HHS Public Access

Author manuscript

Helicobacter. Author manuscript; available in PMC 2018 August 01.

Published in final edited form as:

Helicobacter. 2017 August ; 22(4): . doi:10.1111/hel.12386.

Survival of *Helicobacter pylori* in gastric acidic territory

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Abstract

Helicobacter pylori is well adapted to colonize the epithelial surface of the human gastric mucosa and can cause persistent infections. Its pathogenic effects consist of gastritis, peptic ulcers and increased risk for the development of gastric cancer. In order to infect the gastric mucosa, *H. pylori* has to survive in the gastric acidic pH. *H. pylori* has well developed mechanisms to neutralize the effects of acidic pH. The exact role of many bacterial factors as well as environmental factors still remains unsolved and how these factors involve in the acid mediated survival of bacterium is unknown yet. In this review, we have discussed and summarized the various information published in scientific literatures regarding functional and molecular aspects by which the bacterium can combat and survive the adverse effects of stomach acidic pH in order to establish the persistent infections.

Keywords

Acid acclimation; gastric acidic environment; *Helicobacter pylori*; survival

Introduction

Helicobacter pylori (*H. pylori*), a Gram-negative helical bacterium, has been estimated to infect at least half of the world's population. This bacterium was first identified by two Australian scientists Barry J. Marshal and Robin Warren in 1982. British scientist Stewart Goodwin further investigated this bacterium and reported that it was present in patients with chronic gastritis and gastric ulcers, previously not believed to be bacterial cause [1].

H. pylori has been established as the causative agent of human gastric mucosal infection with several gastro-duodenal diseases such as chronic gastritis, gastric ulcer, duodenal ulcer and increased risk for gastric cancer [2]. The International Agency for Research on Cancer (IARC) classified *H. pylori* as a group I carcinogen in 1994 [3] and based on the epidemiological data the carcinogenic behavior of *H. pylori* was reconfirmed in 2009 [4]. Despite of the several attempts the clear mechanism of development of cancer is not

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disclosure: The authors declare that they have no any conflict of interests.

understood clearly [5, 6]. This bacterium possesses several virulence factors such as outer membrane proteins (OMPs) for adhesion, *cag* pathogenicity island (PAI), VacA, gGT and DupA; however the pathogenicity of *H. pylori* depends on its ability to survive in the harsh gastric environment characterized by high acidity [7]. After transmission, the organism is reached and lodged in the stomach; the ecological niche of the bacterium where the pH tends to be very low. Therefore, to establish the infectious process *H. pylori* must survive in the high acidic environment of stomach.

Ecological niche

Although the exact means of transmission of *H. pylori* is not elucidated clearly, evidences suggest that the bacterium is often acquired during childhood via oral-oral or faecal-oral routes from family members [8, 9]. After transiting to the stomach it localizes to specific locations; the corpus and antrum simultaneously within the stomach [10, 11]. In the stomach *H. pylori* is highly adapted to the challenges encountered [8]. Once the persistent infection is established, the bacterium stays within the stomach for lifetime and it becomes the dominant inhabitants of the stomach [12]. In the gastric lumen the pH is around 2.0 and more in the mucus layer. However, *H. pylori* stays for short duration within the lumen and enters the mucus layer, the habitat of the *H. pylori,* where the pH ranges from 4.5–6.5; the bacterium is highly capable to neutralize the gastric lumen acidity [13, 14].

Many studies have revealed that *H. pylori* has several mechanisms to overcome the lethal effects of gastric acidic pH. This review gives the concise overview about the utilization of different factors by which *H. pylori* maintains the survival capability in acidic environment (Table 1).

Acid stress (Urease dependent) survival of *H. pylori*

The essential colonization factor urease and its associates

Several bacterial species including normal flora and non-pathogens requires the enzymatic activity of several proteins such as urease and hydrogenase to colonize the acidic niche. Urease, a high molecular weight multi-subunit metallo-enzyme, has been demonstrated as a potent virulence factor for some species like *Proteus mirabilis* [15], *Staphylococcus saprophyticus* [16] and *H. pylori* [17]. *H. pylori* produces large amount of intracellular (cytoplasmic) urease, around 10% of total bacterial proteins; however, *H. pylori* also contains urease on the bacterial surface due to the lysis of some organisms [18, 19]. The environmental pH regulates the synthesis of intracellular urease that acts to increase the periplasmic pH and membrane potential allowing synthesis of urease at low pH [20, 21]. In a recent study by Schoep et al., it was found that the surface properties of *H. pylori* urease complex play an important role for persistence during gastric colonization, directly interacting with host components that underlie *H. pylori* persistence [18]. Actually, the urease complex is multi-functional and the surface property of this protein is distinct from the urealytic activity. Uberti et al. [22] demonstrated the involvement of *H. pylori* urease in pro-inflammatory and neutrophil activation activity different from the urealytic activity. Urease, the essential component of colonization, is a nickel containing enzyme and it requires efficient acquisition of nickel from the environment for its activity [23, 24]. One

active urease molecule requires 24 nickel ions for full enzymatic action [25]. The nickel molecules found as trace molecules in the blood are taken by uptake proteins of bacteria, FecA3 and FrpB4, anchored in outer membrane [26]. After entering the outer membrane the nickel molecules are transported to the cytoplasm through the protein channel NixA, a monomeric high affinity nickel uptake protein localized in the cytoplasmic (inner) membrane of the bacteria [27, 28]. Since, the activation of urease system requires nickel, therefore, when cytoplasmic nickel availability is insufficient, the urease system cannot be fully activated and this inactivation impairs survival of *H. pylori* at acidic pH [29]. Another ribbon-helix-helix (RHH) family of DNA binding nickel-responsive regulatory protein (NikR) mediates its repressor function via nickel-dependent binding to a palindromic sequence in the promoter region of the *nik* operon [30, 31] resulting in the expression of the Nik system only when nickel is insufficient in the cell [32]. Since, *H. pylori* shows reduced growth at higher concentration of environmental nickel and in the absence of nickel, NikR has been suggested to function as the main nickel-responsive regulatory system for acid responsive induction of urease expression [33–36]. Too little entry of nickel impairs urease activity and acid survival while too much nickel generates reactive oxygen species leading to cell damage; therefore, entry of nickel needs to be well regulated [37]. NikR regulates the nickel responsive genes including *ureA, ureB, nixA, frpB4*, and *fecA3* [26, 36, 34]. NikR binds to the promoter regions of *frpB4* and *fec3A* genes in high concentration of nickel leading to the depression of these genes and decreased nickel uptake [26].

