Chapter 19
Antimicrobial Resistance and Drug Efflux Pumps in *Helicobacter*

Jennifer Li and Xian-Zhi Li

Abstract *Helicobacter* spp. play important etiological roles in the pathogenesis of gastroenteric diseases such as in the case of *Helicobacter pylori*. Despite wild-type strains of *H. pylori* being generally susceptible to multiple antimicrobial agents, increasing prevalence of antimicrobial resistance in this species constitutes a key risk factor that affects the effective therapy of *H. pylori* infections. Resistance to anti-*H. pylori* agents is mainly mediated by multiple drug-specific mechanisms. However, drug efflux systems, represented by the Hef pumps of the resistance-nodulation-cell division superfamily, are implicated in both intrinsic and acquired multidrug resistance as well as in bile salt/nitrosative stress response and gastric colonization of these pathogens. This chapter provides an overview of antimicrobial resistance and mechanisms in *Helicobacter* with an emphasis on drug efflux systems.

Keywords *Helicobacter pylori* • Antimicrobial resistance • Efflux • RND pumps • Outer membrane • Stress response • Amoxicillin • Clarithromycin • Metronidazole • Bile salts • HefABC • HefDEF • HefGHI

19.1 Introduction

Helicobacter spp. are Gram-negative bacteria belonging to the *Epsilonproteobacteria* class. The representative species, *Helicobacter pylori*, is believed to infect at least 50 % of the world's human population [1]. Although most individuals with *H. pylori* infection do not experience any clinical complications, these infections are often

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implicated in the development of chronic gastritis, peptic and duodenal ulcers, as well as gastric cancers [1, 2]. Indeed, *H. pylori* has been identified as a carcinogen [3]. *H. pylori*, along with additional non-*H. pylori Helicobacter* species, can be divided into three groups (i.e., gastric, enterohepatic, and unsheathed flagella) based on 16S rRNA sequence similarity [4]. Although many of these species have primary animal hosts, some are also known to be associated with gastroenteric and/or hepatic diseases in humans and include, for example, *Helicobacter hepaticus*, *Helicobacter bilis*, and *Helicobacter cinaedi* [4–8]. Furthermore, the clinical significance of *Helicobacter* spp. in the development of gastrointestinal diseases has been supported by microbiome data generated for gut microbiota [9, 10]. *Helicobacter* species can persist and cause chronic inflammation in human gut, thus contributing to the pathogenesis of various gastroenteric diseases [7, 8, 10, 11]. Antimicrobial therapy is needed for the eradication of these bacterial infections; however, the antimicrobials available for the treatment of these infections are quite limited, and a combination therapy is required to achieve optimal clinical effectiveness. Moreover, increasing prevalence of antimicrobial resistance has been observed in *H. pylori* against agents used in *H. pylori* treatment emerges as a crucial issue when tackling *H. pylori* infections [12]. Drug efflux pumps are one of many mechanisms increasingly recognized to play an important role in the emerging resistance in *H. pylori* and other species. Drug efflux pumps of various known transporter families are inherently encoded in *Helicobacter* genomes. In this chapter, we examine the current status of antimicrobial resistance in this genus with an emphasis on drug efflux pumps.

19.2 Antimicrobial Susceptibility, Therapeutic Options, and Resistance Prevalence

Antimicrobial susceptibility studies of *Helicobacter* spp. have been mostly limited to *H. pylori*, which displays significant *in vitro* susceptibility to a number of antimicrobial agents including β-lactams, macrolides, fluoroquinolones, nitroimidazoles, nitrofurans, and tetracyclines [1, 13, 14]. Wild-type strains of *H. pylori* generally have greater susceptibility to antimicrobial agents compared to *Escherichia coli* and *Pseudomonas aeruginosa*, with certain exceptions such as polymyxins, glycopeptides, nalidixic acid, sulfonamides, trimethoprim, and streptogramins (Table 19.1) [13, 17]. The lowered pH within the gastrointestinal tract, the habitat of *H. pylori*, has a negative impact on antimicrobial activity of β-lactams, macrolides, tetracycline, and fluoroquinolones, with values of minimal inhibitory concentrations (MICs) decreased by 4- to 130-fold with pH changes from 7.2 to 5.5 [13]. Susceptibility data for non-*H. pylori Helicobacter* species are very limited, though several isolates of *H. hepaticus* show significant intrinsic resistance to amoxicillin with MIC values of 8–64 μg/ml (cf. with values of ≤0.5 μg/ml for *H. pylori*) [28].

Despite the high *in vitro* susceptibility of *H. pylori* to numerous agents (Table 19.1), *in vivo* therapy of *H. pylori* infections may not correlate well with expectations based on *in vitro* data [13]. The harsh environment within the stomach

Table 19.1 Antimicrobial susceptibility of wild-type strains of *H. pylori*

Antimicrobial	MIC (μg/ml)	Antimicrobial	MIC (μg/ml)
Amoxicillin	0.008[a], 0.06[b]	Gentamicin	1[a]
Ampicillin	0.015[a], 0.06[c]	Tobramycin	0.25–0.5[i]
Mezlocillin	1[b]	Metronidazole	2[e]
Penicillin	0.03[d], 0.12[c]	Furazolidone	0.06–0.25[i]
Piperacillin	0.125[a]	Nitrofurantoin	1[e]
Aztreonam	4[a]	Linezolid	8[a]
Cefaclor	0.5[a]	Novobiocin	0.1[c], 2[a]
Cephalexin	2[d]	Rifampin	0.25[j]
Cefuroxime	0.5[b]	Rifabutin	0.008[j], <0.015[k]
Cefotaxime	0.02[c], 0.125[a]	Tetracycline	0.03[c], 0.125[a], 0.19[l]
Ceftazidime	0.5[a]	Doxycycline	0.19[l]
Ceftriaxone	0.125a, 0.5b	Minocycline	0.19[l]
Nalidixic acid	32[a]	Tigecycline	0.015[d]
Ciprofloxacin	0.12[c], 0.25[a]	Polymyxin B	5[c]
Gemifloxacin	≤0.006[d]	Polymyxin E	8[c]
Levofloxacin	0.25–0.5[e]	Streptogramin A	4[h]
Moxifloxacin	≤0.25[d]	Streptogramin B	8[h]
Nemonofloxacin	≤0.12[d]	Amixicile	0.5[m]
Clarithromycin	0.008[a], 0.03[g]	Bismuth subcitrate	16[e]
Erythromycin	0.06[e], 0.25[a], 0.5[c]	Ethidium bromide	8[a]
Chloramphenicol	0.5[c], 4[a]	Glutaraldehyde	1–10[n]
Clindamycin	1[a], 32[h]		

The data are derived from: [a][15], [b][16], [c][17], [d][18], [e][13], [f][19], [g][20],[h][21], [i][22], [j][23], [k][24], [l][25],[m][26], and [n][27]

poses a challenge for drug selection among orally administered antimicrobial agents. Indeed, therapy has been limited to certain individual agents of various classes which include amoxicillin, clarithromycin, furazolidone, fluoroquinolones, metronidazole, rifabutin, and tetracycline [13, 29]. Therapeutic regimens require combination therapy or sequential therapy with the abovementioned antimicrobials [13, 29–31]. The recommended first-line therapy for the treatment of *H. pylori* infections consists of a standard triple-drug therapy with any two of three antibiotics (amoxicillin, clarithromycin, and metronidazole) and either a proton pump inhibitor (e.g., esomeprazole) or ranitidine bismuth citrate for a duration of 7–14 days [13]. Proton pump inhibitors and bismuth salts possess anti-*H. pylori* activity at high concentration levels [13]. A second-line therapy consists of a quadruple regimen of tetracycline, metronidazole, a bismuth salt, and a proton pump inhibitor [13, 29–31]. Third-line treatment regimens and other rescue therapies are based on the antimicrobial susceptibility profile of the specific strain in question and may include fluoroquinolones, tetracyclines, rifabutin, and furazolidone [32–35].

Antimicrobial resistance is increasingly being recognized as a risk factor affecting treatment efficacy against *Helicobacter* infections [31, 36–39]. A review from 20 years ago has documented a variable but overall high prevalence of 10–70 %

metronidazole resistance [1]. Antimicrobial treatment failure has been linked to increased prevalence of resistant isolates [40]. In a recent study that tested around 340 isolates (including those from patients with up to three treatment failures), the MIC values of amoxicillin varied from <0.015 to 4 μg/ml, with higher prevalence of amoxicillin resistance in isolates from the treatment failures [41]. The rates of resistance to clarithromycin, a major agent for first-line therapies, have increased from 9 % to 18 % in 1998–2008 in Europe and from 7 % to 28 % in 2000–2006 in Japan (reviewed in reference [42]). A recent report has described the rates of resistance to clarithromycin (18 %), levofloxacin (14 %), and metronidazole (35 %) in Europe, and the increased prevalence of resistance was attributable to the increased use of fluoroquinolones and macrolides in clinic [43]. Similarly, a study conducted in China showed increased rates of resistance to clarithromycin (9 % in 2000 to 21 % in 2009) and levofloxacin (10 % in 2000 to 33 % in 2009) with stable rates of about 40–50 % for resistance to metronidazole within a 10-year period [44]. Yet resistance to amoxicillin, furazolidone, or tetracycline was not detectable and/or rarely occurred [44]. A surveillance of nearly 18,000 isolates that were sampled in China between 2009 and 2012 revealed resistance rates of ca. 21 % for clarithromycin and levofloxacin and 94 % for metronidazole with only 0.1 % for amoxicillin, furazolidone, and gentamicin [45]. Resistance to rifabutin remains generally low with the rates of 1.4 % in Germany and 0.24 % in Japan [24, 46]. A German study identified simultaneous resistance to three or four agents in 15 % of isolates contributing to unsuccessful antimicrobial treatment [47]. Furthermore, a Canadian study has also suggested a general increase in resistance to clarithromycin, ciprofloxacin, levofloxacin, and metronidazole beginning from the early 2000s. Together, these data also suggest variable prevalence of resistance in different regions and countries [31]. Newer agents such as finafloxacin and linezolid have been tested for their activity against *H. pylori*, but their implications for therapy require clinical trials [48, 49]. Lastly, heteroresistance, a circumstance in which subpopulations of isogenic strains develop varying antimicrobial susceptibilities [50], was also observed in isolates from the same patients (even before antimicrobial treatment) [51, 52]. Resistance identification can be hindered by heteroresistance with an undesired consequence of selecting more resistant isolates via antimicrobial therapy [50, 51, 53].

It is also noteworthy that the combinatory use of antimicrobials for treating *H. pylori* infections can have an adverse long-term *in vivo* impact on resistance development and persistence in the gut microbiota. For instance, a short-term clarithromycin-metronidazole combination regimen dramatically reduced the diversity of gut microbiota and resulted in a 1,000-fold increase in the *ermB* gene (encoding the macrolide target-modifying RNA methylase), which then persisted in the gut microbiota for at least 4 years [54]. This observation is consistent with an earlier study showing the persistence of *ermB*-mediated resistant enterococci for 1–3 years following an anti-*H. pylori* treatment regimen [55]. Additionally, by modifing lipid A and biofilm formation, *H. pylori* can adapt *in vivo* to resist the antimicrobial activity of calprotein, which is a component of the host innate immune system and is present during the inflammatory response [56].

19.3 Mechanisms of Antimicrobial Resistance

H. pylori displays intrinsic resistance to multiple-unrelated antimicrobials including glycopeptides and polymyxins (Table 19.1) [13], suggesting that access to drug targets likely contributes to resistance manifestation. Acquired resistance can be further developed. One early study from 1990 showed the *in vitro* selection of resistant mutants by antimicrobials at the levels of 4× or 8× MIC, with spontaneous resistance frequencies in the range of 10^{-8}–10^{-6} for ciprofloxacin, erythromycin, metronidazole, and tobramycin [22]. Another study in 2001 reported the frequencies of the *in vitro* spontaneous mutants resistant to clarithromycin, ciprofloxacin, metronidazole, and rifampin being 3×10^{-9} to 7×10^{-8}, while no mutants were recovered for amoxicillin [57]. Development of increasing resistance in *H. pylori* has prompted the investigation of resistance mechanisms. Table 19.2 lists the identified mechanisms of resistance to the major antimicrobials used in the treatment of *H. pylori* infection. Although antimicrobial target changes are a major form of resistance for *H. pylori*, the role of drug efflux systems should not be underestimated.

19.3.1 Amoxicillin Resistance

Resistance to amoxicillin in *H. pylori* appears to occur less frequently [36, 44, 57]. This phenomenon is attributable to mutations in the genes encoding penicillin-binding proteins (PBPs) [41]. *H. pylori* possess three to four major PBPs [58]. Amoxicillin-resistant mutants show significant reduction in the affinity of PBP1 to amoxicillin [59]. Furthermore, amino acid substitutions were observed in PBP1 of resistant isolates [41]. Although mutations in the *pbp2* gene were also noted with those in the *pbp1*, they did not affect amoxicillin resistance [85]. In addition to mutations in PBP1, other unidentified mechanism(s) are likely also needed for high-level amoxicillin resistance [85]. A cysteine-rich protein named HcpA (encoded by *HP0211*) was earlier suggested to not only be a PBP but also a β-lactamase of *H. pylori* that slowly hydrolyzes penicillin derivatives [86]. However, more recent studies only demonstrated HcpA as a bacterial virulence factor triggering the release of a concerted set of cytokines [87, 88]. No further studies support HcpA as a typical β-lactamase. Indeed, typical β-lactamase activity is not detectable in *H. pylori* [89], although it is well known that PBPs generally may have certain β-lactamase activity. This is consistent with the observation that the *H. pylori* genome does not contain genes encoding typical β-lactamases, whose production constitutes the predominant mechanism of β-lactam resistance in Gram-negative bacteria. However, given that many β-lactamase genes are located on plasmids and that *H. pylori* has a strong natural transformation capability, it is not surprising to see the report of a high-level amoxicillin-resistant isolate (≥256 μg/ml amoxicillin) carrying the bla_{TEM} gene [60]; it remains unclear whether this gene was plasmid borne or chromosome encoded.

Table 19.2 Mechanisms of resistance to antimicrobials used for the treatment of *H. pylori* infection

Drug (class)	Mode of action	Resistance	References
Amoxicillin (β-lactams)	Inhibition of cell wall synthesis by targeting penicillin-binding proteins	Mutations in PBP1 with reduced affinity to amoxicillin; reduced porin production; drug efflux	[16, 58–61]
Clarithromycin (macrolides)	Inhibition of protein synthesis by binding to 23S rRNA	Mutations in genes *rrn*, *infB*, and *rpl22* encoding 23S rRNA, translation intuition factor IF-2, and ribosome protein L22; RND efflux pumps	[15, 17, 20, 57, 62–65]
Furazolidone (nitrofurans)	Inhibition of DNA synthesis by cross-linking to DNA	Mutations in nitroreductase genes *porCDAB* and *oorDABC* (*nfsA* and *nfsB* in *E. coli*)	[66–69]
Levofloxacin (fluoroquinolones)	Inhibition of DNA synthesis by targeting DNA gyrase	Mutations in DNA gyrase genes (*gyrA* and *gyrB*)	[32, 57, 70, 71]
Metronidazole (nitroimidazoles)	Production of superoxide radicals and interaction with DNA	Decreased prodrug reduction due to the mutations in *rdxA*, *frxA*, and *frxB*; reduction of superoxide radicals (due to the mutations in ferric uptake regulator); efflux pump overexpression	[72–75]
Rifabutin (rifamycins)	Inhibition of RNA synthesis by targeting the DNA-dependent RNA polymerase	Mutations in *rpoB* gene	[23, 24, 57, 76, 77]
Tetracycline (tetracyclines)	Inhibition of protein synthesis by preventing aminoacyl-tRNA association to ribosome	Mutations in 16S rRNA *rrnA*/*B* genes; drug efflux pumps	[25, 78–84]

Interestingly, two studies have revealed that amoxicillin-resistant/multidrug-resistant mutants accumulate less penicillin, chloramphenicol, and/or tetracycline than susceptible strains [59, 89]. Yet, the accumulation of penicillin and tetracycline by resistant strains was not affected by the ionophore proton conductor, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) [59, 89]). Thus, a reduced accumulation of drugs was explained by investigators as due to reduced uptake and not active efflux. However, the involvement of drug efflux systems needs to be carefully assessed before a solid conclusion is made regarding additional mechanisms of amoxicillin resistance. The outer membrane permeability barrier alone cannot sufficiently explain

drug accumulation differences in the steady state (e.g., within 30–60 min of accumulation). These drug molecules are expected to cross the outer membrane barrier in less than a second [90]. Based on various drug accumulation assays, the steady state of drug levels in intact cells should generally be reached within 30 min [59, 89, 90]. Indeed, the contribution from porin alterations and efflux pumps to amoxicillin resistance in *Helicobacter* spp. has been observed [16, 28] as discussed in the next section. This finding explains a phenotypic relationship between high β-lactam resistance and low- to moderate-level multidrug resistance [89].

19.3.2 Clarithromycin Resistance

Macrolides inhibit bacterial protein synthesis by targeting 23S rRNA. Mutations in the 23S rRNA genes reduce the binding of macrolides to the 23S rRNA [62–64, 91] and are the major mechanism of resistance to macrolides (and particularly clarithromycin for *H. pylori*) [42]. Major mutations include A2142G and A2143G transitions and an A2142C transversion [62, 92] with additional mutations in the 23S rRNA genes reported in the literature [19, 36]. Mutations within other genes, including those in *infB* (encoding translation initiation factor IF-2) and *rpl22* (ribosomal protein L22), were also found to cooperate with 23S rRNA gene mutations in raising resistance level [20]. Lastly, macrolides are often the substrates of multidrug resistance or macrolide-specific efflux pumps in various bacteria [93, 94], and indeed efflux pumps also mediate resistance to clarithromycin, as described in the next section.

19.3.3 Metronidazole Resistance

As a nitroimidazole agent, metronidazole requires reduction by oxygen-insensitive NADPH nitroreductase (RdxA), NADPH-flavin oxidoreductase (FrxA), and ferredoxin-like enzymes (FrxB) to be activated from its prodrug form. Thus, mutations in relevant encoding genes (*rdxA*, *frxA*, and *frxB*) are responsible for metronidazole resistance [66, 85, 91, 95, 96]. Annotated mutations in *rdxA* consist of frame shift mutations, missense mutations, deletions, and insertions [36, 72]. The *rdxA* mutations are also better correlated to clinically relevant resistance (metronidazole MIC >8 μg/ml) than those in *frxA* [73]. Mutations in *frxA* alone may not be sufficient in generating metronidazole resistance [53, 96]. In addition to confirming the role of *rdxA* and *frxA* mutations, a recent study also identified, via whole genome sequencing and natural transformation approaches, mutations in another gene, *rpsU* [97]. The *rpsU* gene encodes ribosomal protein S21; mutations *rpsU* alone do not produce sufficient resistance levels but instead cooperate with *rdxA* mutations to achieve high resistance [97]. Inhibition of superoxide dismutase production in strains with mutations in the ferric uptake regulator is also known to be involved in metronidazole resistance [74]. The contribution of efflux mechanism to metronidazole

resistance is discussed in the next section. Interestingly, the loss of metronidazole resistance occurs under low oxygen conditions (that mimic *in vivo* microaerophilic situation) or in the presence of chloramphenicol [98], suggesting multiple factors affecting metronidazole susceptibility.

19.3.4 Fluoroquinolone Resistance

Fluoroquinolones act on DNA gyrase (A_2B_2 complex) and topoisomerase IV. However, *H. pylori* strains lack the gene encoding topoisomerase IV [42]. Thus, resistance occurs mainly as a result of mutations in the quinolone resistance determining region of the gyrase A gene (e.g., Asn87Lys; Asn87Tyr; Asp91Gly, Asp91Asn, or Asp91Tyr) [42, 70, 91]. Mutations in the gyrase B gene were have also been noted [70]. Furthermore, although fluoroquinolone resistance frequently occurs as a result of efflux pump overproduction in Gram-negative bacteria [94], no efflux pumps affecting quinolone susceptibility have been identified in *H. pylori* to date.

19.3.5 Furazolidone Resistance

The broad-spectrum furazolidone inhibits DNA biosynthesis by crossing-linking to DNA molecules [67]. Mechanisms for furazolidone resistance in *Helicobacter* spp. are not well understood. However, resistance to nitrofurans in *E. coli* has primarily been linked to mutations in genes encoding nitroreductases such as *nfsA* and *nfsB* [99]. A recent study further demonstrated involvement of these mutations in furazolidone-resistant *E. coli* [68]. Pyruvate/flavodoxin oxidoreductase (PorCDAB) and 2-oxoglutarate oxidoreductase (OorDABC) act as nitrofuran nitroreductases in *H. pylori* [66], and mutations in the *porD* and *oorD* genes have been noted in all furazolidone-resistant (>2 μg/ml furazolidone) clinical isolates of *H. pylori* that were obtained from patients previously treated with metronidazole [69].

19.3.6 Rifabutin Resistance

Rifabutin acts on the β-subunit of the DNA-dependent RNA polymerase encoded by the *rpoB* gene. Amino acid substitutions resulting from point mutations in *rpoB* confer high-level resistance to rifampicin and rifabutin (with >128-fold MIC increases) [23, 24]. These resistance levels are dependent on the amino acid substitutions with four distinct regions identified in *rpoB* [76]. Similar to many other species, rifamycin resistance in *H. pylori* occurs more frequently than resistance to other agents [57]. The history of rifamycin use has been linked to the emergence of rifabutin-resistant isolates including those from cases with treatment failure [24, 46, 77].

19.3.7 Tetracycline Resistance

Tetracyclines inhibit protein synthesis by binding to the 30S subunit of the ribosome and preventing association between aminoacyl-tRNAs with the ribosome [78, 79]. Mutations in the 16S rRNA *rrnA*/*rrnB* genes reduce drug binding to the ribosome [100] and yield high-level tetracycline resistance (>40-fold MIC increases for tetracycline, doxycycline, and minocycline [25, 80, 81]). However, other types of tetracycline-resistant isolates were found to lack any mutations in the 16S rRNA genes and instead rely on the altered uptake or efflux [82, 83]. One study has shown proton motive force-dependent efflux of tetracycline in clinical isolates without identifying specific pump(s) [84]. The requirement for multiple mutations in the development of tetracycline resistance may explain its low prevalence in clinical resistance [101, 102]. The involvement of efflux pumps (HP1165) in tetracycline resistance [83] will be discussed in the next section.

19.3.8 Molecular Methods for Resistance Detection

Molecular methods have been developed to detect resistance caused by the specific gene mutations, and have been facilitated by advances in technology such as whole genome sequencing [92]. Commercially available molecular methods for detection of antimicrobial resistance in *H. pylori* also exist as reviewed in the reference [42]. The GenoType HelicoDR test is able to identify point mutations in the *rrn* and *gyrA* genes that are linked to clarithromycin and levofloxacin resistance, respectively [103], but this application has limited in its sensitivity and specificity, apparently making it infeasible for clinical applications [104]. Overall, genetic molecular methods are only applied to known resistance mechanisms for certain genes, as mutations can also be independent of the resistance phenotype [13]. Molecular approaches for examining genetic mutations alone will not be sufficient in characterizing resistance attributable to efflux mechanisms, particularly because multiple regulatory genes can impact efflux gene expression and ultimately the resistance phenotype.

19.4 Outer Membrane Permeability Barrier and Drug Efflux Systems

In Gram-negative bacteria, the outer membrane permeability barrier and drug exporters affect the influx and efflux of antimicrobial agents, respectively, and thus play a role in determining the susceptibility phenotype [94]. A large number of outer membrane and efflux proteins are encoded in the *Helicobacter* genomes [105–107], although the sizes of several known *Helicobacter* genomes are relatively small (only about 1.7–2.0 Mbp) [105, 108–110]. For example, at least 32 outer membrane proteins

[111] and 27 proven or putative drug transporters [112] have been identified in *H. pylori*. These transporters belong to one of the following superfamilies or families [94]: (i) resistance-nodulation-cell division (RND) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the multidrug and toxic compound extrusion (MATE) family, (iv) the small multidrug resistance (SMR) family, and (v) the ATP-binding cassette (ABC) superfamily (Table 19.3).

19.4.1 Outer Membrane Permeability Barrier

The outer membrane consists of a lipopolysaccharide-containing lipid bilayer with water-filled porins and serves as an effective barrier in limiting the influx of antimicrobial molecules [117]. Small hydrophilic agents, such as amoxicillin, cross the outer membrane via the porin channels, while large or hydrophobic agents require penetration of the outer membrane lipid bilayer [94]. Many outer membrane proteins of *H. pylori* have been studied for their role in infection pathogenesis [107, 118, 119], with five proteins (HopA to E) investigated for their channel-forming activity [120, 121]. The HopA to HopD porins form similar pores with relatively small channel size [120], while the less abundant HopE protein forms a larger nonspecific channel *in vitro* [121]. The presence of these porins explains the high susceptibility of *H. pylori* to small hydrophilic antimicrobials such as amoxicillin, which is expected to enter the periplasm through the porin channels. Indeed, mutations in HopB and HopC proteins render cells less susceptible to β-lactams (two- to eightfold reductions in the MIC values) and cooperate with PBP1 mutations to raise levels of β-lactam resistance (16- to 64-fold amoxicillin MIC reductions) [16]. Alterations in outer membrane protein profiles were observed in high-level amoxicillin-resistant isolates [89]. The outer membrane permeability of *H. pylori* to the small hydrophobic agent, 1-*N*-phenylnaphthylamine, was found to be higher than that of *E. coli* [17], an observation consistent with the low MIC values of many hydrophobic agents (Table 19.1). An increased susceptibility to metronidazole occurred in the presence of aspirin, which enhanced intracellular concentrations of tetracycline, but no significant changes in the transcriptional expression of the genes encoding the HopA, HopB, HopC, HopD, and HopE porins and HefABC efflux system were observed [122]. Hypersusceptibility to several hydrophobic agents (e.g., erythromycin, novobiocin, and rifampicin) was reported for mutants carrying null mutations in the *ostA* (also called *imp*) and/or *msbA* genes [27], which encode an organic solvent tolerance outer membrane protein and a lipopolysaccharide lipid precursor exporter, respectively – both of which are involved in the biogenesis of lipopolysaccharide [107, 123].

19.4.2 RND Pumps

Multiple putative RND pumps have been identified, based on protein homology, in several *Helicobacter* spp. (as presented in Table 19.3). The total numbers are fewer than those found in *E. coli* (which contains six RND pumps) or *P. aeruginosa* (>12 RND

Table 19.3 Confirmed and putative drug efflux transporters in *Helicobacter* spp.

Species/ transporter family	Transporter	Membrane fusion protein	Outer membrane protein	Affected drug susceptibility and functions	References
H. pylori 26695					
RND	HefC (HP0607)	HefB (HP0606)	HefA (HP0605)	Amoxicillin, aztreonam, cefotaxime, ceftriaxone, ceragenins, clindamycin, deoxycholate, erythromycin, ethidium bromide, novobiocin, penicillin, piperacillin, and tetracycline; stress response to bile salts	[15, 17, 61, 105, 112]
RND	HefF (CznA; HP0969)	HefE (CznB; HP0970)	HefD (CznC; HP0971)	Metronidazole, cadmium, nickel, and zinc; urease activity modulation; gastric colonization	[17, 105, 112, 113]
RND	HefI (CzcA; HP1329)	HefH (CzcB; HP1328)	HefG (CrdB; HP1327)	Copper; potentially in nitrosative response	[17, 105, 112–114]
RND	HP1487	HP1488	HP1489	Ethidium bromide	[112]
MATE	HP1184			Ethidium bromide	[112]
MATE	HP0759				[105]
MFS	HP1165			Tetracyclines	[83]
MFS	HP1181				[105, 115]
ABC	CadA (HP0791)			Cadmium, zinc