Outer membrane phospholipase A’s roles in *Helicobacter pylori* acid adaptation

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**Abstract**

**Background:** The pH of the human gastric mucosa varies around 2.5 so that only bacteria with strong acidic stress tolerance can colonize it. The ulcer causing *Helicobacter pylori* thrives in the gastric mucosa. We analyse the roles of the key outer membrane protein OMPLA in its roles in acid tolerance.

**Results:** The homology model of *Helicobacter pylori* outer membrane phospholipase A (OMPLA) reveals a twelve stranded β-barrel with a pore that allows molecules to pass with a diameter up to 4 Å. Structure based multiple sequence alignments revealed the functional roles of many amino acids, and led to the suggestion that OMPLA has multiple functions. Besides its role as phospholipase it lets urea enter and ammonium exit the periplasm. Combined with an extensive literature study, our work leads to a comprehensive model for *H. pylori*’s acid tolerance. This model is based on the conversion of urea into ammonium, and it includes multiple roles for OMPLA and involves two hitherto little studied membrane channels in the OMPLA operon.

**Conclusion:** The three-dimensional model of OMPLA predicts a transmembrane pore that can aid *H. pylori*’s acid tolerance through urea influx and ammonium efflux. After urea passes through OMPLA into the periplasm, it passes through the pH-gated inner membrane channel UreI into the cytoplasm where urease hydrolyses it into NH₃ and CO₂. Most of the NH₃ becomes NH₄⁺ that is likely to need an inner membrane channel to reach the periplasm. Two genes that are co-regulated with OMPLA in gastric Helicobacter operons could aid this transport. The NH₄⁺ that might leave the cell through the OMPLA pore has been implicated in *H. pylori*’s pathogenesis.

**Keywords:** *Helicobacter pylori*, Acid tolerance, Multifunctional OMPLA, Urea pathway

**Background**

*H. pylori* survives in the human gastric mucosa

Most bacterial proteins require a distinct pH to function correctly, and that optimal pH is usually much higher than the gastric pH of around 2.5 [1]. Some bacteria, however, thrive in the acidic gastric mucosa [1]. Bacterial cell walls and membranes generally are leaky [2], which would, in the stomach, rapidly lower their cytosolic pH if they had no effective acid stress relieve mechanism [1]. The fact that the stomach pH fluctuates depending on food and liquid intake [3] puts additional constraints on this acid stress relieve mechanism.

The *Helicobacter* genus comprises a versatile group of species that is found in different hosts, usually colonizing the intestine, liver, or stomach [4]. They can be divided into gastric- and enterohepatic-*Helicobacters*, with different morphology and genetic diverse lineages [5]. *H. pylori* is the most prevalent species observed in the human gastric mucosa [6].

When *H. pylori* enters the acidic gastric lumen, it migrates towards the epithelial surface. The pH in the epithelial surface is approximately 5, but it can fluctuate from pH 6 to pH 1 [1]. When *H. pylori* is in an acidic environment [7] pH-dependent transcription factors like Fur, NikR, and ArsRS [8] trigger overexpression of proteins involved in motility and ammonia production. The increased expression of motility genes allows the bacteria to move away from the acidity [7], while the ammonia
will protect the bacterium by buffering the influx of protons [1].

**H. pylori OMPLA implicated in colonization**

Outer membrane (OM) proteins often are multifunctional and involved in maintaining the membrane integrity [9]. They form the first line of defence by detecting possible attacks on the membrane for which purpose many OM proteins function as signal transducers [9].

An intact *H. pylori* outer membrane phospholipase A (OMPLA) has been suggested necessary for colonization of the human gastric ventricle [10, 11]. Only *H. pylori* with intact OMPLA will survive in acidic environments in vitro, whereas phase variants with truncated OMPLA can survive at neutral pH. At neutral pH the bacterial variants with intact OMPLA, have an altered lipid composition. The switch between intact and truncated OMPLA is explained by a phase-variable DNA slippage in the homopolymeric tract of the OMPLA gene, *pldA* [12]. Phase-variable proteins are often implicated in roles involving bacteria—environment interactions [13]. At pH 5 only *H. pylori* with intact OMPLA (OMPLAON-variant) is selected in vitro, even though the enzymatic activity at pH 5 is turned off because the pH optimum for enzymatic activity is 7.