Urea, the substrate for urease enzyme, after entering the outer membrane through porins can passively diffuse across the bacterial inner membrane and upon exposure of *H. pylori* to high acidity; the influx of urea becomes faster. Active influx of urea is acid dependent property that is mediated by urea specific influx protein, permease (UreI) an inner membrane localized, proton gated urea channel [38, 39]. However, the urea concentration in gastric content is around 1 mM at physiological condition and it is assumed that this concentration of urea is not sufficient to provide the enough ammonia and acid survival benefit especially during the starvation period when the pH is around 1.0 [40]. Therefore, the mechanisms either producing the urea endogenously or promoting the other mechanisms are of most importance for the survival in vitro and during starvation period. Therefore, the urea can also be produced endogenously by arginase in vitro, the highly active enzyme of the urea cycle, encoded by *rocF* gene. The endogenously produced urea also provides concomitant acid survival activity in vitro. In a study, the *rocF* knock-out mutants were found to express increased susceptibility to acid treatment in vitro [41]. This observation indicates the importance of the enzyme arginase in the survival of bacterium to acidic pH in vitro and this could be also important for in vivo survival in case there is inadequate level of urea at the site of colonization. Arginine being a substrate of arginase enzyme can protect the *H. pylori* from the adverse effect of acidity by raising the pH in vitro. Urea (endogenous and exogenous) is hydrolyzed by urease resulting in the production of ammonia (NH_3) and carbamate. The carbamate spontaneously decomposed to give ammonia (NH_3) and carbonic acid. The carbonic acid is broken down to CO_2 and H_2O (Figure 1). Both ammonia and CO_2 participate in the lowering of pH. Ammonium (NH_4^+), the protonated form of ammonia neutralizes the stomach acidity, plays an important role to promote favorable environment for *H. pylori* survival in the stomach [42]. However, despite of the role of acid survival

advantage, the ammonium produced must be metabolized or effluxed because its presence within the bacterial cells is counterproductive to the goal of rising pH while maintaining a viable proton motive force. It is unknown how NH_4^+ rapidly exits across the inner membrane as the *H. pylori* lacks the homologs to known NH_3/NH_4^+ transport system. However, Miller and Maier [43] in 2014 demonstrated the role of ammonium assimilating enzymes, glutamine synthetase (GS), glutamate dehydrogenase (GDH) and the ammonium evolving periplasmic enzymes glutaminase (Ggt) and asparaginase (AsnB) in metabolism and effluxing of NH_4^+. The assimilating enzymes GDH and GS convert the NH_4^+ in to glutamate and glutamine respectively. Glutamine can diffuse to the periplasmic space or can be further converted to aspergine and diffused to periplasmic space. In the periplasmic space glutamine and aspergine are converted to NH_4^+ catalyzed by Ggt and AsnB respectively and involves in the hydrolysis of urea inside the cell or the expulsion/assimilation of ammonium elaborating the link between urea-derived ammonium and urease-mediated acid resistance. After exit from the bacterial cell, the NH_4^+ neutralizes the acidity and creates a nearly neutral micro-environment for survival of *H. pylori* (Figure 2).

In summary, the nickel, transportation mediated by NixA and NikR, acts as the cofactor of urease enzyme that catalyzes the hydrolysis of urea, the entry of which is enhanced in acid exposure. The NH_4^+ produced is transported outside of bacterial cell which creates a neutral microenvironment.

Acid acclimation

The acidic pH of the mammalian stomach is important for killing the potentially injurious bacteria that transit to the intestine. However, *H. pylori* can survive the acidic gastric environment by expressing some acid tolerance genes [44]. The CO_2 and NH_3 produced by the hydrolysis of urea, diffuse to the periplasmic space and provide the two major arms of the acid acclimation. The CO_2 in periplasmic space converted in to bicarbonate (HCO_3^-) and H^+ by periplasmic alpha-carbonic anhydrase. The HCO_3^- provides one arm of the acid acclimation maintaining the periplasmic pH close to 6.1. The protons (H^+) produced by alpha-carbonic anhydrase and exogenously entered to the periplasmic space are neutralized by NH_3 [45]; another arm of acid acclimation. Hence, both the NH_3 and CO_2 produced by the intracellular urease activity enables the acid acclimation mechanism in *H. pylori* and can maintain periplasmic pH at 6.1 in the face of an external pH below 2.5 [39, 45]. However, acid acclimation in *H. pylori* is a multifactorial mechanism for effective acid neutralization and the *H. pylori* is able to up-regulate acid acclimation and other group of genes under acidic pH [46]. Marcus et al. demonstrated the role of bacterial inner membrane protein ExbD in acid acclimation mechanism [47]. ExbD, a product of gene *HP1340* in strain 26695 is an inner membrane protein and a part of a 3 protein complex (ExbB/ExbD/TonB) [48]. After exposure of bacteria to the acidic pH, it needs more urea and urease production to help combat the lethal effect of acidic pH. The ExbB/ExbD/TonB complex anchored to the inner membrane transfers energy to the outer membrane in the form of conformational changes in TonB for the active transport of essential molecules that are found in trace amounts in environment such as iron and nickel [49, 50]. Recently, the role of ArsR and ArsS was demonstrated to function in acid acclimation. ArsR is involved in regulation of urease gene cluster expression whereas ArsS is involved in regulation of urease gene transcription and

recruitment of urease protein to the cytoplasmic membrane to augment the acid acclimation during acute acid exposure. ArsR and ArsS work through phosphorylation-independent and phosphorylation-dependent regulatory mechanisms to impact acid acclimation and allow gastric colonization [51]. It has been also concluded that the *arsS* mediate acid survival regulated by transcriptional up-regulation of *arsS* and by an enhanced capacity to sense the pH change in environment [52]. Urease, catalyzing the hydrolysis of urea to carbon dioxide and ammonia, is also critical in the development of diseases caused by *H. pylori* [53]. In this way *H. pylori* is capable of maintaining a near neutral periplasmic pH, the cytoplasmic pH experiences relatively small excursions from its optimal pH in the acidity as if it were in a neutral environment (Figure 3). Therefore, taking en mass, the data depict that the breakdown of urea whose entry is enhanced by ExbD and UreI after acid exposure produces CO_2 and NH_3 that provides the major pathways of acid acclimation for the neutralization of acidity in periplasmic space where pH rises to 6.1.

