chromosome segregation, but not cell shape

To our surprise, it was possible to generate a deletion of the \textit{mreB} gene, through a replacement of the gene with a chloramphenicol acetyltransferase cassette. Therefore we inactivated the \textit{mreB} gene in three different strains indicating that this result was not strain dependent. The generated mutant cells were able to grow, albeit at strongly reduced growth rate compared with wild type cells. To rule out an effect on the downstream \textit{mreC} gene, we isolated total RNA from \textit{mreB} mutants from different \textit{H. pylori} strains and performed dot-blot hybridization with probes specific for the \textit{mreC} gene, showing that \textit{mreC} is expressed in \textit{ΔmreB} cells like in wild type cells (Fig. 7A), ruling out a polar effect of the disruption of \textit{mreB}. \textit{MreB} mutant cells were obtained at a similar

\begin{figure}[h]
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\caption{Localization of Ccrp proteins in different strains as indicated underneath the panels. A–E) Ccrp59-GFP. A) Cells expressing Ccrp59-GFP as sole source of the protein are helical like wild type cells, B–C) White triangles indicate possibly connected Ccrp59-GFP signals, C) 4 images taken in different planes as indicated, after 3D deconvolution, grey triangles indicate signals present in some planes and absent in others. D) Ccrp59-GFP in strain 1061, E) Time lapse with numbers indicating minutes between exposures, white triangles indicate distinct signals that do not move during the experiment. F) model for the possible localization pattern of Ccrp59 along the long axis of the cells. G) strain 1061 1143GFP expressing a fusion of Ccrp1143 with GFP. Note that the GFP fusion has a strong influence on cell shape. Grey bars 2 μm.
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\end{figure}
frequency compared with many deletions of non essential genes generated in our laboratory [23], strongly arguing against the generation of secondary suppressor mutations.

The growth rate of mreB mutant cells was severely decreased compared with wild type cells for strain 26695 (Fig. 7B) and for KE88-3887 and 1061 (data not shown). The growth curves of all wild type strains showed a lag phase of about 8 hours and an exponential increase in cell density until at least 25 h, whereas all mreB mutants (i.e. in the 3 different strains) displayed a highly decreased growth rate.

Interestingly, there was no change in cell morphology of mreB mutant cells other than cell elongation in comparison with wild type cells (Fig. 8, compare A with D for 26695, B with E for 1061 and C with G for KE88-3887). MreB deleted cells were still helical and had the same average cell diameter of 0.78 μm (n>100 cells) than that of wild type cells. Cell elongation can be easily seen in Fig. 8F. Interestingly, mreB mutant cells showed a strong defect in the segregation of chromosomes. In contrast to wild type cells of all strain, which contained one, two, or (rarely) three well defined nucleoids (Fig. 8A, B and C), mreB mutant cells contained brightly staining bilobed nucleoids and large DNA free cell regions (Fig. 8D, E and G). Similar to C. crescentus smc mutant cells that have a strong segregation defect [24], no anucleate mreB mutant H. pylori cells were observed. Fluorescence intensity of the bilobed nucleoids in mutant cells was similar to that of separated nucleoids in large wild type cells, while the length of the bilobed nucleoids was twice of that of single segregated nucleoids in wild type cells, showing that the nucleoids in mutant cells contain two largely duplicated chromosomes demonstrating a separation delay. MreB mutant cells from strain 1061 frequently contained a single extended non-segregated nucleoid in spite of the large cell size (Fig. 8E), showing that loss of MreB strongly affects chromosome segregation in H. pylori cells. MreB mutant cells from strain 1061 could reach more than 3 times the normal cell size (Fig. 8F).

