Helicobacter urease: Niche construction at the single molecule level

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The urease of the human pathogen, *Helicobacter pylori*, is essential for pathogenesis. The ammonia produced by the enzyme neutralizes stomach acid; thereby modifying its environment. The dodecameric enzyme complex has high affinity for its substrate, urea. We compared urease sequences and derivative 3D homology model structures from all published *Helicobacter* genomes and an equal number of genomes belonging to strains of another enteric bacterium, *Escherichia coli*. We found that the enzyme's architecture adapts to fit its niche. This finding, coupled to a survey of other physiological features responsible for the bacterium's acid resistance, suggests how it copes with pH changes caused by disease onset and progression.

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1. Overview

Bacteria provide many fascinating examples of "niche construction", phenomena that involve modification of the environment by the organism. These include photosynthesis, decomposition and formation of microbial mats in oceans. One particularly important example is niche creation by bacterial pathogens. A well-studied case is that of the gastric pathogen *Helicobacter pylori* within its human host. *H. pylori* and the related *Escherichia coli* are motile, Gram-negative pathogens that colonize the gastro-intestinal and urinary tracts. The pH in the stomach is acidic (pH 2–3), in contrast to the colon or other organs (e.g. liver) where the pH is neutral.

Urease is a multi-subunit, metalloenzyme that breaks down urea to ammonia and water. This activity is essential for colonization of gastric mucosa (Mobley *et al.* 1995). The *H. pylori* urease atomic structure has identified the residues essential for catalysis and for dodecameric assembly (Ha *et al.* 2001). Here, we use a comparative structural genomic analysis of urease to evaluate the significance of these features for the bacterium's acid resistance. Taking the available urease atomic structures as starting material, we examined the conservation patterns of residues important for catalysis and assembly across 10 genomes, involving 5 strains each of *Helicobacter* and *E. coli*. In addition to the structural subunits; urease activity is dependent on additional proteins that either transport its co-factor, the metal ion nickel; its substrate, urea, or mediate its assembly. Their genes are organized in operons. Shift to acid pH increases transcription from some operons and decreases it from others. We review variations in these proteins as well as other determinants for pH homeostasis and the motile machinery for pH taxis. In many cases, species variations are manifested by presence of absence of one or more genes. However, in other cases a more fine-grained analysis of sequence and structure variations as we have done for urease may be required to precisely identify the processes that allow bacteria to make progressive adjustments to changes they induce in their micro-environment. The changed environment may in turn change the selection processes operating on the organism's genotype. In other cases, however, a genotype though hard-wired may still allow a plastic response at the level of cellular morphogenesis and physiology

2. Methods

Selection and preliminary analysis of selected genes and genomes was done online using the integrated microbial

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genomes (IMG) data management and analysis platform (*http://img.jgi.doe.gov/cgi-bin/pub/main.cgi/*). Genomes and or genes were selected; scanned for paralogs / homologs with an E-value cut-off of 0.1. Gene neighbourhoods and Clustal-W based multiple sequence alignments were performed as described (Markowitz *et al.* 2006). Atomic structures were retrieved from the Protein Data Bank (*http://www.rcsb.org/pdb/home/home*). The program SWISS-MODEL (*http://swissmodel.expasy.org/*) was used to homology model downloaded protein sequences. Model and template structures were displayed and their chemical properties (hydrogen bonding, flexibility, molecular surfaces) examined using Swiss PDB Viewer version 3.7 (*http://swissmodel.expasy.org/spdbv*). The work was done on a Windows XP (Dell computer, 1 Gb, 2.4 GHz) or an OS 10.4 (Macintosh MacBook Pro 2 Gb, 2.16 Ghz) platform.

3. Genome selection

H. pylori, but not *E. coli*, can reside in the stomach as well as the gut. Acid resistance underlies colonization (figure 1). Possible outcomes of colonization include pathogenic ones that change stomach morphology and physiology, in turn inducing genome alterations in the bacterial pathogen. Three of the *Helicobacter* genomes are from *H. pylori* strains isolated from healthy (Tomb *et al.* 1997) and diseased individuals (Israel *et al.* 2001; Oh *et al.* 2006). The HPAGI genome was isolated from patients suffering from chronic gastritis. Loss of the acid producing parietal cells is characteristic of this disease state. Other bacteria invade the neutral gastric juice; while *H. pylori* takes up residence in stem cells that proliferate upon loss of the parietal cells (Oh *et al.* 2005). The other two *Helicobacter* genomes are from species that have evolved to colonize different host and tissues.