Physiological acid adaptation (Urease independent survival) of *H. pylori*

Characteristic role of mucus and helical shape of bacteria

After transit to the stomach the survival in acidic pH is important for *H. pylori* cells during the early stages of gastric infection before colonization to the gastric mucus that provides a protective layer against the acid content of the stomach. *H. pylori* crosses the gastric mucus layer and starts colonizing the gastric mucosa by adhering to the gastric epithelium where necessary nutrients and protection from the host immune system is obtained [54]. The urea hydrolyzing property exhibited by urease enzyme increases the pH in mucus microenvironment and modifies it to less gel-like, facilitating the movement of bacterium [55]. The viscoelasticity property of mucus layer is high at strongly acidic pH whereas decreases as the gastric pH increases and becomes gel like above pH 4.0. The inter-chain disulphide bonds of mucus glycoproteins (mucins) are reduced by thioredoxin system found in *H. pylori* that alters the mucus micro-structure that provides favorable environment and motility through the viscoelastic mucus gel layer [56, 57]. The characteristic helical shape of bacteria also determines to cope and adapt to different environments [58]. The rapid movement of bacterium within less acidic mucus layer is facilitated by its helical shape, allowing the bacterium to escape extremely low pH [59]. The alterations in the cross linking of cell determining layer, peptidoglycan found in periplasmic space of cell wall, has been shown to modify the bacterial shape. In an *in vitro* study, the cell shape mutants were found unable to efficiently colonize when compared with helical shaped bacteria despite possessing motility similar to the wild type [60]. The cell shape role in determination of bacterial cell movement was also elaborated in a recent study where 7–21% reduction in the speed was found in the helical shape determination mutants with straight rod morphology when compared with the helical shape bacteria in a viscous environment [54]. In another study it has been demonstrated that both the helical shape and motility of bacterium is necessary to penetrate the mucus layer in corkscrew like movement in order to colonize the gastric epithelium [61]. Therefore, the mucus protects the bacteria from acid stress and the helical shape of bacteria is of importance which provides the cork-screw like active motility to swim towards the higher pH environment.

Flagella mediating the movement

H. pylori normally possesses about 3 μm long two to six sheathed flagella at one pole (i.e. lophotrichous) that enables the bacteria to move in their ecological niche [62]. The flagella are composed of three structural elements like those of enteric bacteria: a basal body, hook and filament. The filament consists of the two flagellin protein subunits, more abundant FlaA lies in the outer region and minor subunit FlaB localizes to the base of the flagellum and both proteins are necessary for full motility [63, 64]. In a study by Merrell et al., it was found that acid exposure can activate flagellar proteins that leads to enhanced changes in motility of *H. pylori*, as larger percentage of acid exposed bacterial cells displayed motility and moved at significantly higher speeds [65]. It has been also demonstrated by Yoshiyama et al. that flagellar motor is powered by a proton motive force at low pH and *H. pylori* can swim faster at low pH [66]. The number of flagella also seems to play an important role for speed of bacterial cell during movement. In a recent study, the cells having 4 flagella showed 19% increase in speed when compared with cells having 3 flagella in viscous environment [54]. Thus orally up taken *H. pylori* can move quickly towards the epithelial surface by chemo-attractive substances and promptly evade the acidic periphery of the mucous layer. The role of flagella in the attachment to the host cells and in the survival has been evaluated in several bacterial species [67–69]. However, in *H. pylori,* it was shown that the flagellin (*flaA* and/or *flaB)* and flagellar regulator gene (*flbA*) mutants adhered well to the gastric cells [70]. In another recent study, Kao et al. using the experimental model of *H. pylori* J99 strain depicted that those flagella regulator gene (*csrA* or *rpoN*) mutant strains showed decreased bacterial adhesion to AGS cells [71]. Together these data suggest that although the flagella are not involved directly in the attachment, the regulators of flagella related genes do involve in the expression of adhesins in *H. pylori*.

Chemotactic activity

Some chemicals that are continuously secreted from the gastric epithelial cells to the lumen such as urea [72], sodium carbonate [73] and potassium carbonate [74] serve as potent attractants for *H. pylori* towards higher pH. Despite these data, little is known about the chemotactic behavior of *H. pylori* at various pH of the stomach, ranging from highly acidic to pH 4.5–6.5, which is the ecological niche of this bacterium. In a recent study conducted by Tadjrobehkar and Abdollahi about the chemotactic behavior of *H. pylori* showed that the maximum chemotactic activity occurred at pH 5.5 to 6.5, but no chemotaxis was found in solution with pH 3 and they concluded that chemotactic responses of *H. pylori* were severely affected by pH condition [75]. In *H. pylori*, smooth movement in the presence of chemical attractants and increased stopping behavior in the presence of repellent is facilitated by presence of four chemoreceptors, TlpA, TlpB, TlpC and TlpD for sensing the chemicals found in environment and tuft of flagella found at one pole mediate the movement in response to chemo-attractants [76, 77]. Chemoreceptors are trans-membrane proteins of which the TlpB consisting of two domains, periplasmic and cytoplasmic, is the most important for chemotactic behavior of bacterium that belongs to the class of methyl-accepting chemotaxis proteins (MCPs) [78, 79]. Croxen et al. in a study found that *tlpB* knockout mutants were defective for pH taxis and concluded that the receptor TlpB is essential for sensing pH in *H. pylori* [80]. The periplasmic domain mediating the chemical sensing property accounts for about one-third of the total amino acid found in TlpB that

binds to urea and confers its pH sensing function [79]. Therefore, by chemotactic mechanism *H. pylori* senses the environmental pH and drives itself towards the nearly neutral pH that minimizes the bacterial exposure to acidic environment of stomach to survive and colonize for persistent infections.

