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Review

Biofilm of *Helicobacter pylori:* Life Cycle, Features, and Treatment Options

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Abstract: *Helicobacter pylori* is a gastric pathogen that infects nearly half of the global population and is recognized as a group 1 carcinogen by the Word Health Organization. The global rise in antibiotic resistance has increased clinical challenges in treating *H. pylori* infections. Biofilm growth has been proposed to contribute to *H. pylori's chronic* colonization of the host stomach, treatment failures, and the eventual development of gastric diseases. Several components of *H. pylori* have been identified to promote biofilm growth, and several of these may also facilitate antibiotic tolerance, including the extracellular matrix, outer membrane proteins, the coccoid morphology, modulated metabolism, efflux pumps, and virulence factors. Recent developments in therapeutic approaches targeting *H. pylori* biofilm have shown that synthetic compounds, such as small molecule drugs and plant-derived compounds, are effective at eradicating *H. pylori* biofilms. These combined topics highlight the necessity for biofilm-based research in *H. pylori*, to improve current *H. pylori* targeted therapeutic approaches and alleviate relative public health burden. In this review we discuss recent discoveries that have decoded the life cycle of *H. pylori* biofilms and current biofilm targeted treatment strategies.

Keywords: *Helicobacter pylori;* biofilms; planktonic; antibiotic resistance; extra polymeric substance; abiotic/biotic adhesion; dispersion; clinical treatment strategies; anti-biofilm strategies

Introduction

Helicobacter pylori is a gram negative, spiral shaped, bacterial pathogen that colonizes the gastric epithelium (Cellini et al., 1994, Noach et al., 1994 and Dubois et al., 1994, Malfertheiner et al., 2023). *H. pylori* has been globally recognized as a high priority pathogen as it has been associated with various gastric diseases, including peptic ulcers, chronic gastritis (Labenz et al., 1994 and Blaser et al., 1994), gastric mucosa-associated tissue lymphomas (Pereira and Medeiros et al., 2014) and gastric adenocarcinomas (Asaka et al., 1994, Veldhuyzen et al., 1994, Isaacson 1994, Ma et al., 2022). Mechanisms of transmission remain unknown (Ma et al., 2022), but antibiotic therapies used to treat *H. pylori* infection have alarmingly been losing efficacy in regions with high infection burden (Liu et al., 2022). Antibiotic-resistant *H. pylori* was reported to disproportionately affect children in Asian, African, and European countries (Karbalaei et al., 2022), and in underserved communities in the US (Brown et al., 2022). One current perspective is that *H. pylori* in biofilms, a low growth state, may substantially promote antibiotic resistance and persistence in the host stomach (Cellini et al., 2008). Planktonic *H. pylori* were observed *in vitro* to form water-insoluble biofilms which are defined as stationary aggregates of cells encased in extra polymeric substances (EPS) (Mackay et al., 1998 and Stark et al., 1999). Although molecular mechanisms that facilitate *H. pylori* biofilms remain elusive *in vivo*, *H. pylori* in a form consistent with biofilms have been observed in the gastric mucosa of patients with peptic ulcers (Carron et al, 2006 and Coticchia et al., 2006). In this review we discuss recent discoveries that characterize the features of *H. pylori* biofilms, decode processes that regulate biofilm growth *in vitro* and *in vivo,* elucidate biofilm mechanisms that support antibiotic tolerance and current biofilm-based eradication strategies.

General features of *H. pylori* biofilms

H. pylori biofilms consist of stationary aggregates of cells encased by an extracellular matrix composed of proteins (Windham et al., 2018), extracellular DNA (Grande et al., 2011) and polysaccharides (Li et al., 2019). *H. pylori* biofilm formation starts from planktonic cells that adhere to either abiotic or biotic surfaces to form microcolonies with three dimensional structures (Cole et al., 2004; Hathroubi et al., 2020). Biofilm formation is initiated when planktonic *H. pylori* adheres to abiotic surfaces to form microcolonies (Hathroubi et al., 2018); adherence and subsequently biofilm formation were found to occur optimally in conditions lacking fetal bovine serum (Williams et al., 2008, Hathroubi et al., 2018). Aside from biofilm growth on abiotic surfaces, additional studies have also suggested that *H. pylori* can form a microcolony network that adhered and grew between epithelial cell junctions on human cells (Tan et al., 2009; Anderson et al., 2015) and in murine gastric glands (Sigal et al., 2015). Mature *H. pylori* biofilms consist of a multicellular population with a variety of cell shapes, that vary based on conditions. On abiotic surfaces, most cells adopt the coccoid morphology, with the minority displaying a rod shape (Cellini et al., 2008 and Bugli et al., 2016). As found in other bacteria, *H. pylori* biofilm formation exhibits a similar multiple-step process, including bacterial adherence, biofilm assembly, mature biofilm formation and dispersion. In the next sections, we dissect the features of each step in *H. pylori* biofilm growth.

Adherence

Adhesion is an essential process that initiates *H. pylori* biofilm formation and retains a role throughout the lifetime of the biofilm (Azevedoet al., 2006, Ratthawongjirakul et al., 2016, Wong et al., 2016). Prior studies have found that *H. pylori* can adhere to both gastric epithelial cells (Hessey et al., 1990) and abiotic surfaces (Azevedo et al., 2006). *H. pylori* surface adhesion and microcolony formation was first negatively associated with the concentration of supplemented fetal bovine serum (FBS); serum commonly promotes planktonic growth but inhibits surfaces adherence (Williams et al., 2008). It remains elusive which factors of serum impact *H. pylori* surface adhesion as FBS is an undefined medium with a non-homogeneous mix of growth factors (Zheng et al., 2006). Interestingly, *H. pylori* adhesion on gastric epithelial cell surface does not rely on the presence of FBS, suggesting that this bacterium may utilize specialized mechanism for surface attachment (Yonezawa et al., 2017, Senkovich et al., 2011, Lim et al., 2003, Hathroubi et al., 2020). Furthermore, studies have shown that adhesion on various surfaces directly affected the biomass of mature biofilms, a process that is independent of media components (Windham et al., 2018). *H. pylori* surface adhesion is also strain dependent (Wong et al., 2016), a variation that is potentially due to the heterogenicity of regulatory proteins and outer membrane proteins (OMPs) which are predicted to play a critical role in the initial adhesion step (Servetas et al., 2018, Wong et al., 2016). We will focus on discussing recent findings that have implicated flagella and OMPs as necessary components in the adherence process.

