Maintenance of the Cell Morphology by MinC in Helicobacter pylori

Pei-Yu Chiou¹, Cheng-Hung Luo¹, Kai-Chih Chang²*, Nien-Tsung Lin¹,³*

¹Institute of Medical Sciences, Tzu Chi University, Hualien, Taiwan, ²Department of Laboratory Medicine and Biotechnology, Tzu Chi University, Hualien, Taiwan, ³Department of Microbiology, Tzu Chi University, Hualien, Taiwan

Abstract

In the model organism Escherichia coli, Min proteins are involved in regulating the division of septa formation. The computational genome analysis of Helicobacter pylori, a gram-negative microaerophilic bacterium causing gastritis and peptic ulceration, also identified MinC, MinD, and MinE. However, MinC (HP1053) shares a low identity with those of other bacteria and its function in H. pylori remains unclear. In this study, we used morphological and genetic approaches to examine the molecular role of MinC. The results were shown that an H. pylori mutant lacking MinC forms filamentous cells, while the wild-type strain retains the shape of short rods. In addition, a minC mutant regains the short rods when complemented with an intact minC<sub>Hp</sub> gene. The overexpression of MinC<sub>Hp</sub> in E. coli did not affect the growth and cell morphology. Immunofluorescence microscopy revealed that MinC<sub>Hp</sub> forms helix-form structures in H. pylori, whereas MinC<sub>Ec</sub> localizes at cell poles and pole of new daughter cell in E. coli. In addition, co-immunoprecipitation showed MinC can interact with MinD but not with FtsZ during mid-exponential stage of H. pylori. Altogether, our results show that MinC<sub>Hp</sub> plays a key role in maintaining proper cell morphology and its function differs from those of MinC<sub>Ec</sub>.

Introduction

Helicobacter pylori, the etiologic agent of human gastritis, peptic ulceration, and gastric carcinoma, infects at least half of the world’s population with the organism being highly restricted to the gastric mucosa of humans [1]. During infection, the major actively replicating forms of H. pylori cells are spiral-shaped, but they can convert to cocci under environmental stresses, such as starvation and antibiotic treatment. The coccoid form is viable, but not culturable in vitro. It is less virulent than the spiral form; however, it is thought to be crucial in disease transmission and insensitive to antibiotic treatment [2]. Therefore, cell shape is an important pathogenicity factor for H. pylori. So far, the maintenance and establishment of the spiral structure in H. pylori is known to occur through peptidoglycan relaxation and an intracellular scaffold [3,4,5,6]. While cell division accuracy is crucial for maintaining the shape of some bacteria [7], little is known for H. pylori.

The process of cell division involves the spatial and temporal regulation of the septum formation by the cytoskeletal proteins [7]. In Escherichia coli, precise formation of septum between the two newly segregated sister chromosomes is initiated by FtsZ, which assembles into a ring and recruits a number of proteins, such as FtsA, ZipA, and ZapA, to form the septal ring [8]. In addition, Min proteins are required for the correct division site selection, which prevent polar divisions by blocking the Z ring assembly at cell poles [8]. The functions of FtsZ and min operon have been characterized in many bacteria [9,10,11,12,13]. Although the task of these proteins, which are involved in cell division, is almost identical in reported bacteria, the components and precise regulation mechanisms in preventing polar division of Min systems appears to be different among prokaryotic cells. For example, the Min system, which consists of 3 proteins, MinC, MinD, and MinE, and which is composed of an operon in E. coli, can oscillate periodically between the 2 poles of a cell, and MinE destabilized, but only 2 of them, MinC and MinD, are present in Bacillus subtilis. The function of MinE is substituted by an unrelated DivIVA protein, which targets MinCD in division sites and retains them at the cell poles. However, we found that the homologs of minC, D, and E are present, but not contained, in an operon in the sequenced genomes of H. pylori. Moreover, the amino acid sequences of MinC show a low identity with the corresponding proteins of reported bacteria. So far, whether minC plays any role in cell division of H. pylori remains unclear. Therefore, studying MinC’s functions is vital for understanding the cell division and shape-determining factors of H. pylori. In this study, we generated a minC mutant to study its biological characters. Our results show that minC plays a crucial role in maintaining the cell morphology and the movement capabilities of H. pylori.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Strains of E. coli were cultivated in Luria-Bertani (LB) (Difco Laboratories, Detroit, MI) solid and liquid media at 37°C. H. pylori NCTC 11637 was used to construct mutants. H. pylori strains were grown microaerobically at 37°C on blood agar plate.
(BAP) containing Columbia agar base (Becton Dikinson, Franklin Lakes, NJ, USA) and 5% fetal bovine serum (FBS). Bacterial growth was measured by monitoring OD600, while live cells were determined by viable count on BAPs. When required, antibiotics were supplemented: ampicillin (Ap, 100 μg/mL), chloramphenicol (Cm, 30 μg/mL), and kanamycin (Kan, 50 μg/mL).

DNA Techniques

The methods described by Sambrook et al [14] were used for preparation of chromosomal DNAs, restriction digestion, DNA ligation and E. coli transformations. Plasmids were isolated by using High-Speed Plasmid Mini Kit (Geneaid, Taipei, Taiwan). Natural transformation of H. pylori was performed as described elsewhere [15,16].