Recombinational repair of DNA damage

DNA repair is a fundamental process in all free-living as well as pathogenic bacteria and it acts as one of the defense mechanisms that allow the pathogenic bacteria to survive in their hosts. Double strand breaks (DSBs) occur as a result of a variety of physical or chemical insults that modify the DNA. Damage at a single-strand site, if not repaired immediately, leads to a DSB. More commonly, if a replication fork meets damaged bases that cannot be replicated, the fork can collapse, leading to a DSB. The physiological condition found in stomach frequently cause acid stress in *H. pylori* leading to DNA damage. However, *H. pylori* harbors the transformation mediated recombination repair mechanism that can repair the DNA damage caused by stress conditions [81, 82]. Bacteria can repair damaged DNA by using homologous recombination which involves three steps, presynaptic, synaptic and postsynaptic steps. In presynaptic step, the production of single-stranded DNA coated with RecA protein occurs. Synaptic step involves strand exchange and joining of DNA molecule promoted by RecA protein. In postsynaptic step priming of new DNA synthesis occurs. Recombinational DNA repair requires a large number of proteins among which RecA is critical in DNA recombination and repair [83]. In the well-studied bacterial model *Escherichia coli* (*E. coli*), a 3 enzyme complex; the RecBCD mediating the helicase and nuclease activities initiates the recombinational DNA repair of double-stranded DNA (dsDNA) [84]. The RecBCD binds to the damaged duplex DNA end and unwinds it. After unwinding, the both nascent DNA strands are degraded until the recognition of specific sequence (Chi-sequence), the DNA ends are resected to form a 3′-ssDNA overhang that terminates at Chi sequences. Chi (χ)-sequence (Chi = crossover hotspot instigator) is an 8-nuleotide sequence (5′GCTGGTGG-3′), hotspot for homologous recombination [85]. However, in *H. pylori* the homologous enzymes complex AddAB performs the helicase and nuclease activity [86]. A recent study suggests that, Chi-sequence after binding within the AddAB enzyme complex sequesters the 3′-terminated strand and prevents from the action of AddA nuclease domain, thereby resulting in nuclease activity attenuation beyond Chi-sequence [87, 88]. The sequestration of 3′-terminated strand and continued unwinding and degradation of the 5′-terminated strand by the AddB nuclease domain results in the production of a long 3′-ssDNA overhang. The 3′-ssDNA overhang acts as the substrate for the loading of RecA protein for strand exchange [89]. Loading of the RecA protein to the Chi-containing ssDNA overhang leads to the formation of nucleoprotein complex and promoting the search for homologous DNA sequence and DNA strand invasion with the homologous donor duplex. The donor DNA acts as a template for DNA synthesis resulting in the formation of two Holliday junctions that resolves to yield intact duplex products [90]. Thus, the role of RecA is very important for the initial step of the recombinational DNA repair. Similarly, the role of RecN, another protein involved in DNA recombination process was evaluated under low pH condition, as the *recN* knockout mutants were found highly susceptible to the low pH conditions [91]. Although the mechanism of the RecN protein in the recombinational repair in *H. pylori* is unknown it has been shown in *E. coli* that RecA

dependent recruitment of RecN to the damaged dsDNA serving a scaffolding function facilitates a critical role for searching of homology DNA by RecA. Failure to the recruitment of RecN to the damaged strand causes the accumulation of fragmented chromosomes [92].

Marsin et al. [93] identified a novel RecO orthologue by using bioinformatics analysis and suggested the presence of RecRO pathway in the dsDNA damage repair. Subsequently, Wang et al. [94] demonstrated that the RecRO pathway is not responsible for dsDNA damage repair caused by mitomycin C (MMC) in *H. pylori*; however, the proteins RecR and RecO were found responsible for damage repair caused by acid stress. The *recR* and *recO* knockout mutants were unable to survive at pH 3.0 whereas wild-type strains survived well in the study [94]. In *E. coli*, in addition to the RecBCD pathway; the RecFOR (or RecF) pathway can also repair double strand breaks [95]. In RecF pathway, RecJ, a recombinational exonuclease binds to the 5′-ends of the damaged DNA and starts cleaving the strand in upstream direction creating a 3′-overhang. Single-strand binding protein (SSBP) binds to the 3′-overhang [96] and inhibits the binding of RecA protein to the 3′-overhang. The RecO protein facilitates the displacement of SSBP from 3′-overhang and promoting the binding of RecA protein [97]. The RecR, another key component involved in the recombinational damage repair, mediates the loading of RecA on the 3′-overhang [97]. Recently, Wang et al. elucidated the role of *hup,* encoding a histone like protein by suspending wild-type and the *hup* knockout mutants at different acidic pH (pH 7.0, pH 5.0 and pH 3.0). They found that suspending at pH 5.0 for 1 hour killed 90% of the *hup* mutants but caused no significant effect on survival of the wild-type strains while 40% of the wild-type *H. pylori* survived at pH 3.0 for 1 hour, whereas more than 98% of the *hup* mutants were killed by the same treatment. Thus, the *H. pylori* Hup protein has a critical role in the protection from acid stress as the Hup protein has an ability to physically protect DNA from stress damage [98]. The exact mechanism of how the Hup protein involves in the recombinational repair of damaged DNA in *H. pylori* is unknown. However, in *E. coli* the homologous histone like protein HU regulates the expression of genes involved in anaerobic respiration, acid stress and response to DNA damage [99, 100]. Therefore, it has been suggested that Hup protein physically protects the DNA or it may cause expression of genes that involve in the stress resistance. Further study is demanded to understand; how Hup protein interacts with other recombinational protein in DNA repair mechanism. Another DNA replication priming protein, PriA, of *H. pylori* is a component of the priming system which primes the DNA synthesis, has recently been shown to play an essential role in the acid damaged DNA repair and colonization to the epithelial cells. The *priA* knockout mutants were unable to survive in acidic pH and level of infection was much less when compared with the wild-type strains [101]. The mechanism of this protein in recombinational DNA repair due to acid stress in *H. pylori* is also unknown. However, in well-studied bacterial model *E. coli*, the PriA is a key DNA replication protein that involves in the major processes of DNA replication, recombination, damaged DNA repair and restart initiation of the stalked replication fork at damaged DNA sites [102–104].

In summary, the data above mentioned indicate that the homologous recombination is an important mechanism which comes in action after double-stranded DNA damage caused by acid and oxidative stress and involves in the damage repair. The RecA mediated DNA repair

involving homology search in addition involves several other recombinational proteins such as RecN, RecO, RecR, Hup, and PriA in the acid stress DNA damage repair.