H. pylori flagella play important roles in adherence and subsequent biofilm formation. They are made up of four primary components; basal body, hook, filament, and sheath (Gu et al., 2017). Flagella are typically associated with *H. pylori* motility but have been recently discovered to be involved in promoting surface adhesion and maintaining biofilm architecture (Hathroubi et al., 2020). Motility itself is an essential factor required for *H. pylori* to initiate biofilm (Wong et al., 2016). More insight was provided for this observation by examining abiotic biofilm formation of strains that were non-motile but either retained flagella ($Fla^+ Mot^-$) due to deletion of a flagellar basal body gene *motB*, or lost flagella (Fla- Mot-) due to deletion of the flagellar basal body gene *fliM* (Hathroubi et al., 2018). More biofilm biomass was accumulated in the Fla+ mutant as compared with the Fla- strain. Flagella filaments, furthermore, were visible in the biofilm, and appeared to be forming a matrix. $Fla^+ Mot^-$ strains exhibited initial attachment defects on gastric cell surfaces (Hathroubi et al., 2020). These results suggest that motility is likely involved in the attachment phase on diverse surfaces, and the presence of flagella is required for *H. pylori* biofilm formation.

Another type of molecule shown to contribute to adherence are *H. pylori* outer membrane proteins (OMPs), which can be on the cell surface or present in outer membrane vesicle (OMVs) along with virulence factors and eDNA (Olofsson et al., 2010 and Grande et al., 2015). OMPs play important

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roles in bacterial environmental adaptation and modulation of life cycle phases, including structure maintenance, substance transportation, and microbial-host interaction (Qiao et al., 2014, Egan, 2018).

H. pylori has more than 60 OMPs coding genes (Alm et al., 2000), but not all the OMP's functions are understood (Servetas et al., 2018). Beyond inducing the pro-inflammatory responses, some OMPs were also found to promote multiple processes of *H. pylori* biofilm formation, one of which is to promote surface adhesion as discussed (Yonezawa et al., 2011, Tamrakar et al., 2021). *H. pylori* OMPs facilitate both cell-to-cell and cell-to-abiotic surface adhesion in biofilms, based on the observation of OMV localization in *H. pylori* biofilms via scanning electron microscopy (SEM) imaging (Yonezawa et al., 2009).

A common feature of *H. pylori* OMPs is anti-parallel β sheets that compose a β-barrel, highly stable pore-like structure; transmembrane domains of these proteins interact with host cell receptors (Tamrakar et al., 2021), potentially indicating these OMPs may promote bacterial cell-cell and bacterial-host connections.

One family of *H. pylori* OMPs is the Hom family, a group of four proteins encoded by the following genes: *homA, homB, homC* and *homD* (Alm et al., 2000). These proteins have been specifically utilized as a peptic ulcer disease marker (Tamrakar et al., 2021). Interestingly, *homA* and *homB* were found to contribute *H. pylori* biofilm formation as well (Servetas et al., 2018), indicating the potential association between *H. pylori* biofilm and relative pathology. The outer membrane protein *homB* (J99, *jhp0870;* G27 *HPG27_667*), was recently associated with biofilm formation (Servetas et al., 2018). This protein is interesting as it has been proposed as a biomarker of peptic ulcer disease (Oleastro et al., 2006) and gastric cancer (Talebi et al., 2011). *H. pylori* upregulates *homB* transcription via ArsRS, a two-component system, in the initial adherence and assembly phases of biofilm growth, but then levels fall back to those observed in planktonic cells after 72 hours of incubation (Servetas et al., 2018). This variation suggests the importance of HomB during the initial adhesion and later for the next biofilm assembly stages.

HomA and HomB are composed mainly of β-sheets with cysteine resides on surface loops that help to form homodimers and indicating they are potentially key to aggregation and biofilm formation (Tamrakar et al., 2021). Various studies demonstrated that HomB is negatively regulated by a two-component system, ArsRS system (Windham et al., 2018 and Servetas et al., 2018, Hathroubi et al., 2018). Other Hom family members, the *homD* and *homC* genes, are both upregulated during *H. pylori* biofilm formation (Hathroubi et al., 2018). Polymorphism of HomC have been linked to varied levels of biofilm formation in different *H. pylori* strains (Kim et al., 2016). These findings suggest Hom family OMPs are commonly involved into in the initiation steps of biofilm formation.

The outer membrane protein autotransporter is also likely play a role in regulating *H. pylori* biofilm formation. An uncharacterized autotransporter, paralogous to VacA, *vlpC,* was found to cause a defect of *H. pylori* biofilm formation if disrupted (Alm et al., 2000, Hathroubi et al., 2020). Specifically, this mutant was unable to form mature biofilms. *vlpC* has been upregulated in some biofilms, further supporting this factor is important for *H. pylori* biofilm formation (Hathroubi et al., 2018, Hathroubi et al., 2020)

A group of highly conserved laminin binding proteins of another OMP family, called Hop, has also been shown to be involved in *H. pylori* biofilm regulation as well. AlpB, a Hop family member, was implicated in biofilm formation and antibiotic resistance, since the genetic deletion of *alpB* caused less *H. pylori* biofilm formation (Senkovich et al., 2011, Yonezawa et al., 2017). Since AlpB is highly conserved among *H. pylori* strains, it has recently been identified and investigated as a therapeutic target to eradicate *H. pylori* biofilm (Xiao et al., 2022).

These findings highlight the role played aby OMPs and flagella at this stage, while also emphasizing that there is still much to be discovered.

Assembly

After surface adhesion, *H. pylori* starts forming microcolonies or aggregates that are recognized as the pre-mature form of biofilm (Shen et al., 2020, Hathroubi et al., 2020, Hathroubi et al., 2018). Multicellular aggregates have been observed to be formed by different strains in *in vitro* conditions

within hours (Krzyżek et al., 2021) more complex structures as early as one day of incubation (Hathroubi et al., 2020, Hathroubi et al., 2018). *H. pylori* biofilms formation steps have been characterized using confocal microscopy. This work showed that *H. pylori* strain G27 assembles biofilms initially at the liquid air interface at 24 hours, then assembles aggregates both at the liquid air interface and under the liquid air interface as the biofilm assembled; the distribution of EPS, visualized by staining, paralleled this growth trend (Windham et al., 2018). SEM further revealed that flagella play a critical role in maintaining *H. pylori* biofilm structure, as discussed above (Hathroubi et al., 2020 and Hathroubi et al., 2018). Without flagella, *H. pylori* biofilms were slowly assembled (Ratthawongjirakul et al., 2016). Comparative genomics studies further demonstrated *H. pylori* biofilm assemble at rates that are similar among different strains when calculating cumulative frequency and rate of formation (Wong et al., 2016). Additionally, biofilm assembly is not significantly impacted by *in vitro* conditions, such as serial passaging, nutrient compositions, culturing conditions (Windham et al., 2018).