Figure 7. Influence of MreB on growth. A) Dot-blot hybridization of total RNA with probes specific for mreC mRNA. Upper dots show wild type cells, lower dots mreB deletion strains. The integrity of whole-cell mRNA was tested by agarose gel electrophoresis (data not shown). B) Growth of H. pylori strains in BBF broth, 26695 wild-type (rhombuses), wild type after addition of the solvent DMSO (squares), wild type after addition of 10 μg/ml A22 (black triangles, note that these cells already show a defect in chromosome segregation) and 50 μg/ml A22 (open triangles), and the isogenic mreB mutant (circles) was determined by measuring the OD600. The data represent mean values from three independent determinations. Standard deviations are indicated. doi:10.1371/journal.ppat.1000669.g007
Figure 8. Deletion of \textit{mreB} leads to a defect in chromosome segregation. A, B, D) overlays of DNA (green) and cell membrane (red). A–C) wild type cells of strains indicated on or underneath the image, D–G) \textit{mreB} deletion strains as indicated at the images. E) membrane stain of \textit{mreB} mutant cells showing cell elongation and presence of polar flagella (right panel). H–I) Membrane or DNA stain of cells 2 h after addition of A22, which inhibits MreB polymerization. J–M) Phase contrast images of cells 4 hours after addition of A22. J) 26695 wild type cells, K) 1061 wild type cells, L) 26695 HP0059 deletion cells, M) 1061 HP0059 deletion cells. Grey bars 2 μm.

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Blocking of chromosome segregation leads to a delay in cell division in bacteria such as *E. coli* or *B. subtilis* [25], suggesting that most likely, the elongation of cells lacking MreB is due to the defect in chromosome partitioning.

To obtain further insight into the function of MreB, we treated *H. pylori* cells with A22, which was reported to mediate disassembly of MreB filaments in *vitro* [26]. Addition of a low amount of A22 (10 µg/ml) to exponentially growing *H. pylori* cells led to the formation of abnormally shaped nucleoids (Fig. 8H), and the addition of 50 µg/ml A22 resulted in a similar phenotype than the mreB deletion: only 2 doubling times after addition of A22, nucleoids no longer separated into two (Fig. 8I), and growth proceeded at an extremely slow rate (Fig. 7B, open triangles). Resuspension of cells after A22 treatment into fresh growth medium fully restored growth of cells, showing that interfering with MreB function transiently and rapidly leads to disturbance of the cell cycle, but does not kill the cells under experimental conditions. The fact that A22 treatment resulted in a phenotype that closely resembles that of an mreB deletion reinforces the idea that the phenotype is not masked by a secondary mutation but is due to the inactivation of MreB activity.

The lack of MreB did not interfere with the formation of polar flagella (Fig. 8F, right panel). Our data suggest that MreB plays a direct or indirect role in the progression of the cell cycle, but not in cell shape determination.

We also examined the possible link between Ccrps and MreB, because the effect of the lack of MreB on cell shape could potentially be masked by the presence of Ccrps. Therefore, we treated HP0059 mutant cells with A22, and visualized the effect of inhibition of MreB on cell shape. While wild type cells of strain 26695 (Fig. 8A) or of strain 1061 (Fig. 8B) remained helical during addition of A22, mutant cells of strain 26695 (Fig. 8A) or of strain 1061 (Fig. 8B) remained straight and rod shaped like the non-treated cells, and displayed the same degree of growth retardation as the wild type cells. These data support the findings that cell shape is not affected by the loss of MreB activity, but is determined by Ccrp proteins.

Deletion of mreB leads to a decrease in urease activity

The persistence of *Helicobacter pylori* in the hostile environment of the human stomach is ensured by the activity of urease. Urease catalyses the hydrolysis of urea into carbon dioxide and ammonia, which are buffering compounds essential to raise the pH in the microenvironment surrounding the cell [27] and to maintain the pH homeostasis in the bacterial cytoplasm [28]. Therefore, enzyme activity is essential for both early colonization events and for virulence [29,30]. To test whether this major pathogenicity factor is affected by cytoskeletal elements, we determined urease activity in mreB mutant cells, and found a statistically significant (p<0.01) 2.5 fold decrease in activity in strain KE88-3887 (Fig. 9A), and a ~6 fold decrease in strain 26695 (data not shown). Interestingly, addition of 1 mM NiCl2 restored urease activity up to wt level (data not shown). Western blot analysis showed that the urease level in the mreB mutant strain is similar to or even higher than that in the parental wild type strain (Fig. 9B). It is unclear how MreB exerts its effect on urease activity, but clearly, loss of this cytoskeletal element compromises *H. pylori* pathogenicity.