OMPLA is found in several Gram-negative species [14], and it has been suggested that OMPLA activity is triggered by diverse events, such as temperature shift or heat shock, toxin release, or membrane instability. OMPLA activity in *Escherichia coli* is related to loss of membrane integrity [9]. *E. coli* OMPLA is activated under a wide variety of conditions (e.g. membrane perturbation) by calcium-induced dimerization [15]. OMPLA may be activated under various stress conditions in different species a where phospholipase activity contributes to cell wall degradation [9, 11], but *H. pylori* OMPLA is not likely to be activated by acidity since the bacterium needs an intact OM to survive the harsh conditions in the gastric mucosa.

**The *H. pylori* acid defence mechanism hypothesis**

Intact OMPLA is required for survival of *H. pylori* in acidic environments despite the fact that OMPLA is enzymatically inactive at low pH [10, 12]. Combining these findings with experimental work on five novel isogenic pairs of OMPLA variants, and in silico analyses of OMPLA sequence and structure data augmented with a literature study, we arrive at a hypothesis for a functional mechanism for this acid protection. This hypothesis is depicted in Fig. 1, and a detailed overview of the six steps in Fig. 1 can be found in Additional file 1. All details of this model and the corroborating evidence are presented in the remainder of this article.

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**Results**

Our bioinformatics work, combined with an extensive literature study, and backed up by experimental work on
variants with non-functional OMPLA is summarized in Fig. 1. The remainder of this article discusses all aspects of this model.

**Survival of *H. pylori* OMPLA variants in acidic environment**

Sequencing the variants revealed classical phase variants displaying shorter OMPLA sequences in euBL, euAP, and euBF; see Table 1. Two variants (euBZ and euBB; see Table 1) have missense mutations resulting in a non-functional OMPLA. Under prolonged growth in acidic pH conditions, only those with an ON phase and/or without missense mutations survive [12]. Thus, OMPLA OFF is non-functional OMPLA due to either a truncated OMPLA or a missense mutation. All five OFF variants grow at neutral pH, lacking OMPLA activity and they do not survive at all at low pH.

The existence of these variants further emphasizes the importance of OMPLA for survival at low pH.

**H. pylori OMPLA 3D structure model**

A total of 3084 sequences were included in the OMPLA Multiple Sequence Alignment (MSA; see Additional file 2). Extracting *E. coli* and *H. pylori* OMPLA sequences from the MSA yielded the sequence alignment shown in Fig. 2a. This alignment was used to construct the OMPLA 3D structure model. The extracted *H. pylori* OMPLA sequence is compared to the original unaligned sequence (HPeuAN, GenBank Accession ID: AFR51731) in Fig. 2b. *H. pylori* OMPLA has a 38-residues long insert (the so-called ‘not-modelled insert’ that is highlighted yellow in Fig. 2b). This region consists of a sequence not found in the template *E. coli* sequence, and Blastp only finds acid-tolerant gastric Helicobacteraceae containing this sequence element.

The YASARA/WHAT IF twinset predicts that this insert constitutes a loop without any regular secondary structure.

The MSA was used for entropy-variability analyses (EVA), and Fig. 3 shows the resulting EV plot for OMPLA sequences, which were mapped onto the *H. pylori* OMPLA model structure in Fig. 4. There are highly variable (blue) residues located in the exterior loops, while the most conserved (red) residues are located in the trimer interface, the active site, and the calcium-binding site, while a few are located in extracellular loops. The highly conserved extracellular loop-residues, that must have an important function for OMPLA, include two tyrosine residues: Y233 and Y240 in loops 4 and 6, respectively (see Fig. 4a, b).

The resulting 3D structure model of *H. pylori* OMPLA shows that molecules like urea and ammonium could easily pass through the *H. pylori* OMPLA pore which has a diameter of at least 4 Å, which is much wider than the 1.5 Å diameter of the UreI (see Fig. 5; see Additional file 3) pore that is known to let urea pass into the cytosol (see Additional file 1).

The differences in pore size and amino acid composition between the *H. pylori* OMPLA model and *E. coli* OMPLA structure are visualized in Fig. 6.

**Sequence similarities in acid tolerant species of the Helicobacteraceae family**

The not-modelled sequence highlighted in Fig. 2b is found in nearly all gastric Helicobacter species, but is lacking in enterohepatic Helicobacter species (see Table 2; Fig. 7). *H. pylori*’s OMPLA shares the highest sequence identity with other gastric Helicobacter OMPLAs, and we will explore how they differ from enterohepatic Helicobacters (see Fig. 7).