Table 1. Strains and plasmids used in this study.

| Strain or plasmid | Genotype or description | Source |
|-------------------|-------------------------|--------|
| **Strains**       |                         |        |
| E.coli            |                         |        |
| Top10             | F’ mcrA (mrr-hsdRMS-mcrBC) 80loxZ1M15 lacX174 recA1 deoR ardD139Δ (ara-leu) 7697 galU galK ptsL (Strr) endA1 supG | Invitrogen |
| BL21(DE3)         | F’ dcm ompT hsdS (F’ rK- mB-) gal λ (DE3) | Stratagene |
| MG1655            | wild-type               | [30]   |
| H. pylori         |                         |        |
| NCTC 11637        | wild-type, containing the entire minC | ATCC 43504 |
| PY1               | 11637, minC305          | This study |
| PY2               | PY1, hp0405:PminC305 kan | This study |
| PY2-5             | PY1, hp0405: PminC5 kan  | This study |
| PY3               | 11637, hp0405: PminC305 kan | This study |
| PY3-1             | 11637, hp0405: PminC5 kan | This study |
| **Plasmids**      |                         |        |
| pAV35             | chloramphenicol acetyltransferase (cat) cassette; Cm' | [18] |
| pJMK30            | kanamycin resistance (kan) cassette; Kan' | [18] |
| pUC18             | Cloning vector, Ap'     | Fermentas |
| pUC10             | Cloning vector derived from pOK12, replacement of the kan gene with the cat gene from pAV35, Cm' | [19] |
| pTZ57R/t         | T/A Cloning vector, Ap' | Fermentas |
| pET30a           | Cloning and expression vector, Kan' | Novagen |
| pBAD33           | pBAD expression; pACYC184 ori; Cm' | [31] |
| pCHL2            | hp0405: PflaA kan, Kan', Cm' | This study |
| pCPY001          | pUC18 containing minC305, Ap' | This study |
| pCPY002          | pCPY001 with cat inserted into the unique SphI site of minC305, Ap', Cm' | This study |
| pCPY003          | pCHL2 containing minC305 under flaA promoter, hp0405: PflaA-minC305 kan, Kan', Cm' | This study |
| pCPY004          | pET30a containing 6×his-minC305, Kan' | This study |
| pCPY005          | pET30a containing 6×his-minC305, Kan' | This study |
| pCPY006          | pCHL2 containing minC305, hexaP-flaA promoter, hp0405: PflaA-minC305 kan, Kan', Cm' | This study |
| pCPY007          | pET30a containing 6×his-ftsZ, Kan' | This study |
| pCPY008          | pET30a containing 6×his-minD, Kan' | This study |
| pCPY009          | pBAD33 containing minC305, Cm' | This study |
| pCPY010          | pBAD33 containing minC5, Cm' | This study |
| pOC0405          | pOC10 containing hp0405, Cm' | This study |
| pTZ-PflaA        | pTZ57R/t containing H. pylori flagella promoter, Ap' | This study |
| pTZ-PflaAKm      | pTZ-PflaA containing kan from pJMK30, Ap', Kan' | This study |

doi:10.1371/journal.pone.0071208.t001

Cell Length Determination, Immunostaining, and Image Acquisition

H. pylori from overnight liquid cultures was inoculated into fresh Brucella broth to obtain an initial OD600 of 0.05 and grown to an OD600 of 0.6 to 0.8. Cells were examined microscopically on poly L-lysine-treated slides with a thin layer of 1% agarose in LB. Cell length was measured as the axis length from one pole to the other of the cells captured in microscope, using ImageJ version 1.46 (http://rsb.info.nih.gov/ij/). Average cell length was determined using at least 2 independent measurements, each on 200 cells. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) at a final concentration of 1 μg/mL and membrane was stained with FM4-64 (Molecular Probes/Invitrogen) at a concentration of 1 μg/mL. Bacterial viability was determined by staining the cells with SYTO9/propidium iodine.
The oligonucleotide primers used in this study are listed in Table 2. Primers HP1054-F and HP1052-R for a PCR to the nucleotide (nt) –924 to –946, relative to the hp1054 start codon, and nt –269 to –248, relative to the termination codon of hp1052, respectively. A PCR was performed to amplify the fragment, using the H. pylori NCTC 11637 genomic DNA as the template. The amplicon was purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan) and directly sequenced using a 3730 DNA analyzer (Applied Biosystems, CA, USA). The sequence analysis was performed using NCBI packages.

### Sequencing and Identification of the minC Gene

The oligonucleotide primers used in this study are listed in Table 2. Primers HP1054-F and HP1052-R for a PCR to the nucleotide (nt) –924 to –946, relative to the hp1054 start codon, and nt –269 to –248, relative to the termination codon of hp1052, respectively. A PCR was performed to amplify the fragment, using the H. pylori NCTC 11637 genomic DNA as the template. The amplicon was purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan) and directly sequenced using a 3730 DNA analyzer (Applied Biosystems, CA, USA). The sequence analysis was performed using NCBI packages.

### Construction and Complementation of a H. pylori minC Mutant

The H. pylori minC was a PCR amplified from the strain NCTC 11637 genomic DNA using primers minCN and minCC. The PCR product was cloned into the Sphl site of pUC18 to generate pCPY001. To create an insertional mutant of minC in H. pylori, the chloramphenicol acetyltransferase (cat) cassette form pAV35 (kindly provided by J. M. Ketley) [18] was digested with PvuII and inserted into the unique Sphl restriction site 27 bp from the start site of minCHp in pCPY001 to generate pCPY002. The chromosomal minC locus was disrupted through a homologous recombination upon transforming strain NCTC 11637 with pCPY002. A transformant, selected on BAPs supplemented with Cm, was designated H. pylori PY1. The resulting PY1 was confirmed by a PCR analysis using the same primers of minCN and minCC.