**Discussion**

This report provides novel insight into the bacterial cytoskeleton and the function of cytoskeletal elements, and shows that the human pathogen *H. pylori* has a novel type of system for the establishment and maintenance of defined cell morphology. We show that two coiled coil rich proteins (Ccrp) are essential for the maintenance of proper cell shape in *H. pylori*, whereas the actin-like protein MreB is not involved in the generation of helical and/ or rod cell morphology, like in many other bacteria. Deletion of gene HP0059 encoding for protein Ccrp59 resulted in the complete loss of helical cell curvature in strains 26695, KE88-3887, G27 and 1061. Loss of a second Ccrp protein, Ccrp1143, resulted in a mild reduction of cell curvature in strain 26695. However, in strain 1061, lack of Ccrp1143 resulted in a complete failure to maintain cell morphology, mutant cells were round or oval, or irregularly shaped. Thus, Ccrp proteins contribute differentially to cell morphology in different *H. pylori* strains, and are required for the maintenance of cell morphology in *H. pylori*. Intriguingly, in contrast to mreB and ftsZ, Ccrp encoding genes are highly variable both in terms of their length and in sequence between various *H. pylori* strains analysed in this study. It is thus plausible to propose that the great variety of cell shapes of *H. pylori* strains – from small bent cells to large and highly helical cells – stems from the nature of the Ccrp proteins. For example, Ccrp59 is much longer in strain 26695 than in 1061, which produces smaller and less helical cells than 26695. Thus, loss of Ccrp1143 in strain 26695 may be compensated for by Ccrp59, while Ccrp59 of strain 1061 may not be able to do so. Unfortunately, we do not have sophisticated genetic tools at present to test these intriguing ideas, and clearly, the situation is more complicated, because of the differential contribution of Ccrp proteins in different strains.

We show that both Ccrp proteins form extended filaments in *vitro*. Ccrp59 forms bundles of filaments in *vitro*, in the absence of any added cofactor, and is able to form extended filaments in...
macrophage cells, and thus in the absence of any cofactor from \textit{H. pylori}. Ccrp59 bundles clearly consist of parallel stacks of filaments, which appear to be arranged in a staggered fashion, as is proposed to be the case for IF filaments from eukaryotic cells [6]. However, both Ccrp proteins identified in this study are initially soluble proteins when expressed in \textit{E. coli} cells, and Ccrp59 lacks characteristic N and C terminal domains of IF proteins, showing that Ccrps are distinct from IF proteins. Possibly, Ccrp proteins are evolutionarily older versions of IF proteins, or even unrelated to IFs, and possibly a novel class of cytoskeletal elements in bacteria.

Towards a further analysis of Ccrps, we localized Ccrp59 in \textit{H. pylori} cells. We found that a functional Ccrp59-GFP fusion forms distinct foci, whose position did not change over a time of several minutes, along the length of the cells. Thus, Ccrp59 is not a freely diffusing cytosolic protein, but remains at fixed positions, and may thus serve as a cytoskeletal structure that affects cell morphology. Due to the narrow width of \textit{H. pylori} cells and the relatively weak fluorescence of Ccrp59-GFP, it was not possible to unequivocally determine if the foci are arranged in a helical pattern. Because of the fact that Ccrp59 forms extended filaments, which can be longer than \textit{H. pylori} cells, in vitro, and when expressed in \textit{S. coelicolor}, the filamentous structure that affects cell morphology, crescentin forms individual long filaments [19], and not parallel bundles of filaments like Ccrp59. In \textit{C. crescentus} forms a filamentous structure along the short axis of the cell, and likewise actin-like MamK and MamJ in magnetotactic bacteria, which align magnetosomes in a straight line along the short axis of the helical cells [32,33]. On the other hand, the cytoskeletal element CipA, found exclusively in spirochaetes, is part of filaments running along the long axis of the highly helical cells [34], which even persist and retain their helical path when the cells are gently lysed. Interestingly, CipA is also predicted to contain a high degree of coiled coils. It will be important to determine the nature of the foci formed by Ccrp59-GFP within the cells, and to identify factors that interact with Ccrps in \textit{H. pylori}, to find out how the proteins mediate the generation of helical curvature of the cells.

In \textit{C. crescentus}, the IF-like protein crescentin is essential for the generation of cell curvature [19], while MreB is indispensable for the maintenance of rod shape, in striking contrast to \textit{H. pylori}, where cell shape depends on two Ccrps, but not on MreB. Moreover, Ccrp59 is clearly different from crescentin, because crescentin forms individual long filaments [19], and not parallel bundles of filaments like Ccrp59. In \textit{S. coelicolor}, the filament-forming coiled coil rich protein FilP affects cell rigidity, but not cell shape [35], while MreB is involved in differentiation (sporulation), but does not play a role during vegetative growth [36]. Our findings show that \textit{H. pylori} employs a novel concept for the generation of complex cell shape and suggest that Ccrp proteins may set up complex cell shape in many other bacteria that contain MreB (which may serve different functions), and also in bacteria lacking mreB, such as Corynebacterium, which is rod shaped.