Mature Phase

The maximum biofilm mass can be observed after 3 days in vitro incubation (Hathroubi et al., 2020 and Luo et al., 2021), and can last up to 7-days in different culture conditions (Ratthawongjirakul et al., 2016, Bugli et al., 2016, Windham et al., 2018, Wong et al., 2016). Comparing biofilm growth on the surface of polystyrene plates (hydrophobic surface) that were pre-coated with poly-D-lysine (hydrophilic and positive charged) and tissues culture treated polystyrene (hydrophilic, negative charge) revealed that optimal biofilm growth is not solely dependent on surfaces being ionic; tissue culture treated and negatively charged surfaces significantly promotes biofilm growth (Windham et al. 2018). A special feature of *H. pylori* biofilms observed in SEM images are flagellar filaments which were discovered to promote surface cohesion and cell-to-cell connections as mentioned above, together with pili formations sustain the biofilm structural integrity on both abiotic and biotic surfaces (Hathroubi et al., 2018 and Hathroubi et al., 2020).

In the meantime, different *H. pylori* strains and incubation conditions can differentially impact biofilm formation kinetics. *H. pylori* strains with strong and poor biofilm forming abilities in tissue culture plates had consistent biomass accumulation rates during the intermediate assembly phases but had a variant cumulative biomass at the mature phase after 7 days of growth (Wong et al., 2016). In another study, *H. pylori* SS1 strain produced robust biofilms in relatively low FBS conditions after 3 days of growth on polystyrene plates, with most biofilm cells (~80%) being coccoid shaped (Hathroubi et al., 2018). Interestingly, *H. pylori* G27 strain did not rely on low-serum conditions, as biofilm formation was not impaired even at standard culture media, with 10% FBS and produced biofilms with similar morphological features as SS1 (Hathroubi et al., 2020).

In mature biofilms grown on abiotic surfaces, most cells are coccoid shaped (0.4-0.6 um long) with a minority of rod-bacillus (2-3um long) shape (Hathroubi et al., 2020). The coccoid form of *H. pylori* was proposed as a response to the environmental stressors, but the underlying mechanism for this morphology is not fully characterized (Kadkhodaei et al., 2020). A recent study showed that these coccoid cells maintained their membrane integrity and metabolism for up to 70 hours of incubation, which strongly suggests that they are viable dormant bacteria (El Mortaji et al., 2020). A morpho-structural analysis of *H. pylori* biofilms revealed that strongest biofilm producing cells show a dominance of coccoid forms unlike weak biofilm producing cells presented rod-shaped forms that were dominant in mature biofilms (Krzyżek et al., 2022).

Interestingly, *H. pylori* in the coccoid morphology is more tolerant to antibiotic exposure (Krzyżek et al., 2021) which aligns with *H. pylori* biofilms strong tolerance to antibiotics (Hathroubi et al., 2018 and Hathroubi et al., 2020). Viability staining experiments with biofilms grown on abiotic surfaces suggest that live cells and dead cells compose matured *H. pylori* biofilms (Hathroubi et al., 2020). Transcriptomic experiments show that biofilm cells are less metabolically active than planktonic cells due to the downregulation of multiple metabolic genes, such as *atpC*, *atpE*, and *nifU* (Hathroubi et al., 2018). Gastric epithelial cell lines, such as AGS, have been developed to study *H. pylori* biofilm formation on biotic surfaces (Hathroubi et al., 2020, Yonezawa et al., 2009). After co-

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incubating *H. pylori* and AGS cells for days, *H. pylori* biofilms were observed on the surfaces and between conjunctions AGS cells (Hathroubi et al., 2020, Cárdenas-Mondragón et al., 2016). Interestingly, most biofilm cells were spiral/rod shaped, a different outcome than what was observed in biofilms grown on abiotic surfaces (Hathroubi et al., 2020). Other cell lines have been employed as well, particularly mucin producing cells, like MKN-45 cells, which may present a more natural *in vivo*-like state similar to niches in the host. On the MKN-45 cell line, most of the biofilm cells primarily exhibited the coccoid morphology (Attaran et al., 2021), suggesting this cell line can be used as a model to study the effects of mucin on *H. pylori* biofilm formation. Further studies are necessary to dissect whether different incubation conditions, such as serum concentration and incubation period may modulate *H. pylori* biofilm features.

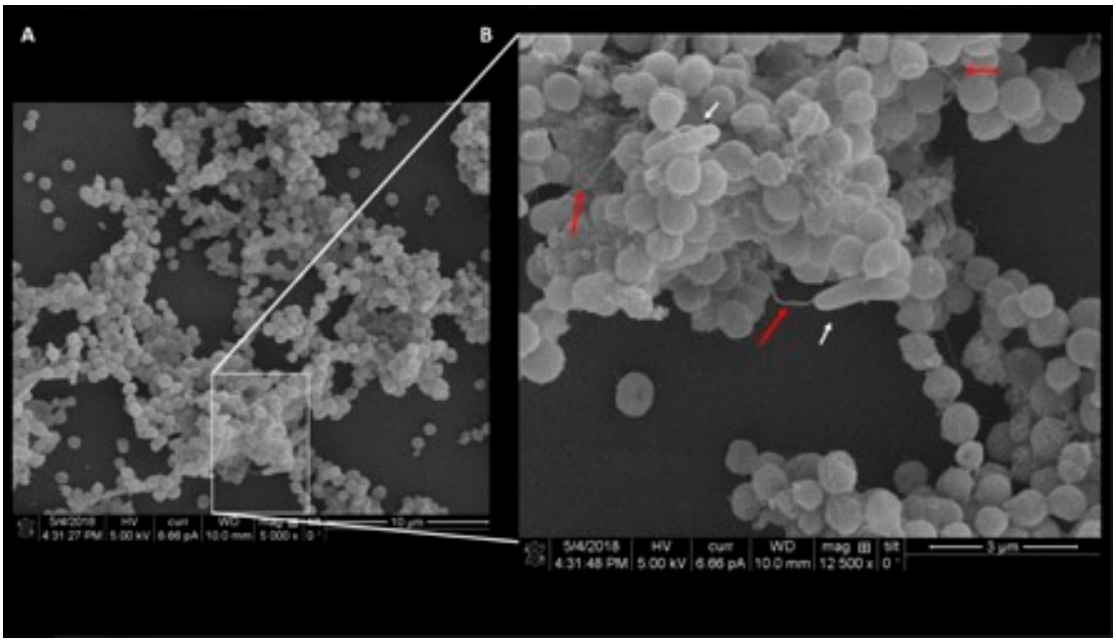

Figure 1. SEM images of mature *H. pylori* biofilms grown on abiotic surfaces. (**A**) Mature *H. pylori* biofilms contain a mixed population of mostly cocoid shaped cells with a minority of spiral shaped cells, (**B**) Higher resolution image showing spiral shaped cells (white arrow) and coccoid cells aggregating via the flagella (red arrow).