To complement H. pylori PY1, plasmid pCHL2 was constructed in several steps to be used as a vector. (1) The hp0405 gene, amplified using PCR with the primers 0405-F and 0405-R, was cloned into the EcoRV site of pOC10 [19] and the fragment between Stul-HincII restriction sites was subsequently removed, which resulted in the vector pOC0405. (2) The H. pylori flagella promoter, PflaA, was amplified using a PCR with primers pflaF and pflaR and ligated with a pTZ39R/t vector to generate pTZ-PflaA. (3) The kan cassette of pJMK30 (provided by J. M. Ketley) [18] was cloned into the XbaI site of pTZ-PflaA to generate pTZ-PflaAKm. (4) PflaA and kan were subsequently cloned into the EcoRI site of pOC0405, generating plasmid pCHL2. The obtained pCHL2 vector possessed a unique StuI for cloning genes of interest between PflaA and kan.

Regarding complementation, minCHp was PCR-amplified from the H. pylori NCTC 11637 genomic DNA, using the primer pair minCN/minCC and was cloned into the unique StuI restriction site of pCHL2 to generate the construct pCPY003. The plasmids were then transformed into mutant PY1 and NCTC 11637 through natural transformation and were selected on BAPs supplemented with Kan. The ectopic integration of the cloned minC in the strain PY2 (PY1-complemented strain) was verified with PCR using the primers minCN and minCC, in which an amplicon of 1.3 kb (the minC gene plus a cat gene) and 0.6 kb (the minC gene only) were obtained. The ectopic integration of cloned minCHp in the strain NCTC 11637 was the designated H. pylori PY3 and was verified with PCR analysis using a pair of primers pflaF-minCC. minCEc, was PCR-amplified from pCPY005 using the primer pair NHis-F/minCec-R and cloned into the unique StuI restriction site of pCHL2 to generate the construct pCPY006. Subsequently, the plasmids were introduced into strain PY1 and NCTC 11637 through natural transformation (described above) and transformants were designated H. pylori PY2-5 and PY3-1, respectively. Finally, the complemented strains were verified with the PCR analysis using a pair of primers, pflaF-minCec-R.

### Plasmids Construction

The minCHp and flzZ gene were amplified by PCR using the genomic DNA of NCTC 11637 as the template, with the primers minCN/minCC and FtsZP-F/FtsZP-R as the primers, respectively. The products were digested with EcoRI and XhoI and cloned into pET30a cleaved with the same enzymes to yield pCPY004 and pCPY007, respectively. The minD gene was amplified by the PCR with the primers PminD1-F/PminD2-R and the amplicon was digested with SacI and HindIII. The SacI-HindIII fragment was cloned into pET30a cleaved with the same enzymes to yield pCPY004, Purified MinCHp, FtsZ, or MinD proteins from E. coli strain BL21(DE3) carrying pCPY004, pCPY007, or pCPY008.
**Figure 1. Genomic organization of min genes in rod-shaped bacteria.** (A) Grey arrows represent the genomic regions surrounding the min genes. White arrows show the localization of min genes. (B) Sequence comparison of *H. pylori* MinC with those of other bacterial MinC protein. The consensus line below the sequence alignment indicates identity (*), strong conservation (:), and weak conservation (.) of amino acid matches. Organisms in the alignment include *H. pylori* NCTC 11637 (KC896795; Hp11637), *Escherichia coli* (NP_415694.1; Ec), *Bacillus subtilis* (NP_390678; Bs), *Neisseria gonorrhoeae* (YP_208845; Ng), and *Thermotoga maritime* (NP_228853; Tm).

doi:10.1371/journal.pone.0071208.g001
were used to raise rabbit anti-MinCHp, anti-FtsZ, or anti-MinD polyclonal antiserum, respectively (Protech Technology Enterprise, Taipei, Taiwan). The \textit{minCEc} gene was amplified by the PCR from \textit{E. coli} MG1655 genomic DNA using the primers \textit{minCec-F} and \textit{minCec-R}. The PCR products were digested with BamHI and SalI and ligated with BamHI-SalI-cleaved pET30a to generate pCPY005. The XbaI-XhoI fragments of pCPY004 and pCPY005 were cloned into XbaI-SalI-cleaved pBAD33 to yield pCPY009 and pCPY010, respectively.

**Western Blot Analysis**

Bacteria cell liquid cultures were centrifuged at 13,000 \( x g \) for 1 min and cell pellets were washed twice in PBS. Pellets were resuspended in sterile water. The bacterial cell concentrations were sonicated using an ultrasonicator (Model XL, Misonix, Farmingdale, NY) to break the bacteria. Total protein concentrations were determined by using the Bio-Rad Dc protein assay kit on samples diluted 20-fold in water and on BSA standards in the same diluted buffer. The equal amounts of cell protein per lane were mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8 containing 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) and heated in a boiling water bath for 10 min. The samples were subjected to polyacrylamide (12%) gel electrophoresis; the protein bands were subsequently transferred onto the polyvinylidene difluoride (PVDF) membranes and probed with rabbit anti-MinCHp antibodies. A peroxidase-conjugated goat affinity-purified antibody against rabbit immunoglobulin G was used as the secondary antibody (Cell Signaling). Immunoreactive bands were detected using enhanced chemiluminescence (Millipore) and X-ray film. Band intensities on blots were measured using ImageJ version 1.46.