We also addressed the question of the function of MreB in \textit{H. pylori}. MreB mutant \textit{H. pylori} cells are viable, but grow much more slowly than wild type cells. Strikingly, mutant cells contained non-segregated but strongly fluorescent (and thus duplicated) chromosome, and were highly elongated. Because a defect in chromosome segregation leads to a delay in cell division, cell elongation in \textit{mreB} mutant cells most likely stems from the delay in cell cycle progression. Thus, in \textit{H. pylori}, MreB affects chromosome segregation, but not cell shape, while in other bacteria, the observed defect in chromosome segregation may be due to an indirect effect caused by the loss of cell shape. Strikingly, \textit{mreB} mutants contain considerably lower levels of urease activity, whereas the amount of urease is unchanged. At present, we have no clear indication as to how MreB might affect the activity of an enzyme. Possibly, MreB affects the activity of membrane proteins such as transporters, and the absence of MreB may thereby change intracellular levels of metals and ions. Indeed, a deletion of \textit{mreB} in \textit{B. subtilis} can be rescued by the addition of high concentrations of magnesium and sucrose [18], and urease activity in \textit{H. pylori} \textit{mreB} mutant cells can be rescued by an increase in the concentration of nickel, which is a limiting factor for the enzyme [37]. In any event, our findings severely alter the spectrum of cellular functions affects by MreB. Because high urease activity is a prerequisite for colonization and persistence of \textit{Helicobacter pylori} in the hostile environment of the human stomach, we establish for the first time a connection, directly or indirectly, between the bacterial cytoskeleton and a pathogenicity factor.

To verify the different contributions of Ccrps and MreB in \textit{H. pylori}, we added the MreB inhibitor A22 to HP0059 mutant cells. The addition of A22 resulted in slow growth in the mutant cells, which however retained their rod cell shape. Therefore, cytoskeletal elements in \textit{H. pylori} strongly affect cell shape (Ccrps) and growth/pathogenicity (MreB), which emphasizes the potential to generate antibacterial chemicals by screening for compounds that affect the assembly of MreB and Ccrp proteins. The study of \textit{H. pylori} at the level of cell biology and the investigation of its cytoskeleton has revealed a novel type of system for cell shape maintenance, and point to additional interesting features of its cell cycle that deserve further investigation.

### Materials and Methods

#### Bacterial strains and growth conditions

Bacterial strains are listed in suppl. Table S1. \textit{H. pylori} strains were routinely cultivated on Dent blood agar in a microaerobic atmosphere as described earlier [37]. Growth experiments were performed in Brucella broth with 5% fetal calf serum (BBF). Bacteria were precultured to an optical density at 600 nm (OD600) of approximately 1.0 in BBF and subsequently diluted 1:150 in test media. Growth rate was assessed by optical density (OD600). All growth experiments were performed in triplicate and were repeated at least three times. \textit{E. coli} strains were grown aerobically at 37°C in Luria-Bertani medium. When appropriate, growth media were supplemented with 50 \( \mu \)g/l ampicillin (Ap) or 20 \( \mu \)g/l chloramphenicol (Cm).

#### DNA techniques and mutagenesis of \textit{H. pylori}

Restriction and modifying enzymes (New England Biolabs, USA) were used according to the manufacturer’s instructions. Cloning was performed in \textit{E. coli} according to standard protocols. Plasmids were isolated with a QIAprep Spin Miniprep Kit from Qiagen (Qiagen 27104). The chloramphenicol-acetyl-transferase gene \textit{catAc} with \textit{Pcat} promoter were amplified by PCR from plasmid pTnMax5 (suppl. Table S1) using primer CATS1 in combination with the primer CATAS1 (suppl. Table S2). The \textit{Pcat} gene were fused to upstream and downstream DNA regions of mutagenized genes by using a modified version of the megaprimer PCR protocol [38,39] as described earlier [23,40,41].
exchange mutagenesis of *H. pylori* was performed by electroporation or natural transformation according to standard procedures [42]. *H. pylori* mutants carrying the *PilA* gene inserted into the chromosome were selected by growth on Dent blood agar containing chloramphenicol (Cm) at concentrations of 20 mg/l. Correct insertion of *cat* and *PilA* was verified by PCR analysis with appropriate primers listed in suppl. Table S2. All fluorescent tag vectors (see Protocol S1) were integrated into the *H. pylori* chromosome via single crossover integration, which was verified by PCR.