Dispersion

Like other bacterial biofilms, *H. pylori* biofilms disperse after reaching optimal growth, indicated by a decrease in crystal violet staining after maximum growth has been reached (Hathroubi et al., 2018, Hathroubi et al., 2020, Krzyżek et al., 2021). Little is known about the signals that lead to *H. pylori* biofilm dispersal, but some evidence suggests that *H. pylori* utilizes a quorum sensing molecule, AI-2, as a signaling molecule to regulate biofilm generation and dispersion (Anderson et al., 2015). AI-2 was initially recognized as a chemorepellent of *H. pylori* sensed by chemoreceptor TlpB (Rader et al., 2011), and this molecule can be expressed by *H. pylori* through *luxS* gene in a cell density-dependent manner (Forsyth et al., 2000, Lee et al., 2006), suggesting that *H. pylori* can efficiently control local density through AI-2 secretion. A later study suggested that AI-2 promoted *H. pylori* biofilm dispersion, as genetic deletion of the *luxS* in *H. pylori* significantly promoted its biofilm formation in comparison to isogeneic WT strain through the lacunarity and fractal dimension analysis (Anderson et al., 2015). The chemotaxis system, in another aspect, was suggested to facilitate *H. pylori* biofilm dispersion by sensing and responding to AI-2, since chemotactic histidine kinase deficient mutant *ΔcheA* exhibited similar biofilm phenotype as the *ΔluxS* mutant (Anderson et al., 2015). Further research is required to decipher the mechanism of how *H. pylori* regulates biofilm maturation and dispersion.

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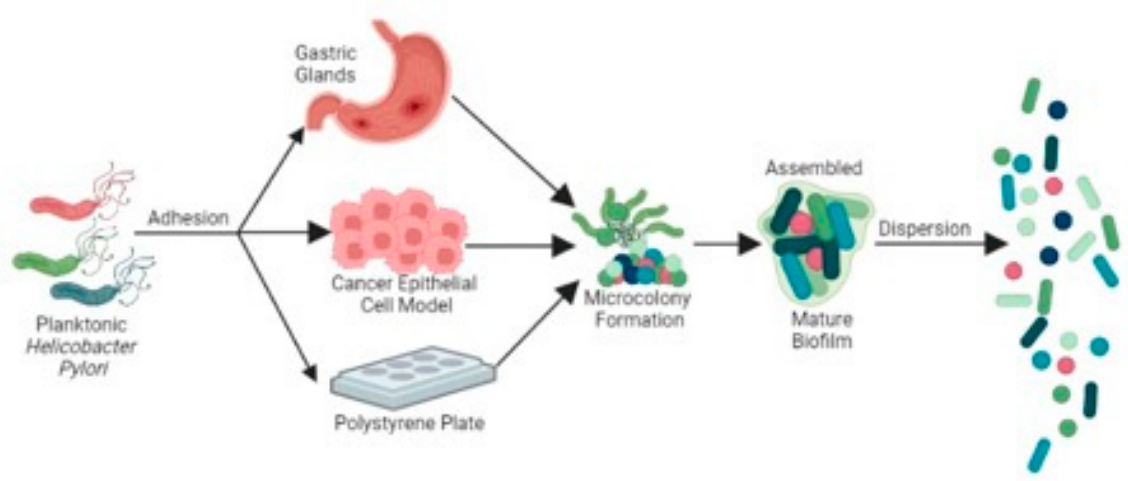

Scheme 1. *Helicobacter pylori* Biofilm Lifecycle. *H. pylori* adheres to both abiotic and biotic surfaces, where it forms microcolonies that susbquently assemble into mature biofilms characterized by the presence of extracellular polymeric susbtances (EPS). Dispersion allows bacteria to colonizes new niches.

H. pylori clinical treatment strategies and the increasing prevalence of antibiotic resistance.

The currently recommended *H. pylori* infection treatment are quadruple therapies that consists of proton pump inhibitor (PPI), bismuth and two additional different antibiotics, including clarithromycin, metronidazole, levofloxacin, or amoxicillin (Liu et al., 2022). However, this classical therapeutic strategy has been being less effective due to the continuing rise of antibiotic resistance. For example, in 2016 a national consensus on Chinese management of *H. pylori* infections reported that metronidazole, levofloxacin, and clarithromycin resistance was 40-70%, 20-50% and 20-50% respectively (Liu et al., 2018). Similarly, the elevation of antibiotic resistance was also noticed in other countries, like Indonesia, that apply the triple-therapy approach consisting of PPI and two antibiotics, (Malfertheiner et al., 2017). The prevalence of resistance to recommended antibiotics metronidazole and levofloxacin increased to 46.7% and 31.2%, respectively; resistance to non-recommended antibiotics remained low however, including amoxicillin (5.2%), tetracycline (2.6%) and clarithromycin (9.1%) (Miftahussurur et al., 2016). In 2020, a case study reported that triple therapies in Indonesia were further decreased to only 67.6% efficient (Fauzia et al., 2020).

To avoid potential therapy failure caused by antibiotic resistance, clinicians have proposed using a tailored treatment approach based on antibiotics susceptibility tests and localized resistance (Fauzia et al., 2020, Pan et al., 2020, Fallone et al., 2016, de Palma et al., 2017 and Li et al., 2022). A clinical study that analyzed the failure of *H. pylori* treatment revealed that patients who were not cured were often infected by diverse *H. pylori* strains that were resistant to different antibiotics (Mascellino et al., 2018). Clarithromycin resistance is attributed to mutations in the 23S rRNA (Redondo et al., 2018); metronidazole resistance was associated to the mutations in *rdxA* and *frxA* loci (Kim and Lee et al., 2017); levofloxacin resistance was caused by *gyrA* and *gyrB* mutations (Miftahussurur et al., 2016). These mutations are naturally occurring, but increased prevalence in the population can occur by exposing strains to sub-MIC levels of antibiotics, such as levofloxacin (Hanafi et al., 2016). Another case study indicated that the tailored treatment approach is necessary and effective in addition to traditional treatment procedures. A total of 112 isolated *H. pylori* strains were genotyped from the *H. pylori* infection prevalent regions that applied quadruple treatment. With the supplementation of the tailored treatment, the dual resistance to metronidazole and levofloxacin was observed in 20.5% of tested strains and triple resistance to metronidazole, clarithromycin and levofloxacin was observed in only ~7% of strains (Liu et al., 2022). The effectiveness of tailored therapies was also evaluated in another clinical trial study in comparison to the traditional bismuth quadruple therapy, and it was

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demonstrated that the tailored bismuth/quadruple therapy was more effective (Pan et al., 2020). Intriguingly, another case study examined 101 clinical *H. pylori* isolates from Indonesian patients with gastritis (91%), peptic ulcer disease (8.9%) and gastric cancer (1%) and discovered that 93% of the isolates formed biofilms (Fauzia et al., 2020). These studies strongly suggest that biofilm formation may play a vital role in facilitating *H. pylori* to acquire high antibiotic tolerance, therefore the eradication of *H. pylori* biofilm is likely a key process for clinical therapy. Nevertheless, there are challenges in clinal therapies: (1) planktonic susceptibility of minimal inhibitory concentration (MIC) may not be a reliable indicator of Minimal Biofilm Eradication Concentration (MBEC) with certain antibiotics (Fauzia et al., 2020; Attaran et al., 2017); (2) isolating clinical strains from infected patients are required the acquisition of gastric biopsies is an invasive procedure (Li et al., 2022). Therefore, it would be very interesting to understand if targeting biofilm formation would enhance *H. pylori* treatment.