**Motility Assay**

Cells of \textit{H. pylori} grown in liquid cultures were stabbed into a soft agar plate containing 0.35% Bacto-agar in Brucella Broth medium and 5% FBS, followed by the incubation of the culture under microaerobic conditions for 3 to 5 days.

**Cell Morphology Analysis**

To test the MinC sensitivity of \textit{E. coli}, the plasmid pCPY009 or pCPY010 was introduced into \textit{E. coli} MG1655. Exponentially growing of strain was serially diluted by 10. Then 3 \( \mu \text{l} \) cultures from each dilution was spotted on a plate with and without arabinose (Sigma) and incubated overnight at 37°C. To study the effects of overexpression, MinCHp, cells harboring pCPY009 were grown overnight in LB medium at 30°C. An overnight culture was diluted 100-fold in an LB medium supplemented with Cm and grown for 2 h at 30°C. The cell cultures were added at various concentrations (0, 0.002, 0.02, or 0.2%) of arabinose and grown at 30°C for an additional 2 h. The cells were spun down, resuspended in LB medium, mixed 1:1 with 2% LB agarose, spotted onto a coverslip, and observed under a light microscope.

**Immunoprecipitation**

Liquid cultures of \textit{H. pylori} were grown to mid-log phase (\( \text{OD}_{600} = 0.6 - 0.8 \)) and harvested by centrifugation at 6000 \( x g \). Cell-free extracts were prepared by suspending cells in a sonication buffer (20 mM Tris-HCl, pH 8.0 containing 300 mM NaCl) and sonicated for 1 min with an ultrasonicator. The resultant suspensions were then centrifuged at 13,000 \( x g \) for 20 min at 4°C and the supernatants containing proteins were collected. The supernatants were mixed with 30 \( \mu \text{l} \) of 20% (w/v) Protein A Sepharose CL-4B (GE Healthcare) for 1 h to remove nonspecifically bound proteins. An aliquot (50 \( \mu \text{l} \)) of supernatant fraction was used as a control for total protein levels prior to immunopre-
Lysate was then incubated with anti-MinChp, anti-FtsZ, or anti-MinD antibodies coupled to Protein A Sepharose beads and incubated with shaking (40 rpm) at 4°C overnight. Beads containing protein complexes were washed 3 times with IP buffer (0.5% NP-40/PBS) and then boiled in sample buffer for 15 min. Samples were subsequently analyzed by SDS-PAGE, followed by immunoblotting.

Nucleotide Sequence Accession Number

The nucleotide sequence of the H. pylori NCTC 11637 minC has been deposited in the GenBank under accession number KC896795.

Results

min Genes are Present in H. pylori, but minC is not Clustered with minD and minE, and Shares a Low Similarity to other Reported Bacteria

As of March 2013, over 40 strains of H. pylori genome have been sequenced and registered in database. Our database search revealed that minC is separated from minD-minE in all sequenced H. pylori strains, different from other bacterial genera in which the corresponding genes are clustered [12,20]. Also, the same genome organization of min occurs in all sequenced Helicobacter spp. Furthermore, genes flanking minC and minDE are conserved in all sequenced Helicobacter spp.: nlpD and lpxC flanking minC gene and
ihC and dpxA flanking minDE (Figure 1A). These findings indicate that these DNA regions are stable.

The minC, minD, and minE genes of H. pylori encode proteins of 195 aa (22.38 kDa), 268 aa (29.31 kDa), and 77 aa (8.92 kDa), respectively. MinD and MinE show high degrees of identity in amino acid sequence to those from other bacteria (48.5% and 43.3% identical to E. coli and B. subtilis MinD, and 29.9% and 14.3% identical to E. coli and B. subtilis MinE/DivIVA, respectively). However, MinC shares low identity with E. coli and B. subtilis MinC proteins, 17.4% and 6.2%, respectively. Therefore, this study further investigates the role of MinC in H. pylori.

Because of the genetic variability of H. pylori, we focused our work on the reference strain NCTC 11637. To investigate the H. pylori minC, the gene was cloned from strain NCTC 11637 by a PCR-based strategy using primers HP1054-F and HP1052-R designed according to the minC flanking sequences of H. pylori strain 26695 (Table 2) for DNA amplification. Sequencing of the amplicon revealed 1,182 bp containing ORF with 195 aa which shared 87% to 95% identity to the MinC from other sequenced H. pylori strains and shared 20%, 13%, 13%, and 14% identity with the homologs from E. coli, B. subtilis, Neisseria gonorrhoeae, and Thermotoga maritima, respectively.

It has been shown that four conserved glycine residues at the MinC C-terminus are essential for MinC functionality as a cell division inhibitor and for the interaction of MinC with other Min proteins in E. coli [21]. Four glycine residues (G104, G122, G129, and G139) were also found at C-terminus of the H. pylori MinC proteins. In addition, a lysine residue at the N-terminus (K3) and an arginine residue (R102) at the C-terminus were also conserved among all known MinC proteins (Figure 1B). These findings suggest that H. pylori MinC is a factor involved in cell division, functioning to interact with MinD.