### Table 1 Characteristic of *H. pylori* colony variants showing altered phospholipase A activity

| *H. pylori* isolates | OMPLA length (amino acids) | Enzyme activity | Truncated OMPLA or missense mutation? | Sequence ID (GenBank ID) |
|----------------------|-----------------------------|-----------------|----------------------------------------|--------------------------|
| euBL (98019)         | OMPLA\_ON 355              | Yes             | –                                      | AFR51755.1               |
|                      | OMPLA\_OFF 263             | No              | Truncated OMPLA                        | N/A                      |
| euAP (29A)           | OMPLA\_ON 355              | Yes             | –                                      | AFR51733.1               |
|                      | OMPLA\_OFF 265             | No              | Truncated OMPLA                        | N/A                      |
| euBF (5A)            | OMPLA\_ON 355              | Yes             | –                                      | AFR51749.1               |
|                      | OMPLA\_OFF 263             | No              | Truncated OMPLA                        | N/A                      |
| euBZ (9B)            | OMPLA\_ON 355              | Yes             | Missense mutation (Pro157Ser)          | AFR51769.1               |
|                      | OMPLA\_OFF 355             | No              | –                                      | AFR51745.1               |
| euBB (53A)           | OMPLA\_ON 355              | Yes             | Missense mutation (Ser235Arg)          | N/A                      |
|                      | OMPLA\_OFF 355             | No              | –                                      | N/A                      |

The isolates used in this article are the same as previously published [14]
In silico pldA operon prediction

Figure 8 shows how the OMPLA gene, pldA, is organized in gastric Helicobacters compared to a series of other bacteria. Gastric Helicobacter species have a common operon organization in which pldA lies downstream of two channels that belong to the COG0733 family that is also known as ‘Na⁺-dependent transporters (channels) of the SNF family’ (AmCI and AmCII). According to ProOpDB pldA is not part of any operon in enterohepatic H. hepaticus (see Fig. 8b). In Table 3 we explore the differences between the gastric and enterohepatic Helicobacter gene organizations. It is clearly seen that the AmCI and AmCII channel are found only in the pldA operon of gastric Helicobacter species, which strongly suggests a role in pH management.

3D modelling of IM channels

The model depicted in Fig. 1 includes two channels to allow NH₃/NH₄⁺ to pass from the cytosol to the periplasm. We constructed 3D models for AmCI and AmCII as described for OMPLA, with the same methodology that was used for the analyses of porins [16]. AmCI and AmCII share a pairwise sequence identity of 31–34% to the template, and ~50% to each other. The results are shown in Fig. 9. Visual inspection of these models reveals that AmCI has a more polar pore and thus might be the
channel for \( \text{NH}_4^+ \), while AmCII pore is more hydrophobic and thus seems more suitable for letting \( \text{NH}_3 \) diffuse to the periplasm. It is also not yet clear if the activity of either of these two channels is a function of either the cytosolic or the periplasmic pH, and we do not (yet) know if AmCI and/or AmCII are sodium symporter or antiporters, or perhaps they even are simple channels and not transporters. Most well characterized sodium or sodium and chlorine dependent transporters have much longer sequences than AmCI and AmCII, suggesting that AmCI and AmCII might miss the co-transporter related domains. Further experiments are needed to answer all these questions, but both the operon structure and the 3D models do not disagree with the idea that AmCI and AmCII help ammonia/ammonium travel from the cytosol to the periplasm.

**Discussion**

The *H. pylori* acid regulation pathway has been well studied and often debated (see Additional file 1 for further details) [1], but all currently available functional models still require unknown OM components for urea influx and ammonium efflux. Our in silico results indicate that *H. pylori* OMPLA can be an OM urea and ammonium channel, while AmCI and AmCII, that are likely co-regulated with OMPLA, can be involved in ammonium efflux from the cytosol. The hypothesis presented in this paper, depicted in Fig. 1, is mainly based on in silico models combined with current literature findings on the urea pathway and *H. pylori* acid tolerance.

Unlike *E. coli* OMPLA, *H. pylori* OMPLA is continuously breaking down membrane phospholipids to lysophospholipids when cultivated at physiological pH [17]. The optimal pH for OMPLA’s enzymatic activity is around 7.0, and this activity is abolished at pH 5.0 or lower, yet this protein is required for in vitro survival at very low pH [12].