Approaches utilized in H. pylori antibiotic resistance detection and prediction.

Due to the global increase in antibiotic resistance with *H. pylori*, efficient antibiotic susceptibility examinations would be expected for appropriate diagnostic and treatment. Currently, two major types of techniques are utilized today, either bacterial viability-based or molecular based technique.

Bacterial viability-based techniques are the standard approach to determine bacterial antibiotic susceptibility and has been utilized to track increasing antibiotic resistance (Midolo et al., 1997, Fauzia et al., 2020), by measuring bacterial viability under exposure to a certain type and amount of antibiotic. Such approaches are further divided into agar or broth dilution methods, Epsilometer test (E-test) methods (Glupczynski et al., 1991), or disk diffusion methods (Tang et al., 2020). These techniques are all capable of quantitatively determining the minimum concentration of an antibiotic that kills *H. pylori* (Mishra et al., 2006). Different methods have specific advantages. E-tests and disk diffusion assays are not a 'one size fits all' approach since the differences in susceptibility to amoxicillin, tetracycline, and furazolidone were observed between the disk diffusion method and E-test method (Tang et al., 2020). For example, the E-test method is easy to apply and time friendly (Midolo et al., 1997), while the *H. pylori* dilution method allows several stains to be tested simultaneously.

As various antibiotic resistance mechanisms have been characterized and the genetic elements have been identified using molecular-based approaches (Saruuljavkhlan & Yamaoka, 2021), these discoveries promote the evolution of more rapid and cost friendly molecular based methods to detect presence of responsible resistant genetic elements and susceptible elements to predict possible antibiotic resistant phenotypes (Tshibangu-Kabamba et al., 2020, Smith & Pellicano, 2019). PCR-based genetic amplification technique and Sanger sequencing approaches together are intensively developed and applied to achieve such goals; these approaches have several advantages including being easily reproducible and time efficient in comparison to traditional bacterial viability-based methods (Mégraud et al., 2007). More importantly, these techniques can be applied directly on bacteria that have not been cultured or are of low abundance including various clinical isolates from gastric tissue or gastric juice (Van Doorn et al., 2001, Schabereiter-Gurtner et al., 2004, Mitui et al., 2014, Nishizawa & Suzuki, 2014). However, this approach has limitations and is only reliable to predict certain types of antibiotics whose resistant mechanism has been specifically characterized, such as clarithromycin and tetracycline, but not for those antibiotics whose anti-mechanism is diversified, such as metronidazole and amoxicillin. To overcome such limitations, next generation sequencing (NGS) technologies have been developed as an efficient tool to detect and predict all potential antibiotic resistance mutations in a bacterial sample (Vital et al., 2022). This type of approach consists of DNA extraction from a given bacterial sample that undergoes whole genome sequencing (WGS) (Fauzia et al., 2021). There are several advantages of this approach compared to the PCR-based molecular approach. With the growing of whole microbial genome data sets, a pan-genome-based machine learning approach was recently developed to predict antimicrobial resistance activities in some bacteria, including *Escherichia coli* (Her & Wu, 2018). This approach uses written algorithms to predict whether a specific stain is resistant to antibiotic drugs by comparing its genome against the accessory part of the pan-genome, to yield the gene clusters that are most crucial to antimicrobial

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resistance activities in *E. coli*. A limitation of this approach is that we may not yet know all antibiotic resistance alleles. Currently, this approach has not yet applied in examining *H. pylori*, but it seems to be a promising one.

Mechanisms of *H. pylori* biofilm-promoted antibiotic resistance.

Biofilm formation may play a significant role in facilitating *H. pylori* antibiotic tolerance (Tshibangu-Kabamba & Yamaoka, 2021). A phenotype of tolerance manifests in that the antibiotic MIC for planktonic *H. pylori* does not accurately reflect the concentration needed to eradicate *H. pylori* biofilm cells. For example, a clinical study compared antibiotic susceptibility of *H. pylori* isolates between the planktonic and biofilm growth and found that *H. pylori* biofilms was more capable of tolerating various antibiotics relative to planktonic *H. pylori*, including up to 1000-fold with amoxicillin, 31.25-fold with clarithromycin, 16-fold with levofloxacin, and 8-fold with metronidazole (Fauzia et al., 2020). *H. pylori* biofilms have exhibited several advantages in facilitating antibiotic tolerance. Studies have proposed the correlation between high biofilm formation capacity in *H. pylori* and the tolerance to clarithromycin, but not however, metronidazole or levofloxacin (Krzyzek et al., 2022). While the reason for the high tolerance of *H. pylori* biofilms is not yet fully understood, several ideas have been proposed including that bacterial cells are protected by the biofilm structure; conjugated bacterial cells within the biofilm increased the chance of genetic exchange. Below we dissect recent mechanisms of antibiotic tolerance employed by *H. pylori* biofilms.

Extracellular polymeric substance matrix reduces the efficacy of antibiotics

H. pylori biofilms are encased in an extracellular polymeric substance (EPS) matrix that maintains the structural integrity of the biofilm, promotes adhesion, and facilitates cell-to-cell interactions (Li et al., 2019). Proteins, polysaccharides and eDNA were confirmed to compose the extracellular polymeric substance matrix in *H. pylori* biofilms (Hathroubi et al., 2018). Immunofluorescence assays with probes specific for proteins, eDNA and polysaccharides show that EPS distribution depends on cell density within the biofilm (Windham et al., 2018). Polysaccharides in the EPS can be stained with FITC-conA which targets mannose groups in polysaccharides. The green fluorescence can be used to visualize the EPS matrix in *H. pylori* biofilms with Confocal Laser Scanning Microscopy (CLSM) (Shen et al., 2020 and Li et al., 2019). The film tracer Sypro Ruby stain targets proteins in the EPS and can also be visualized using CLSM (Hathroubi et al., 2018 and Windham et al., 2018). EPS eDNA in *H. pylori* biofilms can be stained and visualized via CLSM using BOBO-3 (Hathroubi et al., 2018) and propidium iodide (Windham et al., 2018). Enzymatic assays indicate that proteins play vital role in *H. pylori* EPS as proteinase K treatment significantly cause dispersion of *H. pylori* biofilms and reduced antibiotic tolerance (Hathroubi et al., 2018 and Windham et al., 2018). While eDNA and polysaccharides also compose EPS structures, they are predicted to play minor roles compared to proteins, based on the observation that DNase I and sodium periodate treatment targeting the eDNA and polysaccharide respectively, did not cause significant *H. pylori* biofilm reduction (Windham et al., 2018, Hathroubi et al., 2018).