**Mutation of H. pylori minC causes Cell Filamentation and Growth and Motility Defects**

To investigate the role of MinC, a minC mutant of NCTC 11637 was constructed by a marker exchange and designated as PY1. Light microscopic observation of PY1 after gram-staining showed cells with different sizes. As shown in Figure 2, the cell length ranged from 1.6 to 25.7 μm with 64.5% of them falling between 5–10 μm. Comparing to that of the wild-type (2.22±0.75 μm), cell elongation of PY1 was obvious. PY1 exhibited a growth rate similar to that of the wild-type, with a generation time of about 6 h (Figure 3A). The OD₆₀₀ of the wild-type reached the maximum (1.34) at 24 h, while that of the mutant increased to a maximum of 1.88 at 36 h. The OD₆₀₀ of both strains declined gradually following cessation of growth. However, the number of viable cells of the wild-type was about 10 times more than that of PY1 at 36 h (3.8×10⁸ versus 4.5×10⁷ CFU/ml), and the amount of cell protein
of the wild-type was 1.26 times that of the mutant (3.6 versus 2.85 mg/ml).

To compare the cell morphology and membrane integrity, cells of NCTC 11637 and PY1 grown to 24, 48, and 72 h were stained with LIVE/DEAD kit and examined by fluorescence microscopy. All the wild-type cells were in rod form till 24 h, a portion of them (ca. 6%) became coccoid form at 48 h, and then almost all cells were in cocoid form at 72 h. In contrast, all PY1 cells maintained the elongated form throughout the experiment (Figure 3). Both strains appeared alive at 24 h; a large portion of the PY1 cells were dead (75%) at 48 h and almost all of them were dead at 72 h. Compared to the mutant cells, larger portions of the wild-type kept alive at 48 h (63%) and 72 h (48%). Furthermore, PY1 contained clearly segregated nucleoids (Figure 4), indicating that mutation in minC caused no defects in chromosome replication or segregation.

It is known that cell morphology affects the cell motility in numerous bacteria [22]. As motility of H. pylori is crucial in colonizing the gastric mucosa [23], we have evaluated the effects of minC mutation on the motility in this study. Tests were performed on a soft agar plate, comparing the area of spreading zones between the minC mutant and its parental strain. The results showed that the motility activity is reduced by half in mutant strains. As shown in Figure 5, growth of the wild-type cells resulted in a spreading zone of 15 mm in diameter after 72 h (Figure 5), while that formed by PY1 was about 7 mm in diameter (Figure 5). Since cell division related proteins are not involved in flagellar biosynthesis, it appears that the cellular elongation has reduced the cell motility.

Reversal of mutant minC Phenotype in H. pylori by Complementation

To perform the complementation test, we constructed a vector, pCHL2 (Table 1), that allowed for ectopic integration of the plasmid into H. pylori chromosome. The integration, targeted at the locus of hp0405 of H. pylori, was shown to cause no detectable

![Figure 7. Identification of FtsZ and MinD from co-IP performed with MinC during mid-exponential cultures of NCTC11637.](https://example.com/f7.png)

Proteins were eluted after co-IP experiments, samples were separated by SDS-PAGE and detected by Western blotting. Western blots were probed with (A) anti-MinC, (B) anti-FtsZ, and (C) anti-MinD. Lanes L, loading control consisting of whole-cell extract; lanes 1, proteins precipitated with anti-MinC<sub>hp</sub>; lanes 2, proteins precipitated with anti-FtsZ; lanes 3, proteins precipitated with anti-MinD.

doi:10.1371/journal.pone.0071208.g007

![Figure 8. The effects of MinC<sub>hp</sub> and MinC<sub>Ec</sub> proteins on cell length distribution of H. pylori.](https://example.com/f8.png)

(A) Cell length distributions of the PY2-5, PY3, and PY3-1. (B to D) Differential interference contrast (DIC) microscopic images of the three strains shown in panel A to demonstrate the morphology. (B) PY2-5; (C) PY3; (D) PY3-1. Scale bars, 10 μm.

doi:10.1371/journal.pone.0071208.g008
MinC of Helicobacter pylori

| Strain   | Genotype      | Average cell length ± 5D (μm) | Cell shorter than 2 μm (%) | Cell between 2 to 5 μm (%) | Cell longer than 5 μm (%) |
|----------|---------------|------------------------------|---------------------------|---------------------------|--------------------------|
| H. pylori| wild-type     | 2.58±0.70                    | 17.5                      | 82.5                      | –                        |
| NCTC 11637| minCcat       | 7.55±3.86                   | 13.3                      | 26.1                      | 72.6                     |
| PY1      | 11637, minCcat| 2.77±0.80                   | 16.3                      | 84.2                      | 0.5                      |
| PY2-5    | PY1, hp0405::Pflu::minCHp kan | 3.24±3.03               | 46.7                      | 34.6                      | 18.7                     |
| PY3      | 11637, hp0405::Pflu::minCEc kan | 2.99±1.22               | 16.9                      | 76                        | 7.1                      |
| PY3-1    | 11637, hp0405::Pflu::minCEc, kan | 5.31±3.36               | 6.5                       | 86.7                      | 7.1                      |

Effects on the physiology or morphology of *H. pylori* [24,25]. A complemented strain, PY2, with the *minCHp* gene integrated into the locus of *hp0405* of PY1 was constructed. The expression of MinCHp in the complemented strain was confirmed by Western blot analysis (Figure 6). A densitometry analysis indicated that the expression of MinCHp in PY2 was 1.6 times greater than that of the wild-type NCTC 11637. In addition, about 99% of PY2 cells regained normal cell morphology, exhibited a normal cell length distribution (Figure 2D), and restored their motility (Figure 5). This result suggested that elongation of PY1 cells was significant because of the *min* mutation and it was not a polar effect on downstream gene expression.