The OMPLA structure is composed of a 12-stranded transmembrane \( \beta \)-barrel with short periplasmic turns and long extracellular loops. This is consistent with the structure of other outer membrane proteins (OMPs; including that of porins, the largest OMP subfamily) that diffuse molecules through the membrane [16]. The *H. pylori* OMPLA barrel is large enough to have pore activity [16]. Figure 5 supports the idea that there is enough space for urea to pass the OM through OMPLA. The pores of the *E. coli* and *H. pylori* OMPLA vary in shape and amino acid composition, as shown in Fig. 6, supporting our hypothesis that they differ in function. We therefore suggest that *H. pylori* OMPLA is a multifunctional protein with one function being a phospholipase and a second function being acid protection.

Standard sequence alignment methods are reliable for closely related sequences, but often fail when diverse sequences-like OMPLAs- are analysed [18, 19]. The alignment of the *H. pylori* OMPLA sequence with the sequence of the *E. coli* OMPLA template structure (1QD5 PDB) is complicated. To get this alignment we collected as much information as possible about homologous OMPLA sequences and generated a MSA using an iterative profile alignment process. Unlike standard sequence alignment methods, iterative profile alignments can use both structure and function information. The *H. pylori* and *E. coli* OMPLA protein sequences are highly different (see Additional file 2) and their sequence identity is just above the threshold for homology modelling [20] when the not-modelled insert (Fig. 2) is not taken into account.

EV analyses the evolutionary footprint left behind in a MSA and identifies regions in the protein in which conservation and variation relate to various aspects of the protein’s function [16, 19]. The highly variable residues (coloured blue in Fig. 4) observed mainly at the outside facing loops likely are (a-specifically) involved in host evasion [16]. The two highly conserved loop tyrosines (Fig. 4) are located far from the trimer interface, yet they must be functionally important because they are conserved in the MSA.

Most gastric Helicobacters have a sequence insert that is lacking in the enterohepatic Helicobacters (and other species). Table 2 shows two gastric Helicobacters that
are very different and that lack this sequence insert: *Helicobacter mustelae* and *Helicobacter himalayensis*. In phylogenetic analyses, *H. mustelae* cluster with enterohepatic Helicobacters [21]. The genome from *H. mustelae* type strain 12,198 [22] lacks *pldA*, the gene coding for OMPLA. *H. mustelae* possess a nickel-independent urease, called UreAB2 [23]. In the presence of urea this metalloprotein is by itself already sufficient for the bacteria to survive acid shock. It is activated by ferrous ions in the absence of auxiliary proteins [23]. *H. mustelae* has adapted a different mechanism to survive in the stomach of its host, the ferret. The other gastric species that differs, *H. himalayensis* lacks urease genes, which indicates that it lacks the whole urease pathway. Generally, gastric and enterohepatic *Helicobacter* OMPLAs are quite different; enterohepatic *Helicobacters* lack the insert found in most gastric *Helicobacters*, except the OMPLAs from *H. mustelae* and *H. himalayensis*, that seem to have evolved totally different systems to cope with low pH.

The not-modelled insert is similar to that found in the sequence alignment of a subset of sequences presented by Istvan et al. [11]. This not-modelled insert (see Table 2; Figs. 2, 7 for more details) can be of importance for pH-gating as observed in other proteins such as *H. pylori* UreI [24] and *E. coli* OmpG [25]. pH sensitivity has also been detected in the *E. coli* OmpF constriction loop [26].

We do not know the function of the residues in the predicted extracellular loop (the not-modelled insert highlighted in Fig. 2b), but literature has shown that mutating a charged extracellular loop residue can have deleterious effects on acid survival [27, 28]. Our laboratory results (the missense mutations P157S and S235R, see Additional

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**Fig. 4** EVA and *H. pylori* OMPLA model. **a** The most conserved residues are shown as red stick model on a OMPLA monomer model (side view) in which the active site is indicated. **b** As a, but now a OMPLA trimer is used. **c** EVA results mapped on the *H. pylori* OMPLA all-atom trimer (side view) coloured by EV-plot section. **d** As b, but top view. **e** As c, but top view. The blue residues that cover the trimeric hole are periplasmic loop residues (coloured grey in d).
file 5 for residue numbering) revealed the importance of these two OMPLA residues. Ser235 is located in the not-modelled loop insert. Arginine is a positively charged residue and its introduction will lead to electrostatic changes, perhaps resulting in a functionally closed pore. P157 is one of the highly-conserved residues. It is located at the trimer interface (see Fig. 4). This mutation likely destabilizes the β-barrel structure [16, 29, 30], or might disable acidic protection by disturbing the trimer interface that is required for pore-activity [16]. The conserved cysteines, found in the gastric *Helicobacter* OMPLA lie in this extracellular loop. Cys residues are seldom found in OMPs [31] and they are seldom found facing the surface; however, conserved cysteines have a wide range of functions and are usually of great importance to the protein [32–34]. Although protein stabilization would be a likely function for this conserved disulfide bond, further laboratory work is needed to confirm its function. Inspection of the model suggests that Y240 stabilizes the extracellular loops; especially the interactions between the first and third extracellular loop. We have previously predicted that all porins function as a trimer [16], and provided evidence that OMPLA forms a trimeric structure too. The Y233 is located near the putative trimeric hole. This suggests a regulatory role for this residue, but what that role might be remains unclear.