DNA binding protein

HP0119 (HP number comes from protein number of strain 26695) is a histone like hypothetical protein (HLP) found in *H. pylori*. Its role in DNA binding and acid stress tolerance was investigated by Wang and Maier [105]. In their study, the treatment at pH 5.0 and 3.0 for one hour killed 90% and 95% of the *hp0119* knockout mutants respectively but same treatment to wild-type *H. pylori* did not affect the survival at pH 5.0 and about 40% cells were killed at pH 3.0. Thus, the *hp0119* mutants were susceptible to acid stress while wild-type strains were less susceptible [105]. This study indicates the role of HP0119 protein in protection of the bacterium at low pH. The mechanism of HP0119 in protection from acid stress is unknown and remains to be elucidated in *H. pylori*. However, multiple functions of HP0119 have been proposed in acid stress survival. It may possess an ability to physically protect DNA from oxidative and acid stress like *Mycobacterium tuberculosis* histone like protein Lsr2 that protects DNA from stress generated by reactive oxygen intermediates [106]. HP0119 also involves in increased expression of the enzymes that aids the survival of *H. pylori* [105]. It may also act like *Salmonella enterica* histone like protein HU that controls three regulons that coordinate virulence, maintains survival in stress and maintains cellular physiology [107]. Therefore, the DNA binding protein HP0119 may protect the bacteria from acid stress in several ways although the exact underlying mechanism is not known.

Urease independent ammonia production

H. pylori infection is potentiated by the production of ammonia which is of great importance for nitrogen source and for neutralization of gastric acidity. Urease, which is found in abundant amount in *H. pylori* is essential for the production of ammonia from the urea and its survival in the acidic condition [108]. It has been suggested that the large amount of ammonia produced by *H. pylori* can cause tissue damage during colonization and long term ammonia administration induces gastric mucosal atrophy in rat [109]. Short chain aliphatic amides can be used by some bacteria as source of nitrogen for the growth as they possess the ability to hydrolyze the amides to ammonia and the corresponding organic acid, using an aliphatic amidase. For ammonia production, in addition to the urease; *H. pylori* possess two aliphatic amidases and aspartase. AmiE, a classical amidase [110] and AmiF, a new type of formamidase, which was detected when the analysis of the complete genome was sequenced [111]. Apart from these amidases there was also increased expression of *aspA*, an aspartase encoding gene in vivo [112]. In another study it was illustrated that these aliphatic amidases are found in *Helicobacter* species capable of colonizing the stomach epithelia [113].

Histidine kinase protein

H. pylori encounters a range of acidic conditions within the human stomach and the survival in the acidic pH is mediated by regulation of bacterial gene expression in response to pH. Detection and response to environmental signals requires two component signal transduction system (TCSTS) comprised of a sensor histidine kinase and a response regulator [114, 115]. Three histidine kinase proteins ArsS, AtoS (FlgS, FleS) and CrdS [116–118] also designated

as HP0164/HP0165, HP0244 and HP1364 or JHP0151, JHP0229 and JHP1282 are encoded by *H. pylori* genome, respectively (JHP number come from protein number of strain J99). The histidine kinase CrdS is a strain specific which is required to grow at pH 5.0 in strain J99 [41]. In a study, individual histidine kinase gene (*hp0165, hp0244* and *hp1364*) mutant strains were defective to colonize the mouse stomach [119]. It was also reported that the up-regulation of transcription of three genes (*hp0119, hp1432* and *ureA*) in acidic pH was HP0165 dependent as expression was found in wild-type *H. pylori* but not in isogenic *hp0165* mutants suggesting the role of histidine kinase HP0165 as acid sensor [117]. Recently, Loh and Cover also reported the role of histidine kinases HP0165 and HP1364 in the expression of other several genes, including those that encode urease, amidase (HP0294) and formamidase (HP1238) for acid resistance in *H. pylori* [120]. All these enzymes are involved in ammonia generation, are likely to play an important role in acid resistance [35, 121, 122]. Marcus et al. [123] demonstrated that during acute acid exposure ArsS was involved in the recruitment of urease protein to the inner membrane to augment acid acclimation in addition to its role as urease gene transcription as described in acid acclimation (Figure 3).

In summary, the histidine kinase proteins regulating the expression of several other genes maintain the survival advantage of *H. pylori* in acid stress.

Ferric uptake regulator (Fur)

H. pylori is equipped with a repertoire of response regulator gene able to regulate gene expression in response to environmental change as with many other organisms [124]. The regulatory proteins involved in urease, amidase and formamidase regulation are encoded by two genes in *H. pylori*; the HP1027 (*fur*) encoding Fur regulator, which mediates iron responsive regulation of amidases [122] and HP1338 (*nikR*) encoding NikR regulator; which mediates nickel-responsive urease expression [34, 125]. Fur is a best known regulatory protein for regulation of a complicated system that controls the iron uptake and storage in bacterial cell [126, 127]. This regulatory system is accomplished as Fur binds to the promoter region known as Fur box and regulates the expression of Fur-regulatory genes [128]. Fur also regulates gene expression in response to low pH, although, the exact mechanism is not well understood [121, 122, 129]. However, the essential role of Fur in acidic pH adaptation may be partially linked to regulation of *amiE* and *amiF*, the amidases that degrades the amides to produce ammonia [122]. In addition, the critical role of Fur under acid stress may be likely linked to the NikR, which directly regulates the trace elements like nickel availability, can also regulate the iron availability in the cell and Fur expression in the acid response [33, 34]. Indeed, the adaptation to the acid stress in *H. pylori* is a complex process that is mediated by more than one regulatory protein. In a study conducted by Merrell et al. [65] in an experimental model of *H. pylori* 26695, out of 93 genes, 41 were found to be regulated in a Fur dependent manner and 23 genes were identified in both acid and Fur dependent. Bury-Mone et al. [33] also identified 101 genes that showed altered expression during the growth of *H. pylori* in acidic pH. Of these 101 genes, 36 genes were shown to be regulated by NikR and/or Fur for pH dependent changes in gene expression.