Cellular Localization of MinC in *H. pylori*

In *E. coli* and *B. subtilis*, MinC is an effector of the Min system responsible for antagonizing cell division and for preventing the sedimentation of FtsZ [11]. However, consequence of *minC* mutation may not be the same for *E. coli* and *B. subtilis*. To detect the cellular location of MinC, IF microscopy was performed using antibodies against MinC and a secondary antibody tagged to FITC. The results showed that MinC of *H. pylori* was localized at the poles during the late stage of septation in *E. coli*. Interestingly, the MinD protein was precipitated with MinC, but FtsZ proteins co-precipitated by Western blotting (Figure 7). Unexpectedly, the MinD protein was precipitated with MinC, but FtsZ was not (Figure 7, lanes 2 and 3).

MinCEc Interacts with MinD but not with FtsZ during Mid-exponential Stage of *H. pylori*

To examine whether MinC interacts with MinD and FtsZ in *H. pylori*, co-IP was performed using antibodies prepared against MinC, FtsZ, or MinD separately, followed by detection of the proteins co-precipitated by Western blotting (Figure 7). Unexpectedly, the MinD protein was precipitated with MinC, but FtsZ was not (Figure 7, lanes 2 and 3).

The Effects of MinCEc in *H. pylori*

To test whether MinC interacts with MinD and FtsZ, in *H. pylori*, co-IP was performed using antibodies prepared against MinC, FtsZ, or MinD separately, followed by detection of the proteins co-precipitated by Western blotting (Figure 7). Unexpectedly, the MinD protein was precipitated with MinC, but FtsZ was not (Figure 7, lanes 2 and 3).

Discussion

In many bacteria, Min proteins are involved in regulation of cell division. It is known that not all three *min* genes are ubiquitously present in all microorganisms and the entire minCDE cluster appears to be present only in Gram negative bacteria [26]. In this study, we reveal that *H. pylori* possesses homologs of *minC* and *minDE*, except that they are in two loci. Our sequence analysis here shows that residues conserved in other bacteria are all present in *MinCEc* (Figure 1B).

MinC is required for inhibition of septation by FtsZ in many bacteria and deficiency in MinC results in over septation that in turn causes mini-cell formation [8]. In contrast, a MinC deficient mutant of *H. pylori* was found to form elongated cells in this study. Our observations suggest that MinC of *H. pylori* is involved in normal septation that is required for normal cell division. To our knowledge, this is the first report that MinC is required for normal septation instead of inhibiting septation.

In *E. coli*, MinE imparts topological specificity by stimulating MinCD oscillation, thereby ensuring that the concentration of MinCD oscillation, thereby ensuring that the concentration of

Table 3. Cell length measurements.

| Strain   | Genotype      | Average cell length ± 5D (μm) | Cell shorter than 2 μm (%) | Cell between 2 to 5 μm (%) | Cell longer than 5 μm (%) |
|----------|---------------|------------------------------|---------------------------|---------------------------|--------------------------|
| H. pylori| wild-type     | 2.58±0.70                    | 17.5                      | 82.5                      | –                        |
| NCTC 11637| minCcat       | 7.55±3.86                   | 13.3                      | 26.1                      | 72.6                     |
| PY1      | 11637, minCcat| 2.77±0.80                   | 16.3                      | 84.2                      | 0.5                      |
| PY2-5    | PY1, hp0405::Pflu::minCHp kan | 3.24±3.03               | 46.7                      | 34.6                      | 18.7                     |
| PY3      | 11637, hp0405::Pflu::minCEc kan | 2.99±1.22               | 16.9                      | 76                        | 7.1                      |
| PY3-1    | 11637, hp0405::Pflu::minCEc, kan | 5.31±3.36               | 6.5                       | 86.7                      | 7.1                      |

Effects on the physiology or morphology of *H. pylori* [24,25]. A complemented strain, PY2, with the *minCHp* gene integrated into the locus of *hp0405* of PY1 was constructed. The expression of MinCHp in the complemented strain was confirmed by Western blot analysis (Figure 6). A densitometry analysis indicated that the expression of MinCHp in PY2 was 1.6 times greater than that of the wild-type NCTC 11637. In addition, about 99% of PY2 cells regained normal cell morphology, exhibited a normal cell length distribution (Figure 2D), and restored their motility (Figure 5). This result suggested that elongation of PY1 cells was significant because of the *minC* mutation and it was not a polar effect on downstream gene expression.

Cellular Localization of MinC in *H. pylori*

In *E. coli* and *B. subtilis*, MinC is an effector of the Min system responsible for antagonizing cell division and for preventing the sedimentation of FtsZ [11]. However, consequence of *minC* mutation may not be the same for *H. pylori*, because mutation in *minC* gene causes the cell to elongate instead of mini-cell formation observed in *E. coli* and *B. subtilis*. To detect the cellular location of MinC, IF microscopy was performed using antibodies against MinC and a secondary antibody tagged to FITC. The results showed that MinC in the mid-log cells assembled into helix-form structures and located mainly in poles (Figure 6).