OMPLA’s operon structure sheds light on other relevant proteins in the urea pathway (see Fig. 1). The gastric and enterohepatic *Helicobacter* species have different operon organizations, as illustrated in Fig. 8. Genes located in the same operon normally show co-expression and tend to be regulated by the same promoter. Price et al. found that the life-cycle of an operon is under strong selection [35] and genes found in the same operon are likely involved in the same process [36, 37]. Gastric *Helicobacters* have a different gene expression compared to enterohepatic *Helicobacters*, including a higher level of urease expression [23]. The hosts of the gastric *Helicobacter* group are more diverse than the hosts of the enterohepatic group, but their *pldA* operons nevertheless are more similar to each other; some consisting of five consecutive genes (*2 SLC6sbds_Tyt1-Like* genes, *pldA*, *dnaN*, and *gyrB*). The two transporter (channel) genes upstream of the *pldA* gene are generally predicted to lie in the same operon as *pldA* in gastric *Helicobacters*, as shown in Table 3. The gene encoding AmCI is also predicted to be phase variable (see Table 3). The two gastric bacteria *H. himalayensis* (lacking the urease genes) and *H. mustelae* (lacking the *pldA* gene) and enterohepatic *Helicobacters*, have different organization and they have probably evolved different mechanisms for acid adaptation.

Homology searches in clusters of orthologous groups (COGs) database [37] revealed that AmCI and AmCII both belong to COG0733 (*Na*⁺-dependent transporters (channels) of the SNF family). Since genes in an operon are regulated together, the two *Na*⁺-dependent transporters (channels) of the SNF family (AmCI and AmCII) are also likely implicated in acid survival and we suggest that they are involved in ammonium/ammonia transport or diffusion from the cytosol to the periplasm. We do not know why two very similar channels are needed in this process, but visual inspection of their 3D structure models suggests the possibility of a regulatory role for this residue, but what that role might be remains unclear.
### Table 2: Non-modelled OMPLA insert in *Helicobacter* sequences

| Species          | Representative strain | Classification | Predicted loop insert |
|------------------|-----------------------|----------------|-----------------------|
| *H. acinonychis* | Sheeba                | Gastric        | Insert                |
| *H. bizzozeronii* | CII-1                 | Gastric        | Insert                |
| *H. cetorum*     | MIT 99-5656           | Gastric        | Insert                |
| *H. heilmannii*  | ASB1.4                | Gastric        | Insert                |
| *H. pylori*      | ATCC 43504            | Gastric        | Insert                |
| *H. suis*        | H5S (partially sequenced) | Gastric    | Insert                |
| *H. felis*       | ATCC 49179            | Gastric        | Insert                |
| *H. mustelae*    | 12198                 | Gastric        | NA                    |
| *H. himalayensis*| YSI                   | Gastric        | No insert             |
| *H. bilis*       | ATCC 43879            | Enterohepatic  | Short insert          |
| *H. canadensis*  | MIT 98-5491           | Enterohepatic  | No insert             |
| *H. canis*       | NCTC 12740            | Enterohepatic  | No insert             |
| *H. cinaedi*     | CCGU 18818 = ATCC BAA-847 | Enterohepatic | No insert             |
| *H. fennelliae*  | MRY 12-0050           | Enterohepatic  | Short insert          |
| *H. hepaticus*   | ATCC 51449            | Enterohepatic  | No insert             |
| *H. munitarum*   | ST-1                  | Enterohepatic  | Short insert          |
| *H. pullorum*    | MIT 98-5489           | Enterohepatic  | No insert             |
| *H. rodentium*   | ATCC 700285           | Enterohepatic  | No insert             |
| *H. trogontum*   | ATCC 700114           | Enterohepatic  | No insert             |
| *H. typhlonicus* | MIT 97-6810           | Enterohepatic  | No insert             |
| *H. winghamensis*| ATCC BAA-430          | Enterohepatic  | No insert             |

This table lists *Helicobacter* species, strain, classification (gastric vs enterohepatic) and whether the OMPLA sequences contain the predicted loop. The "Predicted loop insert"—column lists those species that have a long, unique insert that could not be modelled, but predicted to be a longer loop. They are typically found in gastric bacteria. The term "short insert" indicates shorter loops, likely to have a different function based on their residue composition. See Additional file 4 for the sequence alignment file.