Fur plays an important role in acid resistance of *H. pylori* independent of urease [121, 127] and independent of its role in iron acquisition [121]. Gancz et al. [129] using a Mongolian gerbil model demonstrated that although a *fur* mutant was able to colonize the gastric mucosa if large infectious dose is given, it does inefficiently at early stage of colonization. The oral ID50 for the *fur* mutant strain was also 100 times higher than that of the wild-type strain. The in vivo colonization defect of *fur* mutant strains can be likely explained by the fact that Fur modulates expression and changes in transcription in response to acid stress of such a large number of genes. Nevertheless, the role of Fur is more important at early stage of colonization. During initial steps the bacteria are challenged to adapt to new environment that requires changes in gene expression to ensure rapid accumulation to the gastric acidic niche. It was evident that the slight decrease in colonization of the *fur* mutant at early time points suggests that Fur is essential for the initial stages of colonization [129]. In another in vivo experiment by Miles et al. [130] using Mongolian gerbil models confirmed the role of Fur during the earliest stages of infection establishments where gerbils were first infected with the *fur* knockout mutants and subsequently super-infected with the wild-type strains at 1, 3, 7, 14 and 28 days after the initial infection. The *fur* mutants were efficiently displaced with the wild-type strains during first week of infection, but this ability progressively diminished overtime. In converse experiment, the *fur* mutants were unable to displace the wild-type strains at any time point tested. When co-infected with fur mutants and wild-type strains and monitored the colonization, a 10-fold and 100-fold colonization defect was found at day 1 and 3 respectively [130]. Thus, the Fur plays an important role in the regulation and expression of some genes in response to the acid stress which provides survival advantage in acid environment. Fur also involves in the colonization of *H. pylori* during the initial stage of colonization.

Role of other virulence factors in acid tolerance

Cytotoxin-associated gene A (*cagA*)

Many virulence factors have been described in association with different clinical outcomes of *H. pylori* infections. CagA is an onco-protein and one of the most widely studied virulence factors [131]. Many studies since last one decade have shown that after secretion, the CagA is injected into the target epithelial cells via type-IV secretory system (T4SS) encoded by the *cag* PAI [132]. After entering to the host cells, phosphorylation of CagA protein undergoes at specific tyrosine residues present in Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs found at the C-terminus of the protein and led to the CagA mediated pathogenicity [133, 134]. However, this virulence factor despite of the role in pathogenicity also involves in the acid susceptibility although the mechanism is not known yet (Table 2). Karita and Blaser first reported that the *cagA*-positive strains pre-exposed at pH 6.0 for 48 hours were more susceptible to re-exposure at pH 3.0 than the *cagA*-negative strains [135]. Subsequently, we also found that the *H. pylori* strains with 5 EPIYA repeats were more susceptible to pH 3.0 than strains having 4 or less EPIYA repeats using Western type strains [136]. Currently, sequences of the EPIYA repeat regions have been annotated according to the segments (20–50 amino acids) flanking the EPIYA motifs (i.e., segments EPIYA-A, B, C or D; C is specific for Western type strains and D is specific for East Asian strains) [137]. Therefore, our previous study indicated that the Western type strains carrying the 2 or less

EPIYA-C motif are more acid tolerable in comparison to strains carrying 3 EPIYA-C motif and this phenomena could be the advantageous for survival in gastric acidic environment for strains possessing ABC and ABCC type CagA strains. Kalaf et al. recently reported that strains containing 2 or more EPIYA-C caused infection in patients with higher mean age rather than early age patients [138]. The in vivo data support our previous data that the elder patients are supposed to produce less amount of gastric acid than early age group. In summary, the CagA negative strains are more acid resistant than positive ones and the Western strains with less number of EPIYA-C motifs are more acid resistant than strains with more number of EPIYA-C motifs.

Role of duodenal ulcer promoting gene (*dupA*)

Duodenal ulcer promoting gene (*dupA*) is located in the plasticity region of the *H. pylori* genome. We first described the role of the gene; one gene that encompassed both *jhp0917* and *jhp0918* called *dupA* was associated with a specific clinical outcome [139]. The prevalence of this gene was significantly higher in strains from duodenal ulcer patients than those from gastritis or gastric cancer patients. We also reported the role of *dupA* in acid survival although the mechanism is unknown yet. We pre-exposed the *dupA* deleted mutants and wild-type strains at pH 6.0 for 24 hours and subsequently re-exposed to pH 3.0 for 20 minutes and found that the *dupA* deleted strains were more susceptible at pH 3.0 than wild-type strains [140]. Further the role of *dupA* in acid shock tolerance was also confirmed by other studies. In a study, Imagawa et al. [140] isolated *dupA* positive strains from patients with significantly higher gastric acid secretion and *dupA* negative strains from patients with low gastric acid secretion. In another study by Abadi et al. [141], 12 *dupA* positive and 20 *dupA* negative strains from gastritis patients were subjected to a pH range from 3.0 to 7.0. All of the *dupA* positive strains were able to grow well at pH 4.0 and 33.3% strains did grow at pH 3.0. In contrast, only 40% and 10% *dupA* negative strains were able to grow at pH 4.0 and 3.0 respectively. The reports of these studies indicate the importance of *dupA* in the survival of organism in gastric acidic pH.

Conclusions

Given the above information currently compiled suggest that the *H. pylori* is well habituated for its ecological niche of gastric acidity at pH below 3.0 to cause the persistent infections. The adaptation mechanism of bacterium to the adverse effects of acidic pH is a complex mechanism involving several factors such as bacterial factors (proteins, enzymes, shape and flagella) and environmental factors (urea, mucus and acid). However, the exact mechanism of several factors involved in the acid toleration still remains unsolved and it demands extensive study to evaluate the exact role in the acid survival.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (DK62813) and the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (24406015, 24659200, 25293104, 26640114 and 15H02657) (YY). It was also supported by the Japan Society for the Promotion of Science (JSPS) Institutional Program for Young Researcher Overseas Visits (YY), the Strategic Funds for the Promotion of Science and Technology from Japan Science and Technology Agency (JST) (YY). SA is a PhD student supported by The Japanese Government (Monbukagakusho: MEXT) Scholarship Program

