Cell

Peptidoglycan Crosslinking Relaxation Promotes *Helicobacter pylori*'s Helical Shape and Stomach Colonization

Laura K. Sycuro,[1,3] Zachary Pincus,[4] Kimberley D. Gutierrez,[2] Jacob Biboy,[5] Chelsea A. Stern,[2,3] Waldemar Vollmer,[5] and Nina R. Salama[2,3,*]

[1]Molecular and Cellular Biology Graduate Program
[2]Department of Microbiology
University of Washington, Seattle, WA 98195, USA
[3]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
[4]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520, USA
[5]Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne NE2 4HH, UK
*Correspondence: nsalama@fhcrc.org
DOI 10.1016/j.cell.2010.03.046

SUMMARY

The mechanisms by which bacterial cells generate helical cell shape and its functional role are poorly understood. Helical shape of the human pathogen *Helicobacter pylori* may facilitate penetration of the thick gastric mucus where it replicates. We identified four genes required for helical shape: three LytM peptidoglycan endopeptidase homologs (*csd1–3*) and a *ccmA* homolog. Surrounding the cytoplasmic membrane of most bacteria, the peptidoglycan (murein) sacculus is a meshwork of glycan strands joined by peptide crosslinks. Intact cells and isolated sacculi from mutants lacking any single *csd* gene or *ccmA* formed curved rods and showed increased peptidoglycan crosslinking. Quantitative morphological analyses of multiple-gene deletion mutants revealed each protein uniquely contributes to a shape-generating pathway. This pathway is required for robust colonization of the stomach in spite of normal directional motility. Our findings suggest that the coordinated action of multiple proteins relaxes peptidoglycan crosslinking, enabling helical cell curvature and twist.

INTRODUCTION

The abundant morphological diversity present among bacteria has long been appreciated by microbiologists. Yet only recently has much progress been made toward understanding the mechanisms responsible for generating distinctive cell shapes (Young, 2006; Cabeen and Jacobs-Wagner, 2007). To date, most studies have focused on a select group of model organisms representing the most common shapes: rod (*Escherichia coli* and *Bacillus subtilis*), coccoid (*Staphylococcus aureus* and *Streptococcus* species), and vibrioid (or curved rod; *Caulobacter crescentus*). The machinery giving rise to cell shape in helical bacteria remains largely unknown. An exception is the *Spiroplasma* cytoskeletal apparatus, composed predominantly of Fib, a protein found in only a few prokaryotic species (Williamson et al., 1991), and MreB, a common prokaryotic cytoskeletal protein homologous to eukaryotic actin (Jones et al., 2001; Bove et al., 2003; Daniel and Errington, 2003). Fib, possibly in conjunction with MreB, forms bundles of filaments in a ribbon-like helix, which attaches to the inner surface of the membrane along the shortest (inner) helical line and twists the cell into a helix (Trachtenberg, 2004; Trachtenberg et al., 2008).

Spiroplasma, however, belong to an unusual class of bacteria, the Mollicutes, which lack the rigid cell wall known as the peptidoglycan (PG, or murein) sacculus. The exocellular sacculus surrounds the cytoplasmic membrane of most bacteria and is composed of stiff glycan strands crosslinked by flexible peptide bridges to form a mesh structure (Vollmer and Bertsche, 2008). PG prevents cell lysis due to turgor pressure and is required to maintain bacterial cell shape. Isolated PG sacculi also retain the morphology of the intact cell (Vollmer and Bertsche, 2008). A group of walled bacteria in which some progress has been made toward understanding helical shape determination is *Spirochetes*. These bacteria house flagella in the periplasmic space between the inner and outer membranes that are sometimes required for helical cell shape (Goldstein et al., 1994). Periplasmic flagella presumably act as force-generating cytoskeletal elements that bend the cell into a helix (Wolgemuth et al., 2006). However, some *Spirochete* species retain helical morphology in the absence of periplasmic flagella and the cellular machinery involved in generating their helicity has yet to be identified (Bromley and Charon, 1979; Ruby et al., 1997).

Here we investigate the helical shape of *Helicobacter pylori*. *H. pylori* is a member of the Epsilonproteobacteria, a class of bacteria composed almost exclusively of helical and curved organisms. *H. pylori*'s habitat is the human stomach, which it colonizes in approximately 50% of the world population. *H. pylori* infection is associated with the development of chronic gastric inflammation that can lead to ulcers and gastric cancer in

a subset of those infected (Kusters et al., 2006). *H. pylori*'s helical cell shape is conserved in human isolates (Goodwin et al., 1985), although the pitch of the helix varies among laboratory strains (L.K.S., unpublished data). This has given rise to the hypothesis that *H. pylori*'s helical shape serves an important function in pathogenesis (Montecucco and Rappuoli, 2001; Cover and Blaser, 2009). The prevailing theory is that helical shape enhances *H. pylori*'s flagellar motility through the viscous epithelial mucus layer in which it resides by a corkscrew mechanism (Hazell et al., 1986).