MinCEc Interacts with MinD but not with FtsZ during Mid-exponential Stage of *H. pylori*

To examine whether MinC interacts with MinD and FtsZ, in *H. pylori*, co-IP was performed using antibodies prepared against MinC, FtsZ, or MinD separately, followed by detection of the proteins co-precipitated by Western blotting (Figure 7). Unexpectedly, the MinD protein was precipitated with MinC, but FtsZ was not (Figure 7, lanes 2 and 3).

The Effects of MinCEc in *H. pylori*

To test whether MinC interacts with MinD and FtsZ in *H. pylori*, co-IP was performed using antibodies prepared against MinC, FtsZ, or MinD separately, followed by detection of the proteins co-precipitated by Western blotting (Figure 7). Unexpectedly, the MinD protein was precipitated with MinC, but FtsZ was not (Figure 7, lanes 2 and 3).

Discussion

In many bacteria, Min proteins are involved in regulation of cell division. It is known that not all three *min* genes are ubiquitously present in all microorganisms and the entire minCDE cluster appears to be present only in Gram negative bacteria [26]. In this study, we reveal that *H. pylori* possesses homologs of *minC* and *minDE*, except that they are in two loci. Our sequence analysis here shows that residues conserved in other bacteria are all present in *MinCEc* (Figure 1B).

MinC is required for inhibition of septation by FtsZ in many bacteria and deficiency in MinC results in over septation that in turn causes mini-cell formation [8]. In contrast, a MinC deficient mutant of *H. pylori* was found to form elongated cells in this study. Our observations suggest that MinC of *H. pylori* is involved in normal septation that is required for normal cell division. To our knowledge, this is the first report that MinC is required for normal septation instead of inhibiting septation.

In *E. coli*, MinE imparts topological specificity by stimulating MinCD oscillation, thereby ensuring that the concentration of
Figure 9. The effects of MinCHp or MinCEc on cell viability and morphology of E. coli. (A) E. coli MG1655 harboring the plasmid pBAD33, pCPY009 and pCPY010, respectively, were serially diluted 10-fold and spotted on LB plates supplemented with the indicated concentration of arabinose at 37°C. (B) DIC micrographs of cells grown in LB with indicated concentration of arabinose at 30°C. Scale bars, 10 μm. (C) SDS-PAGE (left panel) and Western blot (right panel) showing the levels of MinCHp in MG1655(pCPY009). Cells were grown with 0.002% (lanes 2 and 6), 0.02% (lanes 3 and 7), and 0.2% (lanes 4 and 8) of arabinose, respectively, at 30°C to an OD600 of ca. 1.2 for subsequent immunoblot analysis. The
MinCD is highest at the poles [27]. In *B. subtilis*, MinCDJ are localized at the poles or the site of division through polar targeting by DivIVA [28]. In this study, IF microscopy revealed that MinCHp, in the mid-log cells assembled into helix-form structures and located mainly in poles, but do not interact with FtsZ, suggesting that MinCHp-FtsZ interaction is not required for maintenance of cell division. It is proposed that MinCHp interacts with other proteins during different stages of cell division in *H. pylori*. Several studies have shown that Min proteins can function in heterologous background, for examples, the chloroplasts are elongated when minC of *E. coli* is introduced into Arabidopsis thaliana [29], cells of *B. subtilis* transformed with minC of *E. coli* are elongated [10], *E. coli* cells transformed with minC and minD of *N. gonorrhoeae* are elongated [13]. In this study, MinCEc provided in resulting in cell elongation [26]. In contrast, expression of MinCHp trans resulted in elongation of the wild-type cells and was able to polymerize FtsZHp in *H. pylori*, thereby inhibiting cell division and resulting in cell elongation [26]. In contrast, expression of MinCHp in *E. coli* did not cause detectable effects on cell morphology. Since the amino acid sequence of FtsZHp share high degree of similarity with FtsZ (70%) and MinCHp exhibited no co-IP reaction with FtsZ, it appears reasonable to predict that MinCHp cannot interact with FtsZ. Consequently, MinCHp could not inhibit the Z-ring formation in *E. coli*. In addition, based on the observations that i) *H. pylori* and *E. coli* FtsZ have different architecture of filaments, ii) the FtsZ-ring positions at both central and acentric regions in *H. pylori*, and iii) daughter cells show considerably different sizes owing to the asymmetrical division of the cells, Specht *et al.* [9] suggest that FtsZH of *H. pylori* possesses a unique intrinsic characteristic different from that of *E. coli* and the cell cycle of *H. pylori* is clearly dissimilar to that of *E. coli*. Thus, the present observations that i) minC mutation causes cell elongation instead of mini-cell formation, ii) MinC does not interact with FtsZ, and iii) MinCHp causes no effects on cell division when expressed in *E. coli* have confirmed and extended the previous findings in *H. pylori* cell division.

Acknowledgments

We thank Y.-H. Tseng for reading the manuscript.

Author Contributions

Conceived and designed the experiments: PYC NTL. Performed the experiments: PYC. Analyzed the data: PYC KCC NTL. Contributed reagents/materials/analysis tools: CHL KCC. Wrote the paper: PYC NTL.