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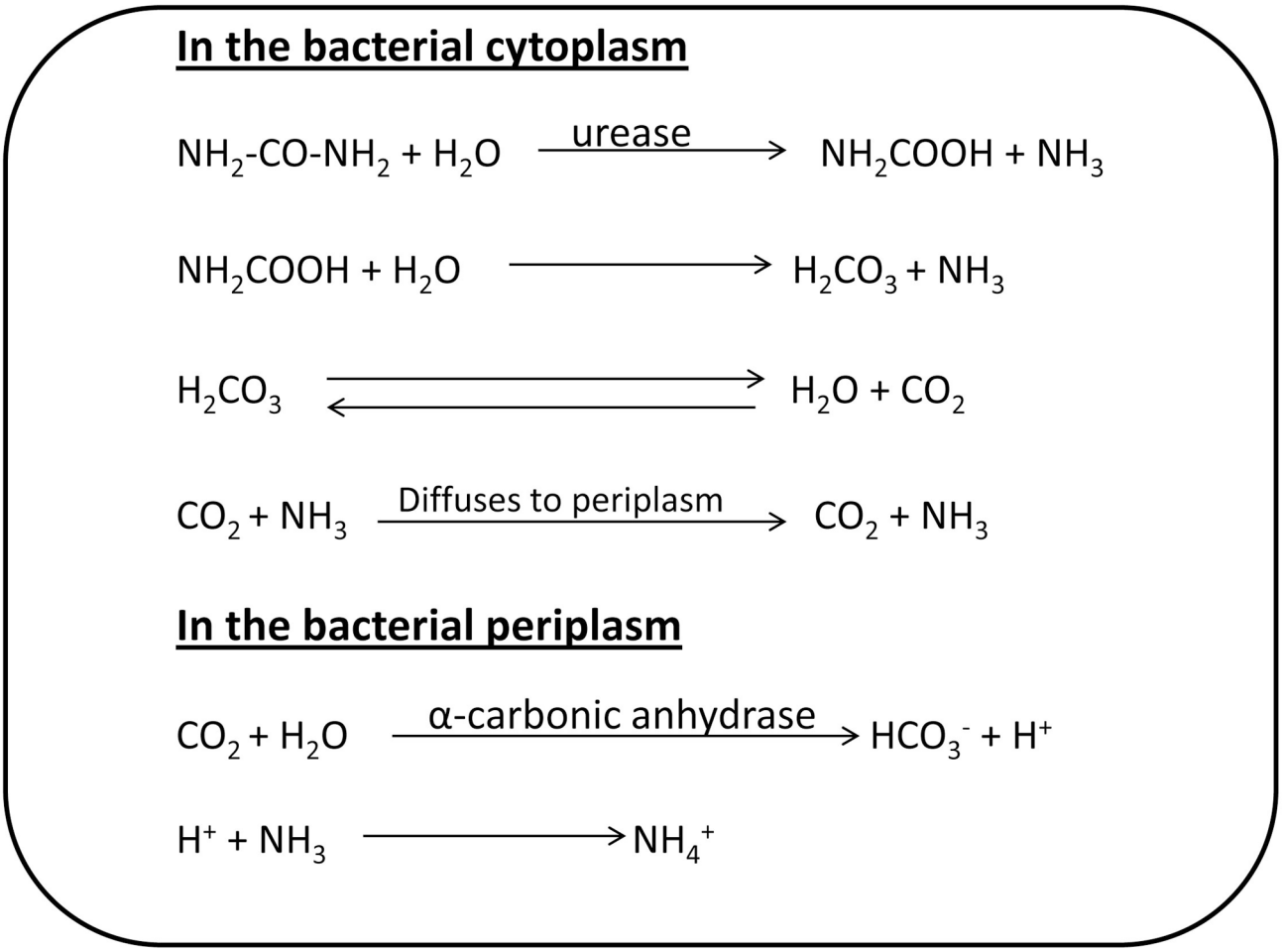

Figure 1.

Hydrolysis of urea by urease. Urease breaks down the urea to form carbamate and NH_3. Carbamate is further decomposed to produce carbonic acid and NH_3. Carbonic acid is broken down spontaneously aqueous to produce H_2O and CO_2. Therefore, one molecule of urea after hydrolysis produces two molecules of ammonia (NH_3) and one molecule of carbon dioxide (CO_2). CO_2 and NH_3 are defused to periplasm where α-carbonic anhydrase catalyzes the breakdown of CO_2 to produce HCO_3^- and H^+. The H^+ produced combines with NH_3 to produce NH_4^+.

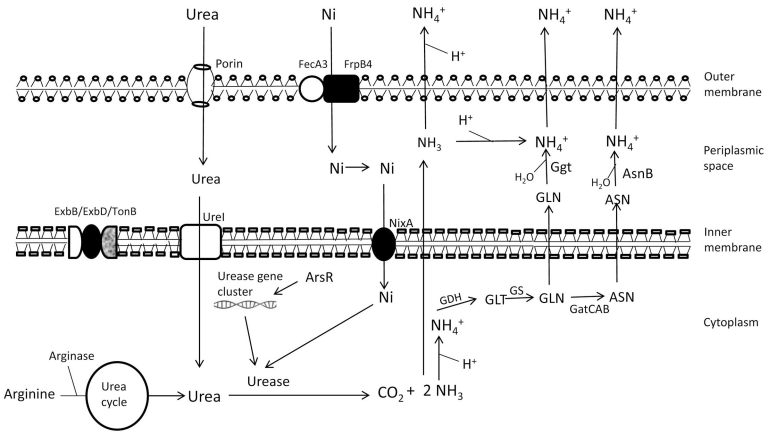

Figure 2.

The metallo-enzyme, urease requires nickel (Ni) for its activity. Ni slowly enters the outer membrane through porin (outer membrane protein) and actively enters through FecA3/FrpB4 proteins anchored in outer membrane and crosses inner membrane through NixA (inner membrane protein). However, in acidic pH more Ni is required for increased activity of urease and for the active transport of essential molecules (Ni), ExbD provides energy to the outer membrane. Urea enters the outer membrane through the porin and inner membrane through UreI (found in inner membrane) which opens in acid exposure of bacteria. Endogenously produced by arginase in urea cycle (during in vitro survival) and exogenously entered urea molecules are hydrolyzed by urease to ammonia (NH_3) and carbon dioxide (CO_2). ArsR regulates urease gene cluster expression. Ammonia (NH_3) produced is diffused out and binds with proton (H^+) leading to the neutralization of acidic pH. However, NH_3 is also converted to ammonium (NH_4^+) inside the cell (cytoplasm and periplasm) which is toxic to the bacteria. The cytoplasmic NH_4^+ is assimilated to glutamate (GLT) by GDH and to glutamine (GLN) by GS. GLN can diffuse to periplasmic space or can be converted to aspergine (ASN) by GatCAB aminoacyl-tRNA amidotransferase (GatCAB) and then can diffuse to periplasmic space. GLN and ASN bind with water molecule and led to the formation of NH_4^+ and catalyzed by Ggt and AsnB respectively. Finally the NH_4^+ exits to the bacterial cell and increases the pH that leads to the survival of bacteria at acidic pH.