In addition to sustaining structural integrity, the EPS may reduce the efficacy of drugs from reaching the interior of the biofilm. EPS itself is minimally affected during antibiotic exposure (Li et al., 2019), supporting the idea that antibiotic treatment does not eradicate *H. pylori* biofilms. Removal of proteins, however, does sensitize *H. pylori* in biofilms to clarithromycin, although it was not demonstrated whether this is EPS or surface protein removal (Hathroubi et al 2020). Therefore, the disruption of EPS of *H. pylori* biofilm may be a highly significant target to effectively eradicate this bacterium (Li et al., 2019).

Coccoid Cellular morphology

Compared to spiral shape that is commonly observed in planktonic *H. pylori* cells, coccoid cells are more commonly found in *H. pylori* biofilm (Hathroubi et al., 2018, Hathroubi et al., 2020). The coccoid cellular shape was recognized to be dormant state of *H. pylori* that contributes to antibiotic

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resistance and disease induction (Reshetnyak et al., 2017, Kadkhodaei et al., 2020). *H. pylori* biofilms, like other bacteria, can sustain the slow growth state (Hathroubi et al., 2018), and promote antibiotic tolerance that specifically target active phase bacterium (Harris et al., 2000, Ikeda et al., 1990). Prior research has shown that significant cell wall alterations occur when *H. pylori* is transitioning to the coccoid morphology (Costa et al., 1999) and has been associated with biofilm growth and antibiotic tolerance (Kadkhodaei et al. 2020).

A couple of genes that modify *H. pylori's* cell wall have been documented to be upregulated in *H. pylori* biofilms and may contribute to the coccoid form and/or antibiotic tolerance. For example, UppS, a putative undecaprenyl pyrophosphate synthase, facilitates *H. pylori* cell wall peptidoglycan modification (Kuo et al., 2007). Transposon inserted of *uppS*, resulted in a defective biofilm formation (Hathroubi et al., 2020). Some naturally occurring cell-wall related mutations may be beneficial for developing antibiotic resistance. For example, recent studies found ethoxzolamide, clinically used sulphonamide drug, can block cell wall synthesis by competitively inhibiting UppS (Modak et al., 2019); however, strains can be become resistant by acquiring mutations in the binding site of UppS (Rahman et al., 2020).

Another cell wall factor found to be important for maintaining *H. pylori* biofilm structure is peptidoglycan deacetylase (PdgA). The *pgda* gene was upregulated in *H. pylori* biofilms (Hathroubi et al., 2018), and was previously associated with host derived oxidative stress (Wang et al., 2010). Oxidative stress induces *H. pylori* biofilm formation (Zhao et al., 2021), which is consistent to a model that PdgA promotes *H. pylori* biofilm formation. In addition, PdgA may play an important role in maintaining *H. pylori* biofilm structure as the *H. pylori* Δ*pgdA* mutant is more susceptible to lysozyme exposure, an enzyme that cleaves the peptidoglycan of bacterial cell wall (Wang et al., 2012).

In addition, another gene *hp0421*, encoding cholesteryl-α-glucoside transferase , was also found to regulate cellular morphology in biofilms (Chou et al., 2017; Qaria et al., 2018). The *hp0421* deletion caused defects in maintaining spiral morphology, an increase in susceptibility to antibiotics and promoted cellular aggregation to form pronounced biofilms faster than the wild-type controls (Qaria et al., 2018) further supporting the important role of coccoid morphology in biofilms. In conclusion, genes that have been implicated in regulating *H. pylori* morphologies and is synchronous with affecting biofilm phenotypes and antibiotic tolerance reveal a key topic that should be investigated to further decode *H. pylori* biofilms.

Downregulated Metabolism in Biofilms

Growing bacterial cells are more easily targeted by certain types of antibiotics, such as ampicillin, that is selected as an essential component of triple-therapy applications for *H. pylori* treatment (Marcus et al., 2012). Recently it has been revealed that *H. pylori* reduces its metabolic activities in the biofilm to mitigate such detrimental effects, along with the trend shifting to coccoid cellular morphology (Hathroubi et al., 2018). A recent clinical study found a positive correlation between strong biofilm former and a general decrease in metabolic rate (Wong et la., 2018). This observation is supported by another *H. pylori* transcriptomic study that suggests biofilm cells are less metabolically active than planktonic cells due to the downregulation of metabolic genes (Hathroubi et al., 2018). Interestingly, *H. pylori* is also able to upregulate specific metabolic enzymes to resist certain natural substrates, functionally as antibiotics. For example, *Combretum mole extracts, an acetone-containing plant commonly consumed in South Africa to alleviate gastric illness,* have bactericidal effects on *H. pylori* (Njume et al., 2011). To tolerate acetone exposure, acetone carboxylase gene *acxA* is upregulated in the *H. pylori* biofilm, indicating the acetone carboxylases is expressed to potentially degrade acetone during gastric colonization. Additionally, *acxA* deletion resulted in a significant biofilm defect (Hathroubi et al., 2020); the *acxA* gene is under regulation of both two-component system under the ArsRS (Loh et al., 2010) and the CrdRS (Allen et al., 2023), which are heavily involved in maintaining *H. pylori* biofilm and promoting gastric gland colonization (Brahmachary et al., 2008, Hathroubi et al., 2020). Both *crdR* and *arsR* regulators were found to be upregulated in biofilms (Hathroubi et al., 2018, De La Cruz et al., 2017, Servetas et al., 2016); *crdR* was found to be upregulated in biofilms on abiotic surfaces (Hathroubi et al., 2018 and De La Cruz et

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al., 2017) and upon adherence to AGS cells (De la Cruz et al., 2017). On the other hand, *arsR* was found to be upregulated in strain 26695 biofilms grown on abiotic surfaces and AGS (De la Cruz et al., 2017). These combined findings suggest that the *acxA* gene is mandatorily expressed and essential to maintain certain functions of *H. pylori* biofilm including protecting *H. pylori* in the host from acetone degradation.