### Non-modelled *Helicobacter* OMPLA insert sequence

| Species          | Insert Sequence                                                                 |
|------------------|---------------------------------------------------------------------------------|
| *H. cetorum*     | EWIGQNVSSTGDSNLGSNGGVRQGRNVR---WGGCRSVSAGQR                                      |
| *H. acinonychis* | EIWAQGQHISNGVGGAECYQPFKNQGPNPQFSPQVYVKNQVNR---WGGCRSVSAGQR                      |
| *H. pylori*      | EWFAGQHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                        |
| *H. bizzozeronii*| EWFAGQHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. felis*       | EWFAGQHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                        |
| *H. heilmannii*  | EWFAGQHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. suis*        | EWFAGQHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. fennelliae*  | ERFAGQHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. bilis*       | ERFAGQHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. munitarum*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. pullorum*    | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. rodentium*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canadensis*  | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canis*       | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. cinaedi*     | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. hepaticus*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. munitarum*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. pullorum*    | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. rodentium*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canadensis*  | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canis*       | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. cinaedi*     | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. hepaticus*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. munitarum*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. pullorum*    | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. rodentium*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canadensis*  | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canis*       | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. cinaedi*     | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. hepaticus*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. munitarum*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. pullorum*    | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. rodentium*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canadensis*  | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. canis*       | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. cinaedi*     | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. hepaticus*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |
| *H. munitarum*   | ERLFAGHISNGVGGACQYQFNKENQNCPFQGVYVKNQVNR---WGGCRSVSAGQR                       |

**Fig. 7** Not-modelled insert sequence. The alignment of the inserted region among the *Helicobacter* species is shown (see Table 2 for more information). The residues highlighted in red constitute the catalytic triad in OMPLA. Gastric species names are in blue; enterohepatic species names are in green. The *H. himalayensis* Y51 genome lacks urease genes, indicating lack of the urease pathway.
that one of the channels can be ammonium specific while the other is specific for ammonia. While the OMPLA family (COG2829) is mainly found in Proteobacteria, Na\(^+\)-dependent COG0733 channels are widespread throughout bacteria and eukaryotes. In order to better understand the possible role of these proteins in the urea pathway (being co-regulated with OMPLA), 3D model structures were constructed for AmCI and AmCII (see Fig. 9). The AmCI and AmCII transporters have closest sequence similarity with Bacillus halodurans MhsT (a BLAST search against the PDB resulted in 31–35% sequence identity for the H. pylori HP0497 and HP0498 sequences). MhsT is a Na\(^+\)-dependent neurotransmitter/sodium symporter and belong to the SLC6 family of Na\(^+\)/Cl\(^-\)-dependent neurotransmitter transporters. These proteins transport small substances, e.g. amino acids or similar structures [38, 39]. The substrates found among SLC6 transporters include glycine, serotonin, dopamine, and norepinephrine [40]. They have a so-called 5 + 5 core helix motif that is embedded in the membrane, albeit that we cannot exclude the presence of more helices; MhsT, for example, has 11 helices, while LeuT has 12 helices [38]. Evolutionary, these transporters adjust quickly to changes; some of them, for example, are voltage-gated channels under certain conditions [41]. We believe that the two putative ammonium channels, AmCI and AmCII, are located in the inner membrane, because helical proteins are seldom found in the outer membrane of Gram-negative bacteria [16]. Likely functions include pH sensing, or solute transport of small substances that are involved in buffering the environment [42–46]. We hypothesize that since they are co-regulated with OMPLA, these transporters are involved in the urea pathway, as shown in Fig. 1. Since there are currently no NH\(_4\)\(^+\) channels known, we believe the two COG0733 are better candidates than the suggested UreI. UreI have an important role in urea influx, but no experiments show a role for Urel in ammonium efflux [42].
Conclusions

OMPs have multiple functions that aid adaptation in rapidly changing environments [16]. Current literature does not explain which *H. pylori* OMP is involved in maintaining higher periplasmic pH level compared to the acidic outside environment. In silico protein structure modelling of *H. pylori* OMPLA indicates a transmembrane pore of 4 Å, and *H. pylori* OMPLA could participate in acid protection through this transmembrane pore. Our group has previously linked intact *H. pylori* OMPLA to an increased risk for ulcer disease [47]. A possible explanation for this could be its role in the urea pathway that concludes with the efflux of ammonium that is implicated in ulcer formation.