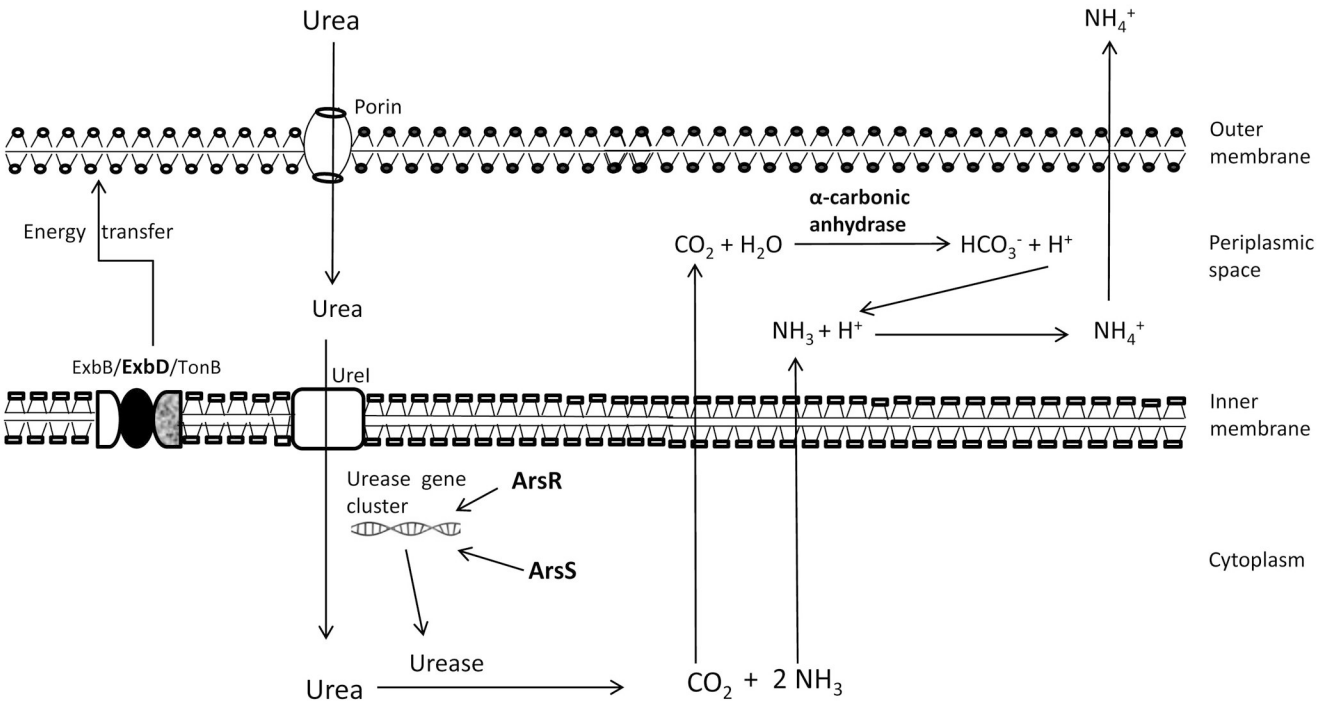

Figure 3. Acid acclimation

Exposure of bacterial cells to acidic pH opens the urea channel, UreI and urea enters the cytoplasm to be hydrolyzed by urease to NH_3 and CO_2. However, the outer membrane proteins (porins) need energy for active transport of essential molecules (urea) which is provided by ExbD located in inner membrane in the form of complex of three proteins (ExbB/ExbD/TonB). ArsR and ArsS regulate the expression of urease gene cluster and increase the urease activity at strong acidic pH. Ammonia (NH_3) and carbon dioxide (CO_2) diffuse to the periplasmic space. In the periplasmic space, CO_2 binds with water molecule to synthesize HCO_3^- and H^+ catalyzed by periplasmic α-carbonic anhydrase. The synthesized HCO_3^- acts as a strong buffer and can maintain the periplasmic pH to 6.1. Protons (H^+), entering to the periplasm and synthesized by α-carbonic anhydrase, are neutralized by NH_3 converting to NH_4^+ and diffuses out of the bacterial cell membrane.

Table 1

Factors involved in the survival of acidic pH.

Factors involved	Mechanisms	Reference
Urease dependent mechanisms		
Urease	Breakdown of urea and production of ammonia that neutralizes the acidity.	17, 42
NixA and NikR	Regulates the availability of nickel molecules for full action of metallo-enzyme, urease.	27, 28, 30, 31, 33–36
UreI	Mediates entry of urea after exposure of bacterial cell to the high acidic pH.	38–39
Arginase	Production of endogenous urea that is hydrolyzed by urease.	41
glutamine synthetase (GS), glutamate dehydrogenase (GDH), glutaminase (Ggt) and asparaginase (AsnB)	Mediates the metabolism and assimilation of NH_4^+ generated by periplasmic alpha-carbonic anhydrase.	43
Alpha-carbonic anhydrase	Conversion of CO_2 to HCO_3^- and maintenance of periplasmic pH close to 6.1.	45
ExbD	Transfers energy to the outer membrane for active transportation of urea and other essential molecules at acidic pH.	48–50
ArsR and ArsS	Regulation, expression of urease gene cluster and recruitment of urease.	51, 52
Urease independent mechanism		
Gastric mucus layer	Gel-like viscoelastic property of mucus facilitates movement of bacteria at acidic pH.	54
Helical bacterial shape	Provides corkscrew like movement that facilitates penetration into mucus layer	58, 60
Flagella	Acid activates the flagellar protein and movement.	65, 66
Chemotaxis and chemo-receptor	Chemoreceptor TlpB senses the environmental pH for chemotaxis	78–80
Recombinational repair proteins; RecA, RecN, RecO, RecR, Hup and PriA	Mediates recombinational repair of DNA damage caused by acid stress.	83, 91, 94, 98, 101
DNA binding protein, HP0119	Histone like hypothetical protein HP0119 physically protects the bacteria at acidic pH	105
AmiE, AmiF and aspartase	Urease independent ammonia production that neutralizes acidic pH	110–113
AtoS and CrdS	Senses acid pH and regulates expression of acid responsive genes.	117–120
Fur	Mediates iron uptake and regulates expression of acid responsive genes.	65, 121, 122, 129

Table 2

Virulence factors involved in acid tolerance

Virulence factors	Role of virulence factors	References
cagA	*cagA*-negative strains were more resistant to low pH than *cagA*-positive strains. Strains with 2 or less EPIYA-C repeats were more acid resistant	135 136, 138
dupA	*dupA*-positive strains possess more acid resistant capability than *dupA*-negative strains	139–141