Efflux pumps involved drug external transportation

Efflux pumps are commonly located on the *H. pylori* cell membrane and facilitate the multiple drugs external transportation (Raj et al., 2021). Efflux pumps have been strongly associated with antibiotic resistant strains and multidrug resistance in recent studies (Liu et al., 2022 and Yonezawa et al., 2019, Attaran et al., 2017) which indicates that they play a significant role in the antibiotic tolerance of *H. pylori* biofilms. Several efflux pumps coding genes, including Hp605 (*hefA*), Hp971 (*hefD*), Hp1327 (*hefG*), Hp1489, Hp1118, Hp1174 (*gluP*), HP0939, HP0497, and HP0471 (KefB), were found to be expressed in both planktonic and biofilm cells, suggesting that efflux pump is essential during *H. pylori* life cycles (Ge et al., 2018, Yonezawa et al., 2019, Cai et al., 2020). Recent studies further revealed that these efflux pump coding genes were significantly upregulated in biofilm to facilitate *H. pylori* antibiotics tolerance (Cai et al., 2020). HPG27_715 (a MATE-family uncharacterized efflux pump), Hp1118, *gluP*, HP1165 (associated with tetracycline resistance), *hefA* were significantly upregulated in biofilms relative to planktonic cells (Hathroubi et al., 2020, Ge et al., 2018, Attaran et al., 2017). *hefA* (Yonezawa et al., 2019 and Attaran et al., 2017), *hefD*, *hefG* and HP1489 were found to be particularly upregulated in biofilms from a clarithromycin resistant strain TK1402 (Yonezawa et al., 2019). *gluP* expression was found to be regulated by *H. pylori* stringent response and genetic deletions of *gluP* cause a biofilm defect and increased susceptibility to different types of antibiotics (Ge et al., 2018). Additionally, genetic deletions in HP0939, HP0497, and KefB also conferred with a biofilm defect (Cai et al., 2020). *hefD* and *hefA* have both recently been associated with multidrug resistance in clinical *H. pylori* strains isolated from Nigeria while no association with *hefG* was detected (Jolaiya et al., 2020). Cumulatively, these findings support the perspective that *H. pylori* utilizes biofilm growth to survive under antibiotic exposure and efflux pumps are a key contributor.

Anti-biofilm strategies

Since chronic infection with *H. pylori* causes various gastric diseases, approaches are being developed to efficiently eradicate this bacterium. Here, we summarize several approaches based on the anti-biofilm treatments including synthetic compounds, natural compounds, and small molecule drugs.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are promising alternatives to antibiotics for combating biofilm infections. One of the advantages of using AMPs is that these molecules are also less likely to induce resistance in bacteria than antibiotics because they target multiple components within the bacterial cell. These small peptides can penetrate the extracellular matrix that surrounds biofilm cells and thus target the bacteria directly.

Another antimicrobial peptide was also recently investigated, Cbf-K16, Cathelicidin-like peptide which showed a good antimicrobial activity against clarithromycin- and amoxicillin- resistant *H. pylori in vitro* and *in vivo* (Jiang et al., 2019). In mouse gastritis model Cbf-K16 demonstrated a 3.9-$\log_{10}$ reduction of bacterial counts in stomach tissues compared to untreated mice group (Jing et al., 2019). Interestingly, treatments with Cbf-K16 significantly downregulated the expression levels of the adhesion-associated genes *alpA* and *alpB* mRNA, both factors play a role in *H. pylori* adhesion and biofilms as mentioned above (Senkovich et al., 2011, Yonezawa et al., 2017, Jiang et al., 2019).

The antimicrobial peptide MSI-78A, also known as Pexiganan, is a 22-amino acid peptide Magainin-2 analogue was reported to have antibacterial activity in solution (Zhang et al., 2015,

Parreira et al., 2019). When surface grafted MSI_78A still demonstrated activity with a high bacterial eradication rate (>90% after 2h) thus not able to proliferate and establish biofilms (Parreira et al., 2019).

Several synthetic peptides were also applied and have been shown to promote biofilm dispersion in *H. pylori*, individually or synergistically with host antimicrobial peptides (Windham et al., 2018). For instance, when *H. pylori* biofilms were treated with synthetic peptides IDR-1018 and DJK-5, it became more susceptible to the host derived anti-microbial peptides (Windham et al., 2018). In addition, DJK-5 is a synthetic short D-enantiomeric peptide designed to be resistant bacterial proteases (de la Fuente-Núñezet al., 2015) and IDR-1018 was designed by altering bactenecin from bovine neutrophils (Mansour et al., 2015). Both DJK-5 and IDR-1018 are capable of degrading a second messenger nucleotide, a stringent response molecule, called (p)ppGpp (de la Fuente-Núñezet al., 2015 and Mansour et al., 2015). Prior *in vitro* studies from several *H. pylori* strains (J99, 26695 and G27) suggested that *H. pylori* utilizes a stringent response at low pH or with poor nutrients to produce significant amounts of ppGpp (Wells et al, 2006). *H. pylori* contains an enzyme called SpoT, a (p)ppGpp synthase and hydrolase, whose genetic deletion causes a defective biofilm phenotype and an increased susceptibility to antibiotics (Ge et al., 2018). DJK-5 and IDR-1018 were tested on *H. pylori* biofilms and were observed to not affect viability of planktonic bacterial viability; biofilm assembly, however, was inhibited only by DJK-5 (dose dependent). In contrast, IDR-1018 reduced mature *H. pylori* biofilms without affecting the bacterial viability within the biofilm matrix (Windham et al., 2018). These findings suggest that synthetic cationic peptides specifically target *H. pylori* in the form of biofilms and that *H. pylori* utilizes mechanisms in biofilms homologous to other bacterial species affected by the same peptides (Windham et al., 2018).

Extracts from Natural Resources

Extractions from natural resources such as plants and other bacteria are commonly applied to treat various microbial infections, including *H. pylori*. Some extractions have been found to be particularly effective in eradicating *H. pylori* by specifically targeting biofilm stability.