**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 the cationic lipid Cellfectin (Invitrogen). Correct insertion of *cat* and *PilA* was verified by PCR analysis with appropriate primers listed in suppl. Table S2. All fluorescent tag vectors (see Protocol S1) were integrated into the *H. pylori* chromosome via single crossover integration, which was verified by PCR.

**Production and analysis of recombinant proteins**

Recombinant versions of the *H. pylori* HP0059 and HP1143 proteins were produced in *E. coli* using the StreptagTM protein expression system from IBA (Göttingen, Germany) according to the manufacturer’s instructions [http://www.iba-go.de](http://www.iba-go.de). The coding sequences from *H. pylori* strain 26695 were amplified using the primer pairs listed in Table S2 and cloned via the *Bgl* restriction sites added as 5’-extensions (underlined) into plasmid pASKIBA-7TM (IBA-Göttingen). The plasmids were transferred to *E. coli* BL21 and expression was induced with 0.2 mg/l tetracycline. The bacteria were harvested by centrifugation and the recombinant proteins were purified to homogeneity on a Strept-Tactin® column according to the manufacturers’ instructions. In case of HP1143 the coding sequence from *H. pylori* strain 26695 was amplified using the primer pairs listed in Table S2 with the Streptag sequence integrated and cloned via the *NcoI* and *PstI* restriction sites into plasmid pETDuet-1 (Novagen). Protein expression was performed according to the manufacturer’s instructions. For protein purification at pH 7, buffer W (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA) as well as the buffer E (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA, 2.5 mM Desbiotin) were 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.

Analysis of urease activity

Urease activity was determined in fresh lysates by measuring ammonia production from hydrolysis of urea, as described previously [37,43]. The concentration of ammonia in the samples was inferred from a standard NH₄Cl concentration curve. Enzyme activity was expressed as μmol Cl⁻ hydrolysed min⁻¹ (mg protein)⁻¹.

**Electron microscopy analysis**

Cloning the Streptag sequence into plasmid pETDuet-1 (Novagene). Protein expression was performed according to the manufacturer’s instructions (http://www.iba-go.de). The recombinant proteins were purified to homogeneity on a Strept-Tactin® column according to the manufacturers’ instructions.

**Production and analysis of recombinant proteins**

Recombinant versions of the *H. pylori* HP0059 and HP1143 proteins were produced in *E. coli* using the StreptagTM protein expression system from IBA (Göttingen, Germany) according to the manufacturer’s instructions [http://www.iba-go.de](http://www.iba-go.de). The coding sequences from *H. pylori* strain 26695 were amplified using the primer pairs listed in Table S2 and cloned via the *Bgl* restriction sites added as 5’-extensions (underlined) into plasmid pASKIBA-7TM (IBA-Göttingen). The plasmids were transferred to *E. coli* BL21 and expression was induced with 0.2 mg/l tetracycline. The bacteria were harvested by centrifugation and the recombinant proteins were purified to homogeneity on a Strept-Tactin® column according to the manufacturers’ instructions. In case of HP1143 the coding sequence from *H. pylori* strain 26695 was amplified using the primer pairs listed in Table S2 with the Streptag sequence integrated and cloned via the *NcoI* and *PstI* restriction sites into plasmid pETDuet-1 (Novagen). Protein expression was performed according to the manufacturer’s instructions. For protein purification at pH 7, buffer W (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA) as well as the buffer E (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA, 2.5 mM Desbiotin) were adjusted to pH 7.

Spin down assays were performed as follows: 20 μl of purified protein fractions in buffer W (usually 24 h after elution, with storage at 4°C) were centrifuged at 13000 rpm in a bench centrifuge, after removal of the supernatant, the pellet was resuspended in 20 μl buffer W. SDS sample buffer were added and equal volumes of supernatant and pellet were subjected to SDS PAGE analysis.

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