References

1. Dunn BE, Cohen H, Blaser MJ (1997) *Helicobacter pylori*. Clin Microbiol Rev 10: 720–741.
2. Andersen LP, Wadstrom T (2001) *Helicobacter pylori*: physiology and genetics; In: Mobley HLT MG, Hazell SL, editor. Washington, DC: ASM press. p.58–68.
3. Specht M, Schatzle S, Graumann PL, Waidner B (2011) Peptidoglycan modification networks modulate Helicobacter pylori's cell shape, motility, and colonization potential. PLoS Pathog 7: e1002603.
4. Specht M, Schatzle S, Graumann PL, Waidner B (2011) *Helicobacter pylori* possesses four coiled-coil-rich proteins that form filamentous structures and control cell shape and motility. J Bacteriol 193: 4532–4539.
5. Syucro LK, Pincus Z, Gutierrez KD, Biboy J, Stern CA, et al. (2010) NADPH oxidase activity is regulated by the bacterial cytoskeleton. J Biol Chem 285: 34694–34703.
6. Waidner B, Specht M, Dennewitz F, Haeberer K, Schaezle S, et al. (2009) A novel system of cytoskeletal elements in the human pathogen *Helicobacter pylori*. PLoS Pathog 5: e1000609.
7. Graumann PL (2007) Cytoskeletal elements in bacteria. Annu Rev Microbiol 61: 509–618.
8. Cabeen MT, Jacobs-Wagner C (2010) The bacterial cytoskeleton. Annu Rev Genet 44: 365–392.
9. Specht M, Demppwolff F, Schärtle S, Thomann R, Waidner B (2013) Localization of FtsZ in *Helicobacter pylori* and Consequences for Cell Division. J Bacteriol 195: 1411–1420.
10. Pavlovna N, Muchova K, Barak I (2010) Expression of Escherichia coli Min system in *Bacillus subtilis* and its effect on cell division. FEMS Microbiol Lett 302: 829–833.
11. Lutkenhaus J (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. Annu Rev Biochem 76: 539–562.
12. Ramirez-Arcos S, Raymond C, Hicks LD, Kay CM, et al. (2001) Conserved glycines in the C terminus of MinC proteins are implicated in their functionality as cell division inhibitors. J Bacteriol 183: 4094–4103.
13. Young KD (2006) The selective value of bacterial shape. Microbiol Mol Biol Rev 70: 660–703.
14. Specht M, Pincus Z, Gutierrez KD, Biboy J, Born P, Pincus Z, et al. (2010) Multiple peptidoglycan modification networks modulate *Helicobacter pylori* sequences in *Neisseria gonorrhoeae*. Nucleic Acids Res 20: 5137–5142.
15. Wang Y, Roos KP, Taylor DE (1993) Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance marker. J Gen Microbiol 139: 2483–2493.
16. Akopyan N, Bukanov NO, Westblom TU, Krooswijk S, Berg DE (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res 20: 5137–5142.
17. Lee MJ, Liu CH, Wang SY, Huang CT, Huang H (2006) Characterization of the Soj/SpolJ chromosome segregation proteins and identification of putative parS sequences in *Helicobacter pylori*. Biochem Biophys Res Commun 342: 744–750.
18. van Vliet AH, Woolridge KG, Kelsey JM (1998) Iron-responsive gene regulation in a Campylobacter jejuni mutant. J Bacteriol 180: 5291–5296.
19. Lao CH, Chiao PY, Yang CY, Lin NT (2012) Genome, integration, and transduction of a novel temperate phage of *Helicobacter pylori*. J Virology 86: 6781–6792.
20. Barak I, Wilkinson AJ (2007) Division site recognition in *Escherichia coli* and *Bacillus subtilis*. FEMS Microbiol Rev 31: 311–326.
21. Ramirez-Arcos S, Greco V, Douglas H, Tesier D, Fan D, et al. (2004) Conserved glycines in the C terminus of MinC proteins are implicated in their functionality as cell division inhibitors. J Bacteriol 186: 2041–2053.
22. Young KD (2006) The selective value of bacterial shape. Microbiol Mol Biol Rev 70: 660–703.
23. Ottemann KM, Losev NFC (2002) *Helicobacter pylori* uses motility for initial colonization and to attain robust infection. Infect Immun 70: 1984–1990.
24. Olson JW, Mehta NS, Maier RJ (2000) Requirement of nickel metabolism proteins HyaP and HyaB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol Microbiol 39: 176–182.
25. Olson JW, Agar RN, Johnson MK, Maier RJ (2000) Characterization of the NifU and NifS Fe–S cluster formation proteins essential for viability in *Helicobacter pylori*. Biochemistry 39: 16213–16219.
26. Rothfield L, Justice S, Garcia-Lara J (1999) Bacterial cell division. Annu Rev Genet 33: 423–448.
27. Hu Z, Mikkheitere A, Pichoff S, Lutkenhaus J (1999) The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. Proc Natl Acad Sci U S A 96: 14189–14194.
28. Patrick JE, Kearns DB (2006) MinJ (NPdJ) is a topological determinant of cell division in *Bacillus subtilis*. Mol Microbiol 70: 1166–1179.
29. Tavva VS, Collins GB, Dinkins RD (2006) Targeted overexpression of the Mini segment of *Bacillus subtilis* chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J Gen Microbiol 159: 2483–2493.
30. Blattner FR, Plunkett G, 3rd, Bloch CA, Perna NT, Burland V, et al. (1997) The complete genome sequence of *Escherichia coli K-12*. Science 277: 1453–1462.
31. Guinan LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177: 4121–4130.