Probiotics can inhibit bacterial biofilms and thus play an auxiliary role in bacterial antibiotic therapy. As documented, the effects of different probiotic strains may play a varied role in restricting certain bacterial biofilms, including *H. pylori* biofilm. Probiotic Lactobacillus fermentum UCO-979C was previously found to play a role in inhibiting *H. pylori* biofilm formation (Salas-Jara et al., 2016). Furthermore, another microbial study found that *Lactobacillus plantarum* LN66 cell-free supernatant (CFS) can weaken *H. pylori* biofilm formation, an effect monitored by SEM and confocal laser scanning microscopy (CLSM) (Ji & Yang, 2021). Probiotics combined with other antibiotics was found to increase treatment efficacy for levofloxacin as LN66 CFS facilitate this antibiotic function to inhibit EPS secretion (Salas-Jara et al., 2016, Jin and Yang 2021). Another intriguing finding is armeniaspirols, which is a novel class of natural products isolated from *Streptomyces armeniacus* previously identified as antibacterial agents against Gram-positive pathogens (Dufour et al., 2012). Armeniaspirol A (ARM1) exhibited potent antibacterial activity against *H. pylori* as well by inhibiting *H. pylori* biofilm formation in a dose-dependent manner. In a mouse model to study multidrug-resistant *H. pylori*, dual therapy with ARM1 and omeprazole showed efficient killing efficacy, comparable to the standard triple therapy, and induced negligible toxicity against normal tissues (Jia et al., 2022). Moreover, at acidic pH 2.5, ARM1 exhibited a much more potent anti-*H. pylori* activity than metronidazole (Jia et al., 2022). All these advantages promote the possibility of ARM1 being used in a clinical application.

Plants are another major resource that organic products are extracted from to treat bacterial infections. A variety of materials have been found to efficiently restrict *H. pylori* infection. For example, *Antractylodes lancea* volatile oils were recently found to inhibit *H. pylori* biofilm formation. This oil complex also exhibits a robust ability to reduce *H. pylori* virulence factor CagA translocation into host cells, a finding observed in a cell culture infection model (Yu et al., 2019). Additional screenings were applied to search for natural molecules to target *H. pylori* biofilm stability. Phytochemicals from *Acorus calamus, Colocasia esculenta Vitex trifolia, Azadirachta indica* A. *Juss* exhibited a significant effect on inhibiting *H. pylori* biofilm formation as well (Marina et al., 2022,

Prasad et al., 2019). Among screening tests, *Acorus calamus* exhibited the highest *H. pylori* anti-biofilm activity via a dose-dependent pattern (Prasad et al., 2019). Phytochemicals from the neem tree (*Azadirachta indica* A. Juss) were also previously shown to have bactericidal properties and several other Neem tree phytochemicals (nimbolide, azadirachtin, gedunin) and were tested for toxicity towards *H. pylori* but only Nimbolide was found to kill both planktonic and biofilm *H. pylori* without having hemolytic activity; Nimbolide was effective towards the nine strains of *H. pylori* tested in a time and dose dependent manner under various stressful growth conditions and metabolic activities (Marina et al., 2022). Dihydroatanshinone I, a natural herbal compound, is another agent that clearly inhibits *H. pylori* biofilm in both *in vitro* and *in vivo* studies when combined with omeprazole as a dual therapy, even more efficiently compared to the standard triple therapy approach that includes metronidazole; more interestingly, this compound exhibited negligible toxicity against normal tissues, indicating the potential in its clinical application (Luo et al., 2021). Extracts from hibiscus flowers (*Hibiscus rosa sinensis L. flower*) also showed properties of inhibiting biofilms and bactericidal effects on drug resistant *H. pylori* strains (Trung et al., 2020). Alginate lyases, a compound found naturally in brown algae that degrades the EPS was found to enhance the efficacy of clarithromycin when both components are synergistically used to treat biofilms (Bugli et al., 2016). These recent findings present promising possibilities of discovering compounds in nature that are effective at killing *H. pylori* even in biofilm forms.

Small Molecule Drug and Nanodrugs

Various small molecule-based compounds that facilitate traditionally applied antibiotics, have been found to be effective at treating bacterial infections. These compounds include both organic and inorganic monomers or polymers that target bacterial essential enzymes, pathways, or structure. For example, carvacrol and thymol were found to inhibit *H. pylori* biofilms by inhibiting an enzyme required for biofilm growth, carbonic anhydrase (Grande et al., 2021). Lipid polymer nanoparticles can eradicate *H. pylori* biofilm by enhancing the encapsulation of a given antibiotic, such as clarithromycin, to reduce biofilm viability and structural integrity more efficiently via bypassing the mucus layer and the EPS of the *H. pylori* biofilm (Li et al., 2019). A following study further found that the function of N-acylhomoserine lactonase silver nanoparticles (aka nanodrugs) in inhibiting *H. pylori* quorum sensing system, potentially combats *H. pylori* biofilm formation (Gopalakrishnan et al., 2020). Additionally, synthesized silver ultra-nano clusters (SUNCs) in another study were found to inhibit *H. pylori* biofilm formation when synergized with other antibiotics, like metronidazole (Grande et al., 2020; Huang et al., 2022). Nanodrugs are slightly negative charged/ hydrophilic oral drugs made of berberine derivatives and rhamnolipids (RHL) that penetrate the mucus layer and effectively clear *H. pylori* biofilms *in vitro* and *in vivo* (Shen et al., 2020, Li et al., 2019); RHL is a biosurfactant composed of di and mono-rhamnose sugars attached to fatty acids produced by *Pseudomonas aeruginosa* (Li et al., 2019) and berberine is a quaternary ammonium alkaloid isolate from *Coptis chinensis* that is proposed to enhance the efficacy of triple therapy for *H. pylori* infections (Shen et al., 2020). Nanoparticles modified with mannose were specifically found to be effective towards multi-drug resistant *H. pylori* and their biofilms (Arif et al., 2022). All these studies show that the combination of nanodrugs with antibiotics efficiently disrupts *H. pylori* biofilm and provides a feasible strategy to eradicate *H. pylori* infection.

Conclusion and Perspective

In conclusion, the scientific community has made considerable strides in unraveling the intricate nature of the gastric chronic pathogen, *H. pylori*, and its biofilm formation mechanisms. Notably, studies employing clinically isolated strains have played a crucial role in advancing our understanding and have paved the way for the development of promising biofilm-based approaches for eradicating *H. pylori*.

Nevertheless, there is still a need for more targeted research to comprehensively evaluate the pharmacological effects of the newly proposed treatments on both the host and the effectiveness of *H. pylori* eradication. These investigations should extend beyond *in vitro* experiments and encompass

comprehensive animal models and rigorous clinical trials. By conducting such studies, we can obtain a more accurate assessment of the therapeutic potential of these proposed treatments and their impact on both the host and the pathogen.

Furthermore, it is important to explore the long-term effects of these novel approaches to ensure their safety and efficacy in real-world scenarios. Additionally, investigating potential resistance mechanisms that *H. pylori* may employ in response to biofilm-targeting therapies would be instrumental in designing more robust treatment strategies.

In conclusion, while significant progress has been made in understanding *H. pylori* biofilm formation and developing potential eradication approaches, further research is necessary to evaluate the pharmacological effects, efficacy, and safety of these treatments in animal models and clinical trials. By addressing these research gaps, we can bring us closer to achieving more effective and personalized strategies for combating *H. pylori* infection and its associated complications.

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