Helical cell shape can be thought of as the sum of three morphogenic components: cell elongation, curvature, and twist. Recent studies of the Gram-negative model organisms *E. coli* and *C. crescentus* have provided significant insight into the genes and mechanisms these species use to elongate the cell body and generate curvature. Although *H. pylori* encodes some of these genes, others appear to be absent or too highly divergent for sequence-based identification. Specifically, *H. pylori* encodes all three of the high-molecular-weight penicillin-binding proteins (PBPs) required for PG glycan synthesis (transglycosylation via PBP1) and peptide crosslinking (transpeptidation via PBP1, PBP2, and PBP3) (Tomb et al., 1997; DeLoney and Schiller, 1999). However, low-molecular-weight PBPs with endopeptidase and/or carboxypeptidase activities that contribute to PG hydrolysis and postsynthetic modification of crosslinked and uncrosslinked peptide chains have not been identified in *H. pylori*. In *E. coli* and *C. crescentus*, cell body elongation requires the actin homolog MreB, which, in conjunction with other cytoskeletal filaments and scaffolding proteins (i.e., FtsZ, MreC, MreD, RodA, and RodZ), appears to spatially position PG synthesis in roughly helical bands or patches along the cell body, resulting in rod elongation (Cabeen and Jacobs-Wagner, 2007; Alyahya et al., 2009; Bendezu et al., 2009). Although *H. pylori* encodes all of these proteins, their role in elongating the *H. pylori* cell body has not been confirmed. CreS, an intermediate filament homolog in *C. crescentus*, is required for that organism's cell curvature (Ausmees et al., 2003). Through interactions with MreB, the CreS filament is tethered longitudinally along the inner membrane, creating a growth differential that limits PG synthesis rates along the CreS-lined sidewall and enables faster rates along the opposite sidewall, thus forming a curved cell body (Cabeen et al., 2009). Intermediate filament-like proteins that may influence cell shape maintenance were recently identified in *H. pylori* (Waidner et al., 2009).

Biophysical modeling has recently suggested an alternative pathway to generating cell curvature and twist through local alteration of PG crosslink number or length (Huang et al., 2008). Here we present biological evidence supporting this model with the identification of four proteins that function to generate *H. pylori*'s helical shape through alterations in PG crosslinking. We show that these proteins are conserved in other Epsilonproteobacteria as well as curved and helical Gammaproteobacteria, suggesting this approach to generating cell shape may be common among Gram-negative bacteria. We also examine the fitness of nonhelical *H. pylori* mutants in the mouse stomach and find they are deficient despite apparently normal motility in vitro.

RESULTS

A Putative Metallopeptidase, Csd1, Is Required for the Helical Cell Shape of *H. pylori*

To identify genetic determinants of *H. pylori*'s conserved helical cell shape, we visually screened a library of random transposon insertion mutants constructed in strain G27 (Salama et al., 2004) for clones with shape defects. Of 2000 clones screened, nine with altered morphology were selected, including one with curved-rod (vibrioid) rather than helical morphology. Amplification of the DNA sequence flanking the transposon revealed this clone's insertion site was within HPG27_1481 (Figure 1A), a gene annotated as *tagE* due to its homology to a ToxR-activated gene in *Vibrio cholerae* (Kovach et al., 1994). *V. cholerae* contains multiple TagE homologs, and although one has been crystallized and shown to contain a metallopeptidase active site similar to that of the *Staphylococcus aureus* endopeptidase LytM (peptidase family M23; proteins with this domain will be referred to as LytM peptidases for clarity), their function remains unknown (Ragumani et al., 2008). Protein structure queries (Bennett-Lovsey et al., 2008) revealed HPG27_1481 threaded onto the tertiary structures of the crystallized *V. cholerae* TagE protein (*E*-value $3.9E^{-25}$) and *S. aureus* LytM (*E*-value $7.3E^{-23}$). Using these models, we identified conserved LytM active site residues in the HPG27_1481 protein (see below). Targeted deletion of HPG27_1481 coding sequence recapitulated the morphological phenotype of the transposon mutant (Figures 1D and 1E and Figure S1C available online) and was complemented by reintroduction of HPG27_1481 at a distal chromosomal locus, *rdxA*, which is often used for complementation in *H. pylori* (Smeets et al., 2000) (Figures S2B and S2C). Having identified HPG27_1481 as a putative LytM peptidase involved in producing helical versus curved-rod morphology, we named this gene *csd1* (cell shape determinant).

csd1 Is Part of a Three-Gene Locus Required for Helical Cell Morphology

All sequenced *H. pylori* strains encode a second LytM peptidase (HPG27_1482) immediately upstream of *csd1* that is 53% similar and 29% identical to Csd1 (Figure 1A). We designated this gene *csd2* upon discovering its deletion also yielded cells with curved-rod morphology (Figure 1F) and complementation restored helicity (Figure S2C). The hypothetical protein encoded downstream of *csd1* is a homolog of *ccmA* (curved cell morphology, HPG27_1480, Figure 1A), a gene of unknown function shown to be important for determining straight-rod versus curved-rod morphology in *Proteus mirabilis* (Hay et al., 1999). Deletion of *H. pylori ccmA* again resulted in curved-rod morphology (Figure 1G) and could be complemented (Figures S2B and S2C).

Microscopic examination of the three curved-rod mutants suggested slight variations in the degree of cell curvature, with *csd2* the most curved and *ccmA* the least. No helical cells were seen in any of the mutant cell populations. To distinguish whether the mutants had completely lost the ability to form helices or their helical turns had a longer period such that individual cells only appeared curved, we induced cell filamentation with the drug aztreonam, an inhibitor of septal PG synthesis. In comparison with the regular pitch of helical wild-type cells, there