We propose that urea passes through OMPLA into the periplasm, while ammonium might exit to increase the cytoplasmic and periplasmic pH. Since acid tolerance is observed only when OMPLA is intact, we suggest that OMPLA's role is to maintain an optimal periplasmic pH as modelled in Fig. 9. We hypothesize that AmCI and AmCII, which are co-regulated with OMPLA, are also implicated in acid protection allowing NH₃/NH₄⁺ to move from the cytoplasm to the periplasm. We propose that OMPLA also is involved in the secretion of NH₄⁺ from the cell. That gives OMPLA two transport roles -urea influx and ammonium efflux- which is possible because it is likely constitutively expressed (as observed in other pathogenic bacteria [33]) which suggests that it probably is abundantly present in the outer membrane.

Table 3 Operon prediction

| Species           | Representative strain | Host       | Classification | Upstream          | Downstream          |
|-------------------|-----------------------|------------|----------------|-------------------|---------------------|
| *H. acinonychis*  | Sheeba Cheetah        | Gastric    |                | AmCI and AmCII    | dnaN, gyrB, 2 hypothetical genes |
| *H. cetorum*     | MIT 99-5656 Dolphin   | Gastric    |                | AmCI* and AmCII   | dnaN, gyrB          |
| *H. pylori*      | ATCC 43504 Human      | Gastric    | AmCI and AmCII | dnaN, gyrB        |                     |
| *H. bizzozeronii*| CII-1 Human           | Gastric    | AmCI and AmCII | No                |                     |
| *H. himalayensis*| YS1 Marmota himalayana | Gastric    |                | No                |                     |
| *H. suis*        | H5 Swine              | Gastric    | AmCI and AmCII | No                |                     |
| *H. felis*       | ATCC 49179 Cat        | Gastric    | AmCI and AmCII | No                |                     |
| *H. mustelae*    | 12198 Ferret          | Gastric    |                | No                |                     |
| *H. heilmannii*  | ASB1.4 Cat            | Gastric    |                | AmCI* and AmCII   | No                  |
| *H. bilis*       | ATCC 43879 Human      | Enterohpatic| No          | No                |                     |
| *H. canadensis*  | MIT 98-5491 Human     | Enterohpatic| No          | No                |                     |
| *H. canis*       | NCTC 12740 Human      | Enterohpatic| No          | pseH              |                     |
| *H. cinaedi*     | CCG18818 ATCC BAA-847 | Human      | Enterohpatic   | No                | metE                |
| *H. fennelliae*  | MRY 12-0050 Human     | Enterohpatic| Hypothetical  | SAM-dependent MTase genes |
| *H. hepaticus*   | ATCC 51449 Mouse      | Enterohpatic| No          | metE, and gene encoding NADPH |
| *H. mirdarum*    | ST-1 Rat              | Enterohpatic| No          | No                | ABC transporter gene |
| *H. pullorum*    | MIT 98-5489 Human     | Enterohpatic| No          | No                |                     |
| *H. rodentium*   | ATCC 700285 Mouse     | Enterohpatic| No          | No                |                     |
| *H. trogontum*   | ATCC 700114 Rat       | Enterohpatic| No          | No                |                     |
| *H. typhlicus*   | MIT 97-6810 Mouse     | Enterohpatic| No          | No                |                     |
| *H. winghamensis*| ATCC BAA-430 Human    | Enterohpatic| No          | No                |                     |

Operon prediction comparing gastric and enterohpatic genes upstream/downstream from *pldA* *Helicobacter* species. Operons are predicted by Fgenesb software (Softberry Inc., Mount Kisco, NY, US). COG0377 corresponds to the “Na⁺-dependent transporters (channels) of the SNF family” (this includes AmCI and AmCII). The COG0377 are phase variable genes.

