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Helicobacter pylori Biofilm Formation and Its Potential Role in Pathogenesis

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SUMMARY	1
INTRODUCTION	2
DEMONSTRATION OF <i>H. PYLORI</i> BIOFILMS	2
<i>In Vitro</i> Models	2
Extragastric Biofilms	4
Gastric Biofilms	4
MOLECULAR MECHANISMS OF <i>H. PYLORI</i> BIOFILM FORMATION	5
Omic Approaches	5
Targeted Studies of Candidate Genes Associated with Biofilms	6
Regulatory Proteins Associated with <i>H. pylori</i> Biofilm Formation	7
COMPOSITION OF THE BIOFILM MATRIX	8
IMMUNE EVASION AND TREATMENT FAILURE	9
Studies of <i>H. pylori</i> Biofilms and Antibiotic Resistance	9
Studies of <i>H. pylori</i> Biofilms and Immune Evasion	10
CONCLUSION	11
APPENDIX 1	11
APPENDIX 2	11
ACKNOWLEDGMENTS	12
REFERENCES	12

SUMMARY Despite decades of effort, *Helicobacter pylori* infections remain difficult to treat. Over half of the world's population is infected by *H. pylori*, which is a major cause of duodenal and gastric ulcers as well as gastric cancer. During chronic infection, *H. pylori* localizes within the gastric mucosal layer, including deep within invaginations called glands; thanks to its impressive ability to survive despite the harsh acidic environment, it can persist for the host's lifetime. This ability to survive and persist in the stomach is associated with urease production, chemotactic motility, and the ability to adapt to the fluctuating environment. Additionally, biofilm formation has recently been suggested to play a role in colonization. Biofilms are surface-associated communities of bacteria that are embedded in a hydrated matrix of extracellular polymeric substances. Biofilms pose a substantial health risk and are key contributors to many chronic and recurrent infections. This link between biofilm-associated bacteria and chronic infections likely results from an increased tolerance to conventional antibiotic treatments as well as immune system action. The role of this biofilm mode in antimicrobial treatment failure and *H. pylori* survival has yet to be determined. Furthermore, relatively little is known about the *H. pylori* biofilm structure or the genes associated with this mode of growth. In this review, therefore, we aim to highlight recent findings concerning *H. pylori* biofilms and the molecular mechanism of their formation. Additionally, we discuss the potential roles of biofilms in the failure of antibiotic treatment and in infection recurrence.

KEYWORDS *Helicobacter pylori*, biofilm, regulation, gastritis, antibiotics

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INTRODUCTION

The observation that bacteria reside within communities is centuries old. In fact, Antonie van Leeuwenhoek commented on the presence of “animalcules” in plaque on teeth in 1623, but the biofilm theory was not developed until 1978 (1). Many names were subsequently used in early studies of the bacterial communities that we now know as biofilms, which can be defined as adherent aggregates of microorganisms encased with an extracellular polymeric substance (EPS) (2–4). Biofilms present one of the most successful modes of life; the importance of the microbial biofilm is demonstrated by its presence in the fossil record dating back over 3 million years (4, 5). Today, biofilms have been identified in a myriad of ecological niches, and they are increasingly being recognized for their importance in human health; up to 80% of microbial infections in humans involve biofilm growth (3, 6). Indeed, biofilms are often associated with chronic infectious diseases since they provide an additional level of protection against environmental, host, and antimicrobial assaults (reviewed in references 4 and 6–8). There is a growing body of evidence that indicates that the bacterium *Helicobacter pylori* can establish a biofilm.

H. pylori is considered one of the most remarkable human pathogens. More than 10,000 years of coexistence and current colonization estimates at nearly half of the human population distinguish *H. pylori* as one of the most prevalent global pathogens as well as the most common bacterial infection (9). *H. pylori* infects the gastric epithelium and will persist as a chronic infection unless treated. Rates of infection with this bacterium are strongly associated with socioeconomic status and hygiene conditions. As such, the prevalence of *H. pylori* varies from more than 80% in developing countries to less than 40% in industrialized countries (10). While *H. pylori* colonization is often associated with asymptomatic infections, *H. pylori* is responsible for gastric diseases such as chronic gastritis, peptic and duodenal ulcers, and gastric cancers (11–13). Indeed, *H. pylori* is the number one risk factor for the development of gastric adenocarcinoma, which occurs in approximately 1 to 2% of infected individuals (14, 15).

Since its discovery in the 1980s, studies of *H. pylori* have focused on the planktonic, free-floating, nonattached mode of growth; however, recent evidence suggests that *H. pylori* can also grow in a surface-attached biofilm mode. Studies are beginning to elucidate *H. pylori* biofilm development both *in vitro* and *in vivo* (16–23). While the examination of *H. pylori* biofilms is relatively new, research in this particular field has been slowly gaining momentum over the past few years. Given the chronicity associated with *H. pylori* infection, it is perhaps not surprising that this bacterium has been observed in biofilms.

En masse, the available data support further consideration of the potential role of *H. pylori* biofilms in gastric infections. These considerations are especially relevant given that (i) *H. pylori* is notorious for being recalcitrant to antibiotic treatment and the immune response (24–27) and (ii) biofilms can impact the efficacy of antibiotic treatment and clearance by the host immune response. Indeed, recent U.S. estimates have found that 20 to 25% of infected individuals are not cured by current therapy (28). In this review, we provide insights into current research on the composition of *H. pylori* biofilms and associated genetic factors. Additionally, we discuss how biofilms could be associated with the failure of antibiotic treatment and infection recurrence.

DEMONSTRATION OF *H. PYLORI* BIOFILMS

In Vitro Models

Although the role of *H. pylori* biofilms is unknown, researchers are beginning to develop *in vitro* models to investigate the biofilm phenotype. As such, several studies have demonstrated the ability of *H. pylori* to produce a biofilm *in vitro* (18, 29, 30) (Fig. 1). Indeed, one group demonstrated biofilm production by clinical, laboratory, and mouse-adapted strains at the air-liquid interface on glass coverslips after 3 to 5 days of shaking culture in brucella broth (BB) supplemented with 7% fetal calf serum (FCS) (30). These biofilms were composed mainly of coccoid bacteria within a three-dimensional architecture of stacked layers and holes (30). In comparison, Yonezawa et al. demon-

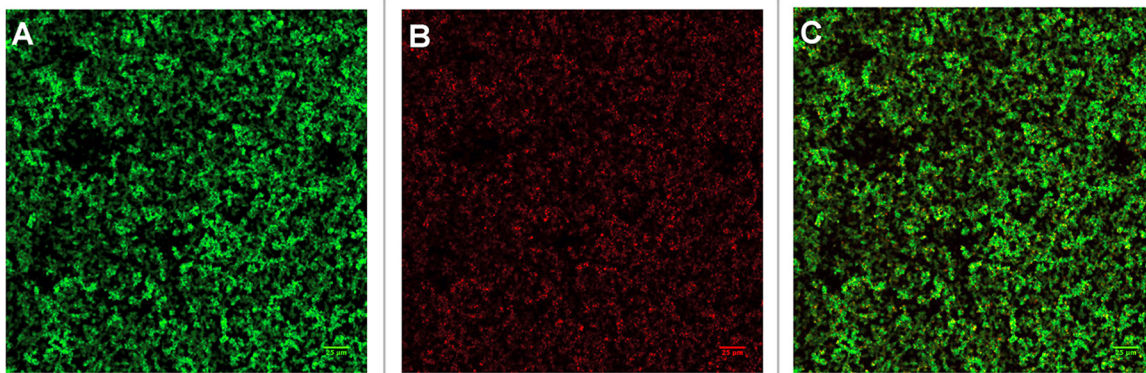


FIG 1 Confocal laser scanning microscopy image of an *H. pylori* G27 biofilm stained with the FilmTracer Live/Dead biofilm viability kit. The biofilm was formed in 96-well plates after 3 days of growth using brucella broth medium supplemented with 10% FBS. (A) Live cells; (B) dead or compromised cells; (C) merge.

strated that *H. pylori* Sydney strain 1 (SS1), a mouse-colonizing strain used extensively in *H. pylori* research, formed relatively little biofilm biomass compared to those formed by ATCC 43579 and other clinical isolates (31). Scanning electron microscopy (SEM) analysis revealed that a biofilm formed by strain TK1402 consisted of thick layers of cells with a bacillary morphology (31). Conversely, biofilms formed by other strains (e.g., SS1) exhibited thin layers of amorphous cells mixed with some autolyzed cells (31). The difference in morphological forms associated with *H. pylori* biofilms is intriguing. The two dominant morphologies adopted by *H. pylori* cells include a bacillary-spiral form and a coccoid form. Both forms have been observed *in vitro* (32–34) and *in vivo* (35, 36). The exact nature of the coccoid form is not fully understood, and little is known about this form in *Helicobacter pylori*. Furthermore, there is currently a split in the *H. pylori* field concerning the significance of the coccoid form; some consider these cells to be dead/degraded, and others consider these cells to represent a viable but nonculturable (VBNC) form (32, 37).

In other species, VBNC cells are metabolically less active, are capable of long-term survival, and resuscitate under suitable conditions (37, 38). In general, various stress signals induce some bacteria to enter the VBNC state. These signals include low pH, osmolarity, nutrient starvation, and antibiotic exposure (30, 39–42). Similarly, in *H. pylori*, the coccoid form is known to dominate after prolonged culture, under starvation conditions, and after exposure to antimicrobials (32, 37).

The observed differences in biofilm-associated cell morphology may be due to differences in growth conditions employed in different studies. Indeed, growth conditions are important factors that have been shown to influence biofilm formation of other species (43–45). Similarly, variations in the compositions of liquid media appear to have a significant influence on *H. pylori* biofilm formation (20). The fastidious nature of *H. pylori* is well known and was in fact a major hurdle during the initial isolation of the bacterium (46). *H. pylori* is microaerophilic and thrives under conditions that include a carbon dioxide-enriched (5 to 10%) atmosphere, high humidity, and a temperature of 35°C to 37°C (46). In addition, cultures require rich medium that is supplemented with specific factors to promote optimal growth. Common *H. pylori* liquid media begin with a complex base, such as BB, brain heart infusion (BHI) broth, or Ham's F-12 medium, which are then supplemented with serum and additional carbon sources (e.g., fetal bovine serum [FBS] or 2,6-dimethyl- β -cyclodextrin [46, 47]). One study assessed the effects of four types of common liquid culture media on *H. pylori* biofilm formation: BB supplemented with 2% FBS, BHI broth supplemented with 2% FBS, and Ham's F-12 medium supplemented with or without 2% FBS. As expected, significant variation in biofilm production by both *H. pylori* ATCC 43629 and clinical strain 9/10 was observed (20). Interestingly, Ham's F-12 medium without serum optimized biofilm formation despite slowing bacterial growth (20). Mechanistically, this is not fully understood but

may involve enhanced EPS production (20). Future studies will be required to understand the role of various medium types in *H. pylori* biofilm formation.

A similar study found that the presence of FBS appears to negatively impact *H. pylori* biofilm development (48). One mechanism by which this response could occur is via the disruption of surface adherence, which is a crucial step in biofilm formation. Indeed, Williams et al. observed that serum negatively affects *H. pylori* adherence to surfaces (48). That group demonstrated that lower concentrations ($\leq 1\%$) of serum were needed to optimally promote *H. pylori* adherence to abiotic surfaces and to inhibit swimming (48). Similarly, Shao et al. demonstrated that the removal of serum induced bacterial aggregation and, thus, biofilm formation (49). These results are intriguing since serum is commonly used to supplement *H. pylori* liquid media to promote planktonic growth; it is thought to provide essential growth factors and to reduce fatty acid toxicity (50). Overall, even if the use of complex and rich media for *in vitro* biofilm growth does not necessarily reflect the natural environment that *H. pylori* encounters *in vivo*, the optimization of growth media and conditions that promote biofilm formation will be helpful for future studies to help gain a better understanding of *H. pylori* biofilms and their potential role in gastric infection.

An additional difference that cannot be overlooked when comparing data from *H. pylori* studies is the differences in strains being studied. Without a side-by-side comparison of strains in various media, it is difficult to tease out whether differences in the morphologies of *H. pylori* within biofilms are due to medium differences or due to inherent differences between strains. Given the well-documented heterogeneity observed among *H. pylori* isolates (51, 52), it is perhaps not surprising that biofilm formation varies between strains. Indeed, several groups have compared multiple strains and observed differences in their abilities to form biofilms (30, 31, 34). For example, among the clinical strains assessed by Yonezawa et al., an isolate (TK1402) from a Japanese patient with duodenal and gastric ulcers showed a significantly higher level of biofilm biomass than those of the other tested strains (31). However, a correlation between their abilities to form a biofilm and gastric ulcers requires further investigation.

Extragastric Biofilms

Few reports have documented the presence of *H. pylori* in the environment (53–56). The fastidious nature of *H. pylori*, the specificity of the gastric niche, and the tendency of *H. pylori* to transition to the coccoid form when stressed all seem contrary to the long-term survivability of extragastric *H. pylori*. However, given that biofilm formation can occur as a response to environmental stressors (4, 7), perhaps *H. pylori* biofilm formation is part of *ex vivo* survival. Indeed, a majority of the early *H. pylori* biofilm studies focused on the possibility that *H. pylori* forms a biofilm as a mechanism to survive in aquatic reservoirs (57–59). The presence of *H. pylori* in water-associated biofilms has been demonstrated by using PCR (29, 58), peptide DNA probes (60), and fluorescence *in situ* hybridization (FISH) (56, 61); however, despite positive indications using all of these methodologies, the gold standard is still culture. Two groups reported positive *H. pylori* cultures from wastewater (56, 62). Although those two groups did not comment on biofilm formation, a separate study presented *in vitro* evidence that suggests that interactions with specific bacteria (for example, *Mycobacterium chelonae*) within a multispecies biofilm may contribute to *H. pylori* viability (63). Together, these findings offer precedent to support the exploration of the ability of *H. pylori* to survive in multispecies biofilms. Additional proposed extragastric environments for *H. pylori* include dental plaque (reviewed in reference 64) and biofilms associated with vegetables (65–67). The existence of live extragastric *H. pylori* is not definitive; however, the potential involvement of biofilms as a means to foster a more favorable colonization niche is worth further exploration.

Gastric Biofilms

Biofilms are often associated with chronic infectious diseases since they provide an

additional level of protection against environmental, host, and antimicrobial assaults (4, 6, 7, 68, 69). Indeed, the ability of *H. pylori* to form biofilms may contribute to the longevity of this infection. Carron et al. and Coticchia et al. provided the first evidence of biofilm formation by *H. pylori* during colonization of the human gastric mucosa (16, 17). Using biopsy specimens and SEM analyses, they demonstrated the presence of dense layers of what appeared to be biofilm-associated bacteria at the mucosal surface of *H. pylori*-positive patients (16, 17). The SEM images from both studies were consistent with a community of organisms embedded in the EPS (16, 17). *H. pylori*-negative patients, in contrast, had smooth mucosa with little evidence of a bacterial community. Biofilm-like growth was seen in over 97.3% of the analyzed mucosal surfaces in *H. pylori*-positive patients, compared to only 1.64% in *H. pylori*-negative patients (17). Recently, Attaran et al. demonstrated *H. pylori* biofilm formation in a mouse model (36). Through the use of SEM and immunofluorescence with a polyclonal anti-*H. pylori*-specific antibody, those investigators found that clinical isolates colonize the mouse gastric mucosa in a manner consistent with a biofilm. This included the presence of an amorphous extracellular matrix at the mucosal surface (36). SEM revealed a dominant proportion of *H. pylori* biofilm cells to be in the coccoid form, which is consistent with previous observations (17, 35). However, it remains unclear whether the biofilm matrix *in vivo* consists of bacterial self-produced polymeric substances, a host-derived matrix (e.g., collagen, laminin, and fibronectin), or both. Further studies will be needed to determine the contribution of the host extracellular matrix.

In addition to colonizing the surface of epithelial cells and the mucus gel layer, the presence of *H. pylori* within the glands of the murine and human gastrointestinal tracts has been observed (70–72). These glands were suggested to be protective niches that could support the growth of *H. pylori*. At the height of infection, around 50% of glands are occupied, and some of them could contain more than 200 bacteria (70, 72). Interestingly, aggregates of *H. pylori* found in the gastric glands may be consistent with it being in the biofilm growth mode (Fig. 2). Studies to evaluate *H. pylori* biofilm development in the gastric glands and the potential contribution of this growth mode to colonization and persistence are warranted.

MOLECULAR MECHANISMS OF *H. PYLORI* BIOFILM FORMATION

Omic Approaches

In support of the theory that biofilms form by similar mechanisms regardless of the bacterial species, the genes demonstrated to be important for biofilm formation tend to fall into a few categories: adherence, quorum sensing, cell wall synthesis, the stress response, carbohydrate metabolism, and cell division (73). Genomics, transcriptomics, and proteomics are high-throughput strategies that have been implemented to identify factors associated with *H. pylori* biofilms (Table 1). One genomic study compared the sequences of wild-type strain J99 and 31 *H. pylori* clinical isolates that each had a range of biofilm-forming abilities as a means to identify genes enriched in those strains that were high biofilm producers (34). The strains were classified as low, moderate, or high biofilm producers based on crystal violet staining intensity. By studying the genetic differences between strains, those authors were able to identify several genes that appear to be associated with biofilm formation. Indeed, three hypothetical genes (K74_10375, K747_09130, and K747_06625) were significantly correlated with biofilm formation (34). K747_06625 is predicted to contain a homing endonuclease and a ParB-like domain, the latter of which is associated with biofilm formation in some bacteria (74, 75). Additionally, four functional genes, coding for a flagellar protein (*jhp_1117*), an alpha-(1,3)-fucosyltransferase (*fucT*), an outer membrane protein (OMP) (encoded by *homD*), and a cytotoxin-associated gene pathogenicity island (*cag* PAI) protein (CagA), were also associated with biofilm formation in *H. pylori* (34). The role of the *cag* PAI proteins in *H. pylori* biofilms was further investigated by the creation of deletion mutations in *cagA* and the entire *cag* PAI. Both mutations resulted in a significant decrease in biofilm biomass compared to that of the wild type (34).

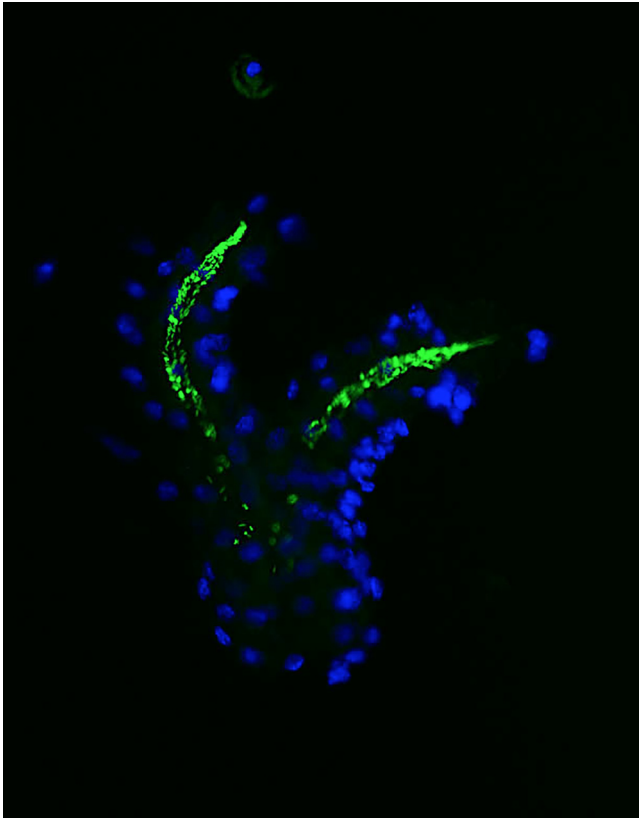


FIG 2 Gastric gland colonization by *H. pylori*. *H. pylori* SS1 was visualized by green fluorescent protein (GFP) expression (green), while gland cells were stained by using DNA Hoechst fluorescent dye (blue).

Other work similarly supported the importance of the *cag* PAI proteins. Using a proteomic approach, Shao et al. observed that two Cag proteins, Cag26/CagA and Cag24/CagD, were highly expressed in *H. pylori* biofilms that had been induced by serum starvation compared to planktonic *H. pylori* cultured under the same conditions (49). Similarly, a study also showed that the creation of isogenic strains lacking CagE altered biofilm formation by *H. pylori*, further supporting the role of the Cag PAI in biofilm formation (30). These results are particularly relevant to pathogenesis, since infections with *cag* PAI-containing strains are often associated with an increased risk for gastric cancer (76).

The proteomics study by Shao et al. described above also revealed 35 other proteins that showed altered expressions between biofilm and planktonic *H. pylori* cultures (49). These proteins are associated with various biological functions, including motility, virulence, signal transduction, and regulation (49). These observations strongly support the facts that biofilm differs from the planktonic mode of growth and that *H. pylori* biofilm cells harbor a distinct proteomic profile. Another group utilized a proteomic analysis to identify biofilm-associated proteins (77). In that study, NapA, a secreted neutrophil-activating exoprotein, was associated with *H. pylori* biofilms (77). NapA expression was significantly upregulated during biofilm formation, and a *napA*-deficient mutant strain produced a reduced biomass and less cell aggregation than did wild-type strains (77).

Targeted Studies of Candidate Genes Associated with Biofilms

In addition to the omics approaches described above, other groups have taken a more targeted approach. Cole et al. evaluated genes known to be associated with biofilm formation in other bacterial species for a potential role in *H. pylori* biofilm development (30). Of the potential biofilm-associated genes, only the loss of *luxS*, a

TABLE 1 Some genes associated with biofilm formation by *H. pylori*

Gene	Gene product	Approach(es)	Reference(s)
K74_10375	Hypothetical gene	Genomic ^a	34
K747_09130	Hypothetical gene		
K747_06625	Hypothetical gene		
<i>fucT</i>	Alpha-(1,3)-fucosyltransferase		
<i>homD</i>	Outer membrane protein		
<i>cagA</i>	Cytotoxin-associated gene pathogenicity island		
<i>cag26-cagA</i>	Cytotoxin-associated gene pathogenicity island	Proteomic ^b	49
<i>cag24-cagD</i>	Cytotoxin-associated gene pathogenicity island		
<i>cagE</i>	Cytotoxin-associated gene pathogenicity island	Mutagenesis	30
<i>napA</i>	Neutrophil-activating protein	Proteomic, ^b mutagenesis	77
<i>luxS</i>	Quorum sensing	Mutagenesis ^d	30, 78
<i>tlpB</i>	Chemoreceptor	Mutagenesis, ^d transcriptomic ^c	78
<i>aibA</i>	Periplasmic binding proteins		
<i>aibB</i>	Periplasmic binding proteins		
<i>arsR</i>	Regulatory response	Mutagenesis, ^d proteomic ^b	23, 49
<i>alpB</i>	Outer membrane protein	Mutagenesis ^d	99

^aWhole-genome sequencing of these 32 clinical strains was performed on the Illumina MiSeq platform.

^bTwo-dimensional gel electrophoresis (2-DE) coupled with the identification of proteins through peptide mass fingerprinting (PMF) by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS).

^cReal-time quantitative reverse transcription–PCR (RT-qPCR).

^dPhenotype confirmed by molecular complementation.

quorum-sensing gene, had a significant effect on biofilm formation (30). The contribution of LuxS is perhaps not surprising given that *H. pylori* has only one known type of quorum-sensing system, autoinducer 2 (AI-2), which is synthesized by LuxS. In comparison, *H. pylori* has multiple ways to sense AI-2. One method is via the chemoreceptor TlpB, which senses AI-2 as a chemorepellent when AI-2 is bound to either the AibA or AibB periplasmic binding protein (78, 79). LuxS-deficient *H. pylori* mutant strains do not produce AI-2 and exhibit increased adherence and biofilm biomass compared to their wild-type counterparts (30, 78). *H. pylori* biofilms from strains lacking AI-2 chemoreception ($\Delta tlpB$, $\Delta aibA$, or $\Delta aibB$) showed enhanced biomass, large microcolonies, cell clustering, and an increased number of attached bacteria (78). Thus, it seems that AI-2 sensing via the chemotaxis system decreases biofilm formation. While this role is in contrast to what is seen in many bacterial species, given that another role of AI-2 sensing is to enhance motility, flagellar gene expression, and morphogenesis (80, 81), perhaps a role in decreased biofilm formation is not surprising.

Regulatory Proteins Associated with *H. pylori* Biofilm Formation

Preliminary investigations into the role of regulatory proteins in *H. pylori* biofilm formation have also been conducted. Recently, the expressions of 16 transcriptional regulators in *H. pylori* 26695 were analyzed under various conditions, including in cells found in biofilms (82). Compared with planktonic cells, biofilm-grown cells showed increased expression levels of all investigated transcriptional factors, with the exception of the heat shock regulator HrcA. Biofilm-induced transcription factors included the response regulator ArsR, two sigma factors (RpoD and FliD), and the metalloregulators Fur, NikR, and CrdR (82). Although the roles that these transcriptional factors play in biofilm formation are largely unknown, the data suggest that a large and complex regulatory network likely controls gene transcription during *H. pylori* biofilm growth. Two regulatory proteins that have been investigated in more detail are the response regulators ArsR (HP0166) and CrdR (HP1365) (23, 30). While the loss of CrdR from 26695 resulted in no difference in biofilm formation (30), mutation of ArsR to a nonphosphorylatable form or deletion of its cognate sensor kinase ArsS in G27 resulted in a dramatic

increase in biofilm formation (23). ArsRS-deficient mutant strains showed increases in cell aggregation and biofilm biomass, which were visible by SEM (23). The ArsRS system is known to regulate OMPs; therefore, one possibility is that the adherence and/or autoaggregation of bacteria is linked to the expression of particular OMPs at the cell surface. These traits in turn control biofilm formation. ArsR was also identified in the proteomic analysis conducted by Shao et al. (49). Numerous candidate genes have now been proposed to play a role in *H. pylori* biofilm formation. However, more extensive studies, including the evaluation of genes in multiple strain backgrounds, are necessary to truly understand how these genes contribute to biofilm formation.

COMPOSITION OF THE BIOFILM MATRIX

As stated in the introduction, biofilms can be defined as adherent aggregates of microorganisms encased in an EPS (83). Several groups have begun to characterize the EPS of the *H. pylori* biofilm. The earliest studies focused on polysaccharide components (18). When *H. pylori* ATCC 43504 (NCTC 11637) was grown in a continuous culture using a glass fermentor, a biofilm formed at the air-liquid interface. The biofilm materials from the wall of the glass culture vessel were dissolved in saturated phenol, and the monosaccharides of both crude and partially purified biofilm material were analyzed by gas-liquid chromatography (18). This method revealed the presence of carbohydrates with a high carbon-to-nitrogen ratio (18). Although the precise composition and structure of the polysaccharide were not identified, several components of *H. pylori* lipopolysaccharide (LPS), such as C₁₄ and C₁₆ lipids, *N*-acetylglucosamine, fucose, glucose, galactose, and glyco-mannoheptose, were identified (18). LPS-like material has been described as a component of the biofilm matrix in other bacteria and suggested to play a role in biofilm formation (84–86).

In a similar study that used nuclear magnetic resonance (NMR), the biofilm matrix of *H. pylori* strain ATCC 43504 was shown to contain mannose-related proteoglycans (proteomannans) and 1,3- or 1,4-mannosyl linkages (77). Proteomannans consist of a beta-1,3/1,6-glucan structure attached to a surface of complex proteoglycans. Interestingly, these same types of molecules also compose the inner layer of the *Candida albicans* cell envelope (87) and have been shown to have immunomodulatory properties (88).

Extracellular DNA (eDNA) has also been found as part of the biofilm matrix of several bacterial species and is thought to contribute to biofilm structure, genetic exchange, and bacterial variability (8, 89–91). Similar to other species, eDNA has been observed as a component of the *H. pylori* biofilm matrix; however, in the *H. pylori* biofilm, the role of eDNA is not fully understood (92). DNase I treatment of a preformed biofilm did not disperse an *H. pylori* biofilm, suggesting that eDNA does not play a role in biofilm architecture or that the eDNA might be protected by other molecules (92). In a follow-up study, it was observed that during biofilm formation by strain ATCC 43629, eDNA was detected in outer membrane vesicles (OMVs) from both biofilm (bOMV) and planktonic (pOMV) fractions (19). OMVs are 20- to 500-nm-diameter bilayer structures that contain phospholipids, proteins, LPS, and DNA (93, 94). OMVs were shown to be part of the biofilm matrix in several Gram-negative bacterial species (95, 96), including that of *H. pylori* (97). Furthermore, the presence of OMVs was observed in biofilms from the strong-biofilm-forming *H. pylori* strain TK1402 but not from weak biofilm producers. This suggests that OMVs might promote *H. pylori* biofilm formation (97, 98). Together, these findings support the hypothesis that eDNA may be protected by OMVs and may avoid DNase I treatment (92). Interestingly, larger amounts of eDNA are associated with bOMVs than with pOMVs (19). However, eDNA was also found at the pOMV membrane surface and the *H. pylori* cell wall, suggesting that *H. pylori* may just generally be covered with eDNA. Free eDNA, as well as that associated with OMVs, could potentially be utilized by *H. pylori* for genetic recombination. Sampling of this DNA by the bacteria could contribute to genetic variability and possibly enhance bacterial survival (89–91). Thus, the precise role of OMVs and eDNA in biofilm formation is still not clear (19).

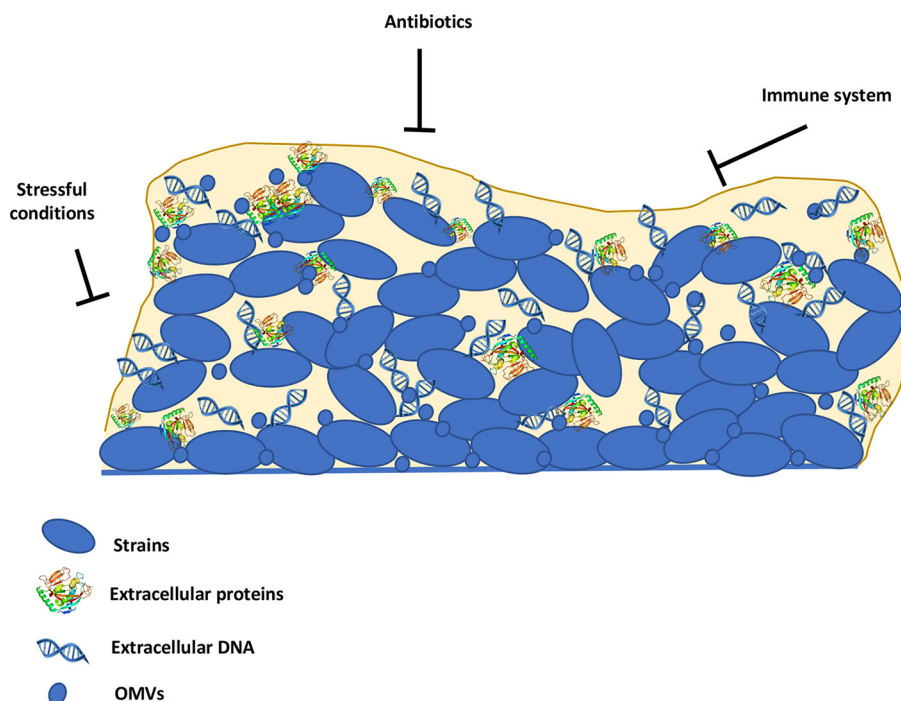


FIG 3 Schematic representation of an *H. pylori* biofilm. Extracellular polymeric substances in *H. pylori*, composed of extracellular DNA, extracellular proteins, and OMVs, may act as a shield to protect the bacterial community from immune cells and antimicrobials.

In addition to eDNA, proteins such as the AlpB OMP have been shown to be associated with OMVs and are thought to contribute to biofilm formation (99). Indeed, a study compared protein profiles of OMVs obtained from a wild-type strong-biofilm-forming strain (*H. pylori* TK1402) to those from isogenic biofilm-defective mutant strains. Those authors observed weaker expression of a 52-kDa protein in the biofilm-defective mutant strains than in the wild-type profile; this protein was identified as AlpB (99). To confirm the role of AlpB in biofilm formation, an *alpB*-deficient strain was further evaluated for biofilm formation. The *alpB* mutant strain exhibited a defect in biofilm formation, suggesting that AlpB may play a role in cell attachment, cell-to-cell aggregation, and, thus, biofilm development (99). It is not currently known, however, whether AlpB needs to be in OMVs to exert this effect.

In summary, current data suggest that the *H. pylori* biofilm matrix contains at least proteomannans, LPS-related structures, eDNA, proteins, and OMVs (Fig. 3). Elucidation of the role of each of these components and their importance in the biofilm matrix will be noteworthy future studies.

IMMUNE EVASION AND TREATMENT FAILURE

Studies of *H. pylori* Biofilms and Antibiotic Resistance

While there are still many unanswered questions regarding *H. pylori* biofilm formation, the implications of biofilm-associated infections are well documented for other species (3, 83, 100). The possibility that *H. pylori* may adopt a biofilm mode of growth during colonization of the gastric mucosa and gastric glands could have a profound impact on antimicrobial treatment. For multiple bacteria, biofilms are known to render infections of multiple bacteria more difficult to eradicate with antimicrobial therapy (3, 8, 83, 100, 101). Bacterial biofilms resist antimicrobial treatments by using a transient and noninheritable mechanism called tolerance, which is related to the physiological state of the biofilm cells and the physical barrier formed by the extracellular matrix (8, 100, 101). In addition to antibiotic tolerance, biofilms often represent a reservoir of genetic diversity by promoting genetic exchange between different subpopulations.

Several studies have documented the increased dissemination of antibiotic resistance genes in biofilms through horizontal gene transfer, integrative conjugative elements, and natural transformation (102–104). Additionally, bacterial cells within biofilms undergo several stressful conditions, including major nutrient starvation, which could increase the mutation frequency and the emergence of antibiotic-resistant mutant strains (8, 69, 105, 106).

Biofilm-associated *H. pylori* was shown to be more resistant *in vitro* to clarithromycin, which is one of the common antibiotics used to treat *H. pylori* infections (107). Specifically, the MIC increased by 16-fold and the minimum bactericidal concentration (MBC) increased by up to 4-fold in biofilm cells compared to planktonic ones (107). *H. pylori* biofilm cells also showed an increased mutation rate, which could promote clarithromycin-resistant mutations (107).

The contribution of *H. pylori* biofilms to the spread of antibiotic resistance has not been fully explored; however, the emergence of resistance to clarithromycin, levofloxacin, and metronidazole urges us to develop new strategies that take into account not only bacterial resistance but also the mode of growth.

While the field of *H. pylori* biofilm research is fairly new, groups have already begun to explore alternative therapeutic approaches that may target and eradicate biofilms. The use of the mucolytic, thiol-containing compound *N*-acetylcysteine (NAC) has shown promise in both humans and mice (21, 108). In early studies that looked at *H. pylori*-infected mice, daily treatment with NAC (120 mg for 14 days) successfully reduced the *H. pylori* load by almost 1 log compared to the nontreated group. Pretreatment with NAC (40 mg/day) significantly reduced the *H. pylori* load but did not completely prevent colonization (108). Furthermore, NAC was also shown to augment the activity of clarithromycin and lansoprazole, a proton pump inhibitor (PPI) dual therapy. *H. pylori*-positive patients that received both dual therapy and NAC showed significantly better eradication of *H. pylori* than did those on dual therapy only (109). The mechanism by which NAC functions in the treatment of *H. pylori* is not clear; however, subsequent studies showed that NAC was effective at dispersing preexistent *in vitro* *H. pylori* biofilms and at preventing their formation (21). NAC is believed to function by disrupting disulfide bonds that cross-link glycoproteins in the mucus. While the exact proteins targeted by NAC are not known, it is possible that NAC targets the proteins of the biofilm matrix and leads to their destruction. In a clinical trial, NAC was demonstrated to similarly disperse *H. pylori* biofilms during *in vivo* infections (21). Indeed, subjects with a history of at least 4 *H. pylori* eradication failures who were treated with NAC before receiving a traditional antibiotic regimen demonstrated better clearance of *H. pylori* (65%) than did the non-NAC group (20%) (21). The exact molecular mechanisms underlying the reported therapeutic effect of NAC are not clear, since this molecule seems to be able to target both *H. pylori* biofilms and the gastric mucus. Further studies are required to decipher NAC's activity against *H. pylori* and its biofilms and to help assess its efficacy for possible future antimicrobial therapies.

Studies of *H. pylori* Biofilms and Immune Evasion

In addition to enhanced defenses against therapeutic interventions, biofilms also defend against the host immune system. Gaddy et al. demonstrated that exposure to subinhibitory concentrations of the host antimicrobial protein calprotectin (CP) resulted in altered lipid A structures, which led to a decrease in surface hydrophobicity and an increase in biofilm formation (22). CP is produced by neutrophils and other myeloid cells (22, 110–113). One function of CP is to sequester essential metals, including manganese and zinc; this sequestration contributes to a process known as nutritional immunity (112). Interestingly, chemical chelation of zinc from *H. pylori* cultures produced biofilm results similar to those seen with the addition of CP. These findings suggest that one mechanism that *H. pylori* may utilize to elude the host response is the alteration of its cell surface and subsequent biofilm formation.

Another mechanism of immune interference associated with *H. pylori* biofilms may be via the proteomannans associated with the matrix. In *C. albicans*, these mannose-

related proteoglycans purportedly display several immunomodulatory roles. These roles include the suppression of B and T lymphocytes (88) and the promotion of mast cell degranulation, a process that releases several mediators (e.g., histamine, serotonin, serine protease, and proteoglycans) that affect the gastrointestinal mucosal barrier (114). Interestingly, the pathogenesis of *H. pylori*-infected gastritis has been associated with mast cell degranulation (115, 116) and regulatory T cells that actively suppress the T cell response (117–119).

Together, these preliminary data suggest that *H. pylori* found within a biofilm may be protected from both antimicrobials and the immune system; however, the role of *H. pylori* biofilms in antimicrobial resistance and clearance by the immune system *in vivo* remains to be elucidated.

CONCLUSION

H. pylori-associated diseases are a scourge for human health and still raise major health concerns worldwide. The increase in antimicrobial resistance, the high infection rates, and the ability of *H. pylori* to subvert the host immune response have made this pathogen one of the most successful human pathogens. Cures against *H. pylori* infection frequently fail, and consequently, multidrug-resistant strains have emerged and become a very serious problem. Evidence suggests that *H. pylori* can form biofilms and that these structures may have a role in persistence, survivability, and/or recalcitrance to antimicrobial treatment within the host or environment. Thus, these structures require further study. However, there are many challenges that researchers must keep in mind to create informative *in vitro* *H. pylori* biofilm models. These challenges include culture conditions, the culture system, and differences between strains. While these challenges may seem daunting, it is important to remember that similar problems have been faced in other biofilm fields, and awareness of these issues will aid in the design of experiments and the interpretation of their results. Hopefully, the recent data that indicate that *H. pylori* bacteria within a biofilm are distinct from planktonic bacteria will inspire future studies and research to demystify the unexplored role of biofilms in *H. pylori* infection and, thus, improve the efficacy of future eradication therapies.

APPENDIX 1

OPEN QUESTIONS

- What conditions or signals drive *H. pylori* to produce a biofilm?
- What are the steps of biofilm formation for *H. pylori*? What genes are required?
- Do biofilms play a role in chronic disease and the persistence of *H. pylori*?
- What role(s) does *H. pylori* biofilms play in the pathogenesis of gastritis, peptic ulcers, and gastric cancer?
- Are biofilms present at any specific points in time or locations during infections?
- If *H. pylori* biofilms form *in vivo*, do they modulate the immune response?
- Does *H. pylori* multiply within a biofilm?
- Do *H. pylori* biofilms play a role in antibiotic therapy failure?
- Are cells in *H. pylori* biofilms dormant or antibiotic tolerant?

APPENDIX 2

H. PYLORI TREATMENTS

Triple therapy for *H. pylori* infection remains the first-line therapy in areas with low clarithromycin resistance (<15%) and consists of a proton pump inhibitor (PPI) (e.g., omeprazole, esomeprazole, or lansoprazole), clarithromycin, and amoxicillin for a duration that ranges from 7 to 14 days (120, 121). However, over the past decade, success rates have fallen to unacceptable levels ($\leq 80\%$) (122). Resistance to clarithromycin has been proposed as the main factor for eradication therapy failure (121, 123).

As a second-line therapy for patients in whom first-line therapies have failed, a bismuth-containing quadruple-therapy regimen is usually recommended (120, 121,

124–126). Bismuth-containing quadruple therapy consists of a PPI, bismuth subsalicylate, metronidazole, and tetracycline for a duration of 10 to 14 days. Bismuth-based regimens offer an effective option as rescue therapy, with cure rates being relatively higher than those with first-line therapies (121, 122, 124). Bismuth is a nontoxic heavy metal with broad-spectrum bactericidal activity (127). Its action against *H. pylori* remains unclear, but like many heavy metals, it may play a role in iron sequestration (127). Similar effects have been observed when *H. pylori* was exposed to either bismuth compounds or iron chelators, which suggests that bismuth may act mainly through an iron deprivation mechanism to inhibit *H. pylori* growth (128). Interestingly, previous reports have demonstrated the efficacy of bismuth against several biofilms (129–131). While the effect of bismuth on *H. pylori* biofilms has not been studied, it is possible that bismuth-containing therapies act through the action of bismuth against biofilms. In such a scenario, bismuth might decrease the *H. pylori* biomass and thus enhance the effect of the antibiotics. The eradication of *H. pylori* is still one of the most difficult challenges in gastroenterology. One route to explore is whether antibiofilm compounds would enhance treatment outcomes.

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