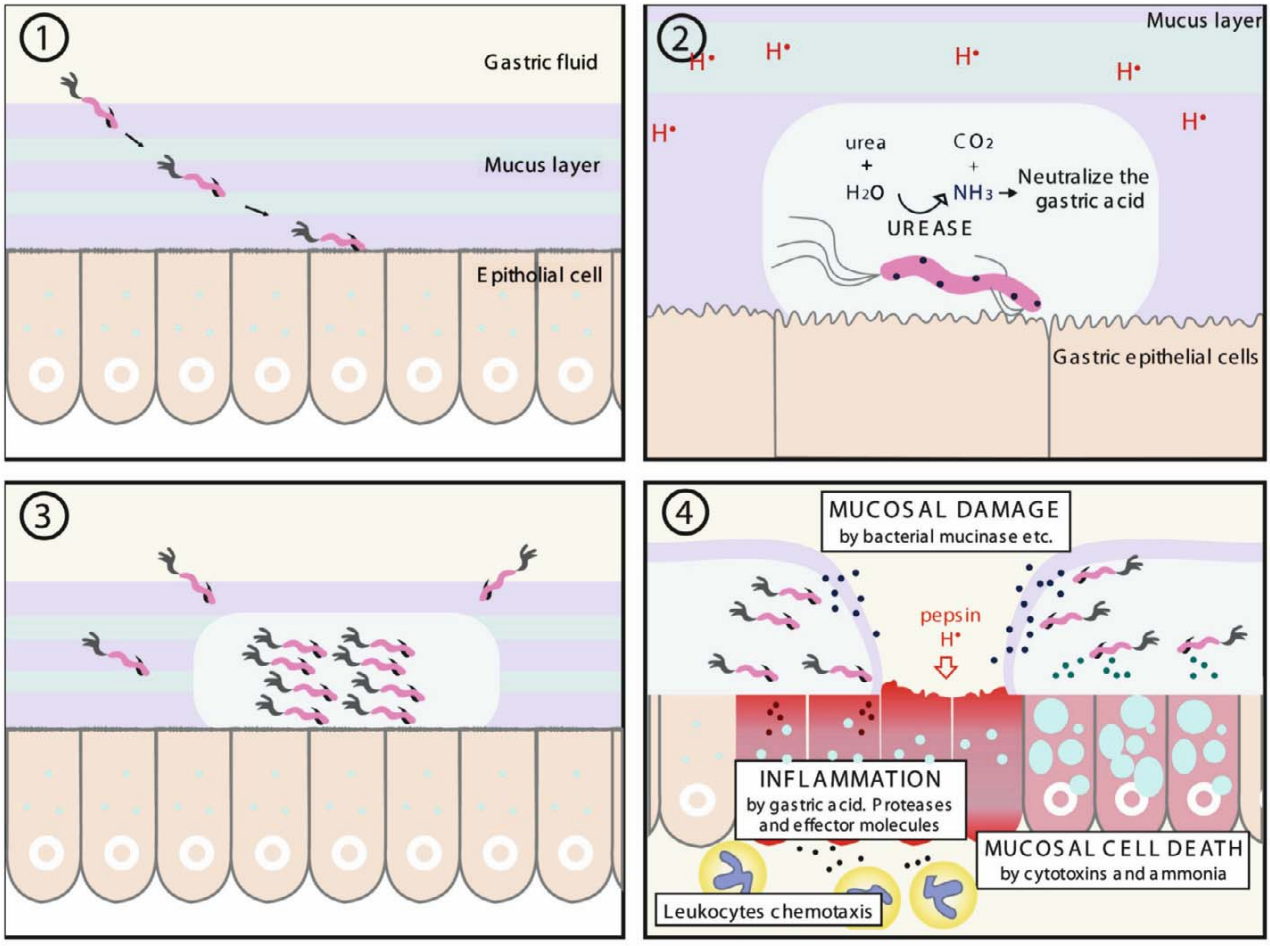

Figure 1. Role of urease in the colonization of gastric mucosa by *Helicobacter pylori* (taken from *http://h.pylori.info/* with permission). Panel numbers denote successive stages in colonization of the host mucosa. Attachment (1) is followed by formation and activity of extracellular ureases (black dots) (2). This leads to aggregation (3) and mucosal cell damage.

H. acinonychis (Eppinger *et al.* 2006) colonizes the gastrointestinal tract of large cats; while *H. hepaticus* colonizes rodent liver and lower intestine (Suerbaum *et al.* 2003). The five *E. coli* genomes include two non-pathogenic K12 strains, two geographically distant isolates of the enterohaemorrhagic strain O157, the causative agent of diarrhea ("hamburger disease") plus the uropathogenic strain CFT073 (Blattner *et al.* 1997; Pernat *et al.* 2001; Welch *et al.* 2002) (figure 2).

4. Niche dependent variations in urease structure

The available X-ray crystal structures reveal that the fundamental structural unit is a dimer (in *H. pylori*), or a trimer (e.g. *K. aerogenes*) (figure 3). Urease is not present in the K12 strains or the uropathogenic CFT073. The urease subunits show high conservation in gene organization, sequence and molecular architecture of the asymmetric subunit in the crystal structures. Sequence alignment reveals that the single *Helicobactor* UreA gene is split into two genes UreA and UreB in *E. coli*. UreB is preserved as UreC. The *Klebsiella* and *Bacillus* enzymes exist as anti-parallel trimers. This trimer design is also used in *H. pylori*. However, four trimers are stapled together to form a much larger dodecameric complex with a prominent central core. The atomic structure has identified elements required for formation of this tetramer of trimers. In particular, the UreB C-terminal loop staples the trimers together (figure 4). This loop is absent in the ureases of *Helicobacter hepaticus* and *E. coli* strain 0157; but present in the feline gastric pathogen *Helicobacter acinocychis*. This predicts that trimes come together to form the dodecameric complex only in the ureases from gastric *Helicobacter* sp. Thus the presence of this feature essential for dodecamer formation correlates with colonization of a particular niche (stomach) rather than species. This implies that it is an advantageous characteristic for niche colonization. This advantage may result from the fact that the active sites face into the central core of the dodecameric assembly. Microbial ureases, including the ones from *E. coli* and *Helicobacter* species, have a pH optimum of near neutrality and are irreversibly denatured by exposure to pH values below 5 (Mobley *et al.* 1995). The isolation of the solution in the core from the bulk phase could allow it to be maintained near the neutral pH optimum due to the ammonia produced as a result of enzymatic activity.

The 1.55 angstrom resolution structure of the *Bacillus pasteurii* urease has given insight into mechanism (Benini *et al.* 2000; Musiani *et al.* 2001). Key features of the urease active site are the nickel coordination residues, the urea binding cavity and the surface flap that controls access to the active site (figure 5A). Eight residues in the c subunit function to co-ordinate the nickel ion and the substrate (Lys220, His137, His139, His249, His275, Gly280, Arg339, Ala366). These are strictly conserved, albeit in slight different sequence positions in *K. aerogenes*, *B. pasteurii* and the selected *Helicobacter* and *E. coli* strains. Nevertheless, there are large differences in the measured turnover number, k_{cat}, and Michaelis constants (K_M's). The k_{cat}'s range from 3450 s^{-1} (*K. aerogenes*) to 1650 s^{-1} (*H. pylori*), while K_M's range from 55.2 mM (*B. pasteurii*) to 0.18 mM *H. pylori* (Ha *et al.* 2001). In addition Ha *et al.* (2001) discuss differences in the flap (residues 309–349) that opens to clamp the substrate in the active site. The flap is a helix-loop-helix segment connected to the rest of the protein by a flexible hinge. In *H. pylori*, the flap seals the empty active site cavity; but weakly so that it transiently opens and closes to wedge in the substrate. In *B. pasteurii*, the flap remains in the open conformation with or without substrate. In *K. aerogenes*, the flap is highly disordered in the crystal structure, even with substrate. The good steric complementarity between the two flap faces ensures that

TABLE 1: GENOMES

SPECIES	SIZE/GENES	PATHOGEN	REFERENCE
Helicobacter pylori 26695	1.67 kb/1,623	?	(Tomb *et al.* 1997)
Helicobacter pylori J99	1.64 kb/1,537	Y	(Israel *et al.* 2001)
Helicobacter pylori HPAGI	1.60 kb/1,586	Y	(Oh *et al.* 2006)
Helicobacter hepaticus ATCC 51449	1.80 kb/1,918	N	(Suerbaum *et al.* 2003)
Helicobacter acinonychis Sheeba	1.55 kb/1,660	N	(Eppinger *et al.* 2006)
Escherichia coli K12	4.6 kb/4,411	N	(Blattner *et al.* 1997)
Escherichia coli 0157:H7 EDL933	5.6 kb/5,552	Y	(Perna *et al.* 2001)
Escherichia coli 0157:H7 Sakai	5.6 kb/5,482	Y	(Hayashi *et al.* 2001)
Escherichia coli CFT073	5.2 kb/5,562	Y	(Welch *et al.* 2002)
Escherichia coli W31110	4.6 kb/4,049	N	(Hayashi *et al.* 2006)

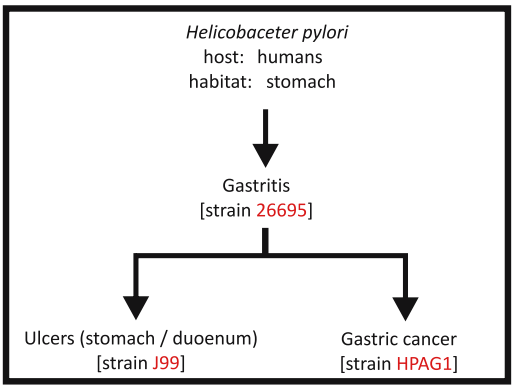

Figure 2. The ten selected genomes listed with size and references to source articles (left). Schematic illustrating how the 3 *H. pylori* genomes relate to each other in terms of disease progression (right).

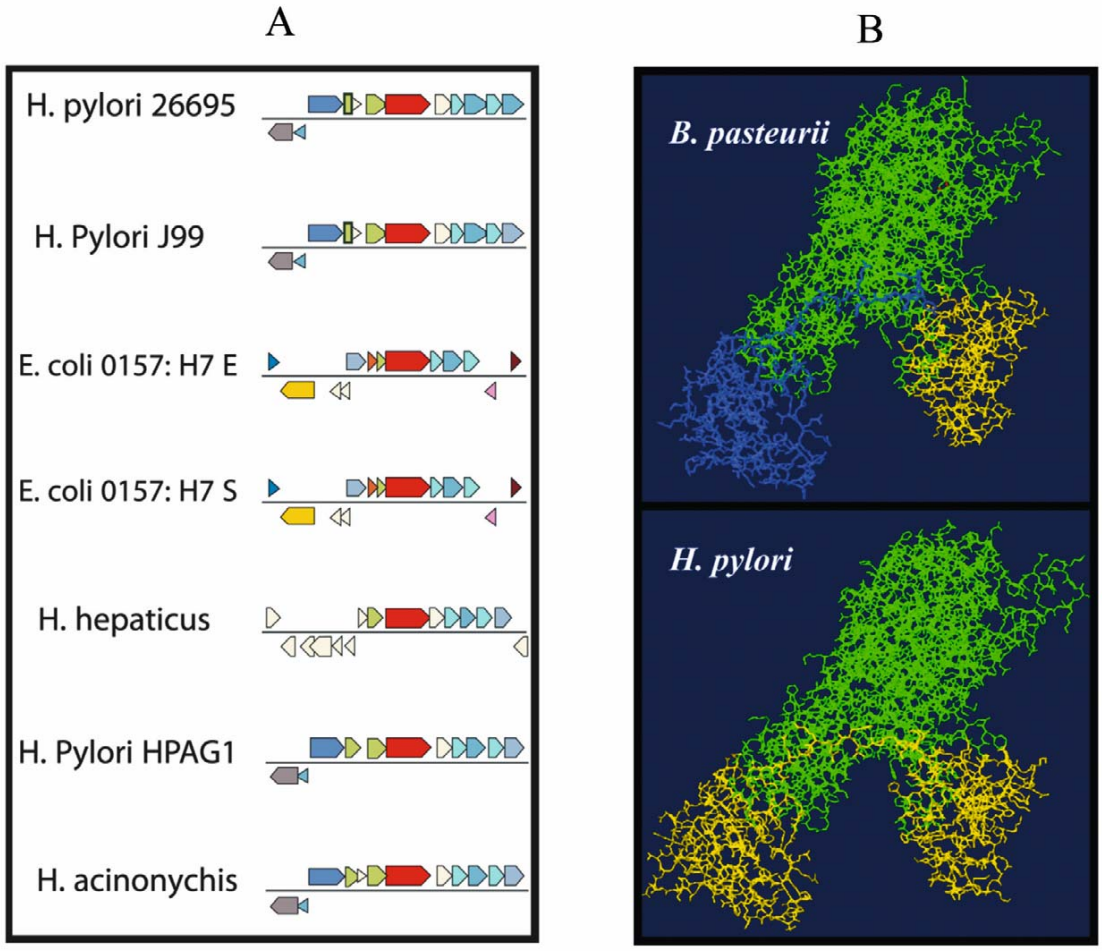

Figure 3. (**A**) Urease structural gene neighborhood. UreA (red), UreB = UreC (*E. coli*) (magenta). The *E. coli* strain lacks UreI (yellow). (**B**) X-ray crystal structures of the *B. pasteurii* and *Helicobacter* (bottom) urease asymmetric unit. UreA (yellow); UreB (*B. pasteurii*) (blue); UreB (*H. pylori*) = UreC (*B. pasteurii*) (green).

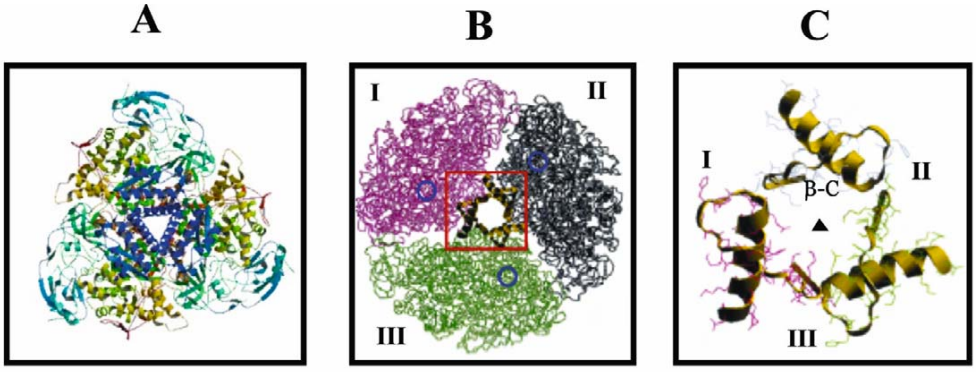

Figure 4. (**A**) *B. pasteurii* urease. The asymmetric unit forms a trimer. (**B**) *H. pylori* urease. Four trimers, arranged as for *B. pasteurii*, associate to form a central core. Red square highlights the C-terminal a-helical segments from each trimer responsible for formation of the tetramer. (**C**) Ribbon representation of the contact helices.

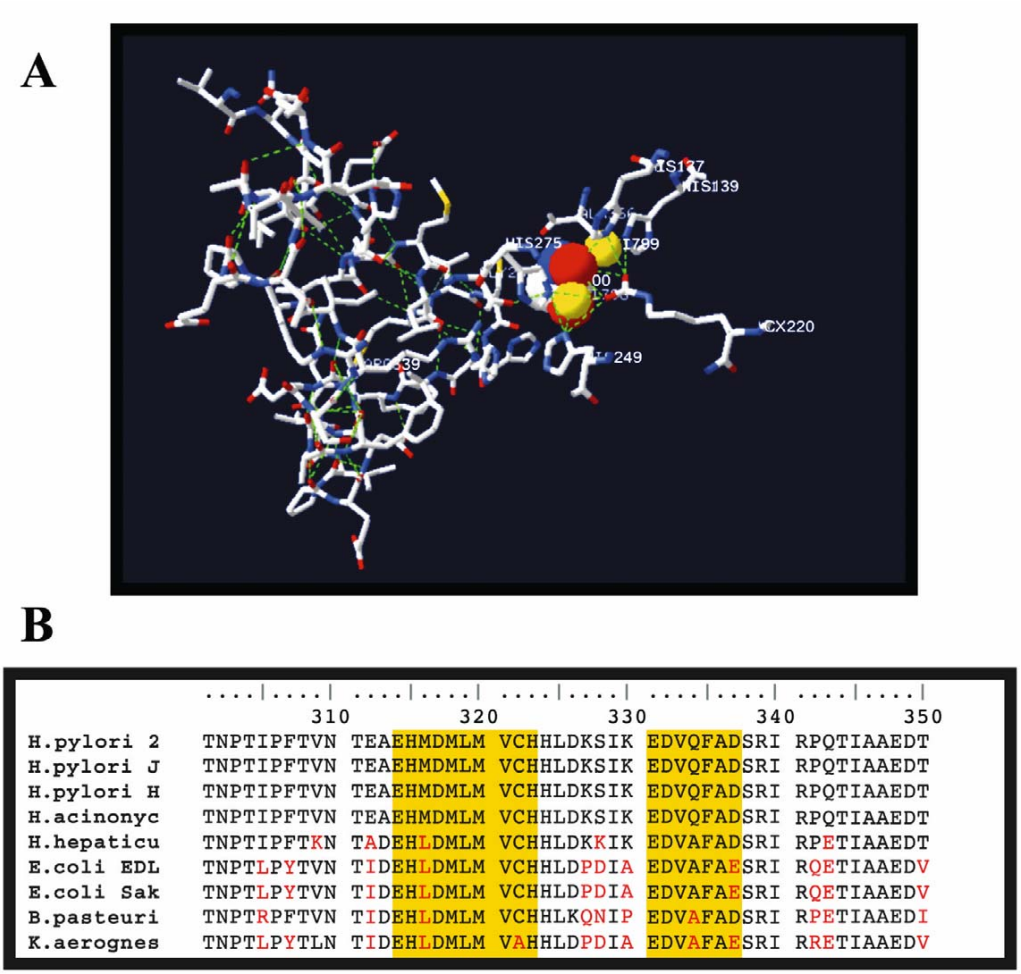

Figure 5. (**A**) *B. pasteurii active* site. Nickel ions (yellow), amino-hydroxy acetamide (red); hydrogen bonds (green). The flap (left) has a double helical architecture and is in the open configuration. (**B**) Sequence conservation of the flap. Residues absent from the gastric *Helicobacters*, but conserved among *B. pasteurii*, *K aerogenes* and *E. coli* O157 are in red. In 5 out of 13 cases (312AI, 317L, 328NKD, 334A, 343E) the latter set also encompasses *H. hepaticus*. α-helical segments are blocked in yellow.

the substrate is well solvated within the active site but does not provide a hindrance to dissociation of the products. In contrast, the crystal structures of the *B. pasteurii* and *K. aerogenes* urease imply that the attachment of the substrate urea will be hindered by competition with water molecules. The difference in K_M's may thus be explained by the expected difference in solvation energies, since K_M reflects the binding constant K and the solvation energy contributes to the free energy difference $\Delta G = RT\{\ln(K)\}$. Sequence alignment reveals that the sequences adjacent to the flap helices cluster into two groups (figure 5B). The gastric *Helicobacters* form one group, while *E. coli*, *K. aerogenes* and *B. pasteurii* form another. The *H. hepaticus* sequences have an intermediate composition; and presumably an intermediate k_{cat}, and K_M.

5. Urease expression and substrate availability

Nickel and urea regulate urease expression, as well as activity. Urea induces urease expression directly via NikR in some *E. coli* strains where the urease is plasmid encoded. The transcription factor, NikR, activates urease expression. In all ten *Helicobacter* and *E. coli* strains (figure 2), it requires nickel for binding promoter DNA of a diverse set of operons involved in nickel uptake and metabolism. The atomic structure of the *E. coli* NikR complex with synthetic promoter DNA reveals the DNA contacting amino acid residues, as well as the positions of the nickel ions (Schreiter *et al.* 2006). The palindromic, consensus motif for the *E. coli* NikR binding site is of the form GTATGA-N_{16}-TCATAC. In contrast, the consensus motif for *H. pylori* NikR has been proposed to be TATWATT-N_{11}-AATWATA,

where W represents either A or T (Ernst *et al.* 2005,. 2006). Comparison of the crystal structure of the *E. coli* NikR DNA complex with that of *H. pylori* NikR (Dian *et al.* 2006) shows that the separation between the two DNA contact domains scales in proportion to the number of nucleotides separating the contacted DNA segments (figure 6). This suggests that NikR associates with the fully extended DNA form.

In *H. pylori*, NikR activates some operons and represses others. The urease operon is activated, but that for *nixA*, the gene encoding the high affinity nickel transporter (Bury-Mone *et al.* 2004), and NikR itself is repressed. In *H. hepaticus*, by contrast, urease expression is not nickel responsive at the transcriptional level, only at the post-translational level. Furthermore, *H. hepaticus* does not exhibit the large (over an order of magnitude) increase in urease activity seen in *H. pylori* upon shift to acid pH (Belzar *et al.* 2005). The increase in *H. pylori*, must be due, in part, to stimulation of NixA mediated nickel transport by acid. The high affinity nickel transporter, NixA, is present in *H. acinocychis*, but not *H. hepaticus* or the *E. coli* strains. The changes in NikR structure induced by nickel occupancy as well as by the DNA do not presently permit an analysis of what features of the NikR-DNA interaction repress as opposed to activate transcription.

The urease operon also codes for accessory proteins. One of these, UreD, is needed to deliver nickel to the apo-urease (Kusters *et al.* 2006). Another, UreI, encodes a urea channel that is activated by extracellular protons (Bury-Mone *et al.* 2001). Interestingly, the enterohaemorrhagic *E. coli* strain O157 lacks *ureI*. Presumably, urea uptake by simple permeation through the membrane is sufficiently rapid so as not to limit its modest urease activity. Instead of NixA, *H. hepaticus* has a system of lower affinity nickel transporters. ABC type nickel transporters are present in all strains. We looked for, but did not find obvious differences in the architecture of these proteins within the three *H. pylori* isolates.

6. Other factors that confer acid resistance

H. pylori remains viable in the highly acidic (pH 3.0) environment of the stomach by two strategies: (i) locomotion from the acid lumen to the more neutral mucosal layers of the stomach wall (figure 7A); (ii) intracellular pH homeostasis by ammonia production and regulation of trans-membrane proton flux (figure 7B).

6.1 *Motility and chemotaxis*

Motility is essential for *H. pylori* pathogenesis. Non-motile mutants fail to colonize the stomach of piglets and mice raised in a microbe-free (gnotobiotic) environment (Wand *et al.* 2006). *H. pylori* have 4-7 complex flagella that are sheathed (Suerbaum 1995). Urease transcription and flagellar biogenesis seem to be linked (McGee *et al.* 2002). The flagellar sheath is continuous with the outer membrane and seems to be an adaptation to combat stomach acid. *E. coli* flagella disassemble when the pH falls below 4.5. Flagellins of the gastric *Helicobacters* are homologous to *E. coli* and would be expectedly to similarly disassemble in acid pH if not shielded by a sheath. In addition, the sheath functions as an attachment organelle. In the *Pylori* strains and *H. acinocychus,* a strain with analogous pathology for large felines, it contains a neuraminyllactose binding adhesin (*hpaA*) that attaches to the stomach wall. Other *Helicobacter*

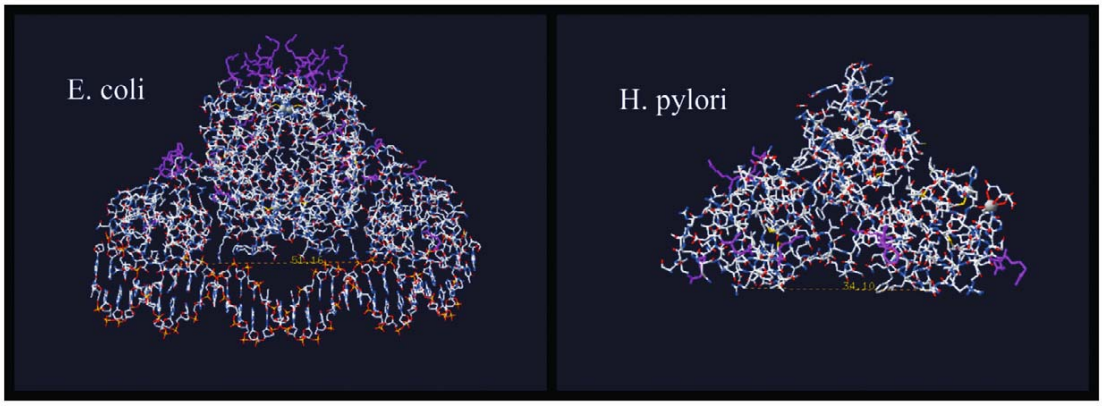

Figure 6. Crystal structures of *E. coli* NikR complexed with operator DNA (left) and *H. pylori* NikR alone (right). Yellow lines denote distances between the two DNA contact domains in the dimer structure. The distances are 51.2 and 34.1 angstroms respectively. Their ratio is the same (34.1/51.2 = 0.6665) as that for the nucleotides separating the recognition sequences in the palindromic consensus motifs (11/16 = 0.6875). This implies that *H. pylori* NikR, like *E. coli* NikR, binds the B DNA form. The differences in the lengths of the recognition motif reflect the span between the B DNA contact domains in the 3D atomic structures of the NikR proteins.

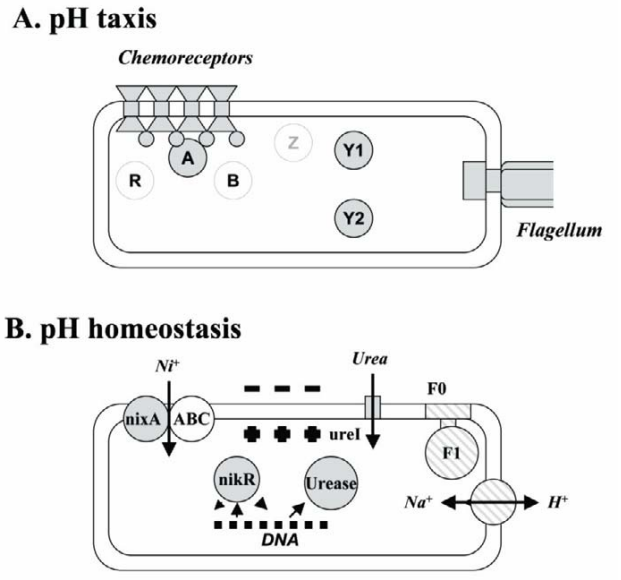

Figure 7. (A) *Helicobacter* pH taxis. Cytoplasmic protein components of the taxis machinery are either present in all ten chosen *Helicobacter* and *E. coli* strains (continuous outline, grey, black letters), absent in gastric *Helicobacters* alone (dotted outline, white, black letters); or absent from all *Helicobacters* (dotted outline, white, grey letter). The *Helicobacters* have a flagellar sheath and 2 CheY proteins (Y1, Y2) in contrast to *E. coli*. **(B)** Urease activity and pH homeostasis in *H. pylori*. In addition to low affinity ABC type nickel transporters (white), the gastric *Helicobacters* uniquely have the high affinity nickel transporter, NixA. NixA and UreI scavenge nickel and urea respectively from the gastric lumen. Both are stimulated by the acid luminal pH. In turn, the increased intracellular nickel stimulates NikR, the urease transcriptional activator, and urease itself (continuous gray). At acid pH, *H. pylori* has an inside out membrane potential, while the F_0F_1 ATP synthase and Na^+/H^+ antiporters are inhibited (chequered gray). Thus urease activity is activated by acid, while membrane proton conductance is decreased (see text).

species in addition to *H. hepaticus* are known to colonize rodents, in organs other than the stomach. A number of these are unsheathed, analogous to *E. coli* (Simmons *et al.* 2000*)*. These facts are consistent with the view that the flagellar sheath has been configured in gastric *Helicobacters* to counteract the acid environment. It prevents flagellar disassembly and causes the bacteria to adhere to the stomach wall, which then functions as an absorber. Once attached to the wall, they act to alkalinize the adjacent mucosal layers by extra-cellular, as well as intra-cellular urease activity, as discussed earlier. pH taxis then ensures that bacteria migrate in the pH gradient thus generated to accumulate on the wall.

As in the *E. coli'*s, pH taxis utilizes the central pH taxis circuitry. The circuitry in the *Helicobacters* lacks CheZ, the facilitator of CheY dephosphorylation. In addition, the gastric *Helicobacters* lack the methyltransferase, CheR, and the methylesterase, CheB, in contrast to *H. hepaticus*. Of the four *H. pylori* chemosensors, TlpB, is required for pH taxis as well as colonization of the gastric mucosa. It has been argued that the TlpB Hamp domain has a charge pattern that is similar to the Tsr Hamp domain of the *E. coli* Tsr receptor that mediates pH taxis (Croxen *et al.* 2006). However this pattern may be determined by other factors (e.g. CheW interaction) that are only indirectly related to signal generation. The Hamp domain sequences of Tsr, the related chemoreceptor Tar that generates an antagonistic signal, and TlpB exhibit high conservation within themselves (alignments not shown). Thus the charge patterns are important even though they are not presently interpretable.

6.2 *pH homeostasis*

The cytoplasmic membrane has a low basal proton conductance (1.6 μS/cm^2 surface area [Maloney 1979]). While the mechanistic basis is debated, *H. pylori* maintains an inside out membrane potential at acid pH (Marais *et al.* 1999). Transmembrane proton flux is controlled by Na^+/H^+ transporters and the F_0F_1 ATPase. Micro-array experiments reveal that both these membrane complexes are down-regulated in *H. pylori* upon shift to pH 3.0 (Bury-Mone *et al.* 2004). The *atp* operon encodes for the F_0F_1 ATPase. Analysis of its gene neighborhood indicates that the *atpI* accessory subunit is absent in the gastric *Helicobacters*. In addition, the operon encodes a gene (*atpX*) homologous with the F_0 b subunit in the position where *atpE*, the gene for the c subunit would normally be. The genes for the F_0 a and c subunits are located elsewhere (McGowan *et al.* 1997). The mechanistic implications of these differences remain to be explored.

7. Prospects

Bacterial pathogenesis involves a complex series of host pathogen interactions that change the bacterial genome as well as the micro-environment of the host organ. Bacterial pathogens have specialized DNA shuffling and horizontal gene transfer mechanisms that provide high genome plasticity (Hofreuter and Haas 2002; Karnholz *et al.* 2006). Genome micro-arrays of globally representative isolates (n = 60) of *H. pylori* and *H. acinonychis* have defined a 1,111 core genome out of a total of 1,531 genes. Variable genes included pathogenicity islands acquired or lost *en bloc* and those encoding restriction/modification enzymes, methylases and adhesins (Gressmann *et al.* 2005). These mechanisms serve to accelerate natural selection and emergence of new species. *H. hepaticus*, for example, lacks the CAG pathogenicity island (Suerbaum *et al.* 2003). Polymorphic diversity in *H. pylori* urease subunit genes (Owen *et al.* 1998) provides a

framework for tracking isolates from geographically diverse origins. In contrast to the gain or loss genes identified by Gressmann *et al.* 2005, most polymorphisms are neutral, resulting in functional ureases. These neutral mutations are obviously maintained over many generations in order to serve as trackers for human population migrations. Thus the urease operon seems to be part of the stable genome, in the *Helicobacters*. By contrast, in the five *E. coli* genomes inspected, the urease operon is absent from the chromosome. It is found, however, on the H7 plasmid DNA of the two pathogenic *E. coli* O157 strains.

The DNA transfer mechanisms noted above allow *Helicobacter* genomes as a whole to undergo micro-evolution on rapid timescales during co-habitation within an individual host. Micro-evolution in patients during disease progression or after therapy has been well-documented (Israel *et al.* 2001; Kauser *et al.* 2005; Prouzet-Mauleon *et al.* 2005; Kraft *et al.* 2006). Much of the observed changes result from horizontal transfer due to mixed colonization within the host (Kraft *et al.* 2006). This combination is essential for ensuring that micro-evolution occurs over the short times set by the finite lifetimes of the human hosts. Our survey of the three *H. pylori* genomes reveals the urease and accessory genes as well as other key components for acid resistance (transporters, F_0F_1 ATPsynthase, motility and pH taxis apparatus) are not targeted during such mico-evolution. Different species (*H. pylori*, *H. acinonychis*, *H. hepaticus*) do display variations that reflect long-term adaptations to different niches (e.g. stomach versus liver). While a more extensive and detailed analysis will be required, we speculate that this is because the *Helicobacters,* as a result of the long co-habitation with their hosts, have evolved a sophisticated, multi-level control circuitry; based on transcription, protein assembly, and ligand induced activity modulation; that adjusts the activity of these components to match changes within the host environment as disease progresses. This reflects an in-built plasticity in *Helicobacter* cellular physiology rather than genetic modification resulting from niche construction. The physiological plasticity seems to be a more viable strategy than gain or loss of encoding genetic elements; given the central position of pH homeostasis in the gastric *Helicobacter* lifestyle. To recap, the sequence shown in figure 1 may now be described as follows. First, a few motile bacteria traverse the viscous mucous layer and attach to the epithelial cell lining via their flagellar sheath adhesins. This occurs perhaps by chance, but more likely because variations in run-tumble bias within a population ensure that a subset of bacteria continue to run (swim) at acid pH. Second, the unstirred mucous layer adjacent to the immobilized bacteria is rendered alkaline by urease activity. The intracellular urease activity is stimulated by nickel/urea accumulation enhanced by the external acid environment; while surface localized ureases exposed to the external medium are resistant to acid pH denaturation due to their dodecameric architecture. Third, the local, neutral pH "niche" thus created by a few leads to mass migration and accumulation of the population to this niche by taxis from acid to neutral pH. Fourth, the immobilized bacteria secrete mucinases to breakdown the mucous layer and cause subsequent death of epithelial acid-secreting parietal cells by exposure to the gastric juice. Proliferation of stem cells to replace the parietal cells completes the construction of the local, neutral pH micro-environment. The bacteria establish intracellular residence in the stem cells by mechanisms that are not presently understood; but which may involve elements of the variable genome such as the pathogenicity islands.

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