* Truncated proteins

Fig. 9 EVA for 4444 COG0733 sequences. EV-plot to the left with the residues mapped onto the MhsT structure to the right (4US3 PDB template file). Each box represents a degree of entropy-variability and is coloured as described in “Methods” section. a) EV-plot. b) EVA mapped onto MhsT.
Methods
Survival of \(H.\) pylori OMPLA variants in acidic environment
We collected \(H.\) pylori clinical isolates in four hospitals in the Oslo region, Norway [12, 14]. 57 isolates were examined for spontaneous colony variants showing altered phospholipase A activity by thin-layer chromatography (TLC) as previously described [47]. Isogenicity of five selected variant pairs were confirmed by amplified fragment length polymorphism (AFLP) as previously described [12]. From each variant, the \(pldA\) gene was sequenced as previously described [47], in order to detect the genetic background for the OMPLA phenotype (Table 1). Survival of the selected variants at pH 3.5 was determined as previously described [12]. See Additional file 1 for more information.

Operon predictions
ProOpDB [36] predicts operon structures of prokaryotic genomes, and presents results in figures in which all genes in a clusters of ortholog genes (COG) have the same colour. We used this software to determine all genes in the same operon as OMPLA. Not all species are represented in this database, so the genes used to analyse gastric vs enterohepatic Helicobacters were analysed in Fgenesb (Softberry Inc., Mount Kisco, NY, US). The gene sequences were manually curated using NCBI’s geneview and BioEdit. We extracted the genes transcribed in the same direction as \(pldA\) and used Fgenesb to find out which genes likely are co-regulated.

In silico protein sequence analyses
Structure-based MSAs were produced using the workflow described by Kuipers et al. [18]. This procedure uses core sequence elements (e.g. \(\beta\)-strands and \(\alpha\)-helices) to generate an initial profile. The sequences of all homologs (found with Blastp [48]) are aligned iteratively to the profile that is updated after each alignment round. The aligned sequences of the model and template were extracted from the final MSA (see Additional file 2) and used to construct the 3D structure model (see Fig. 2a).

In silico protein entropy-variability analyses
The MSA was used to develop an evolutionary model through EVA. The MSA and EVA analyses were performed as described [16, 18] using the YASARA/WHAT IF twinset [49–51]. EVA plots the entropy versus the variability to describe the variability pattern for every residue in the MSA. This plot contains five sectors that each holds residues with common structural and functional characteristic, as shown in Fig. 10 [19].

OMPLA 3D structure modelling
The \(H.\) pylori OMPLA model was built for the NCBI: AFR51731 sequence using the 1QD5 [15] (monomer PDB file downloaded from [http://www.rcsb.org] [52]) as template. The sequence alignment underlying the homology modelling was extracted from the aforementioned MSA. All waters, lipids, and crystallization additives were removed from the template file before modelling, and the remaining, reduced monomer was further subjected to the YASARA Clean function in the YASARA/WHAT IF twinset. The actual modelling was performed with the WHAT IF server [swift.cmbi.ru.nl/servers/html] because the sequence alignment was complicated and this server strictly follows the user-given alignment. The md_runmembranefast script in YASARA/WHAT IF twinset was used to optimize all 3D models. The very putative motif of the not-modelled insert was predicted by constructing the entire \(H.\) pylori OMPLA model automatically in YASARA/WHAT IF twinset, using the hm_build script.
3D structure modelling of two genes in the pldA operon: AmCl and AmCII
The two Na\(^{+}\)-dependent channels of the SNF family (AmCl and AmCII; corresponding to the HP0497 and HP0498 genes in \textit{H. pylori} strain 26695) homology models were constructed with YASARA/WHAT IF twinset, as described for \textit{H. pylori} OMPLA. Both models were constructed using the same PDB file 4US3 as template. The alignments were extracted from a single large MSA that was generated as described below.

In silico protein structure analyses
All structure analyses were performed using the YASARA/WHAT IF twinset. Models and template structures were superposed using different substrates with molecular surfaces shown using YASARA. The OMPLA pore was visualized with different substrates using WHAT IF twinset. Pore sizes were estimated using WHAT IF (as implemented in the YASARA/WHAT IF twinset) where solvent exclusion maps were produced for spherical probes with radius P using the surface map option in WHAT IF (srfmap) [49, 54]. Pore-Walker was used to estimate possible pore paths (see Fig. 2 in Additional file 3) [55].

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its Additional files 1, 2, 3, 4 and 5. The datasets used and/or analysed during the current study are available from the corresponding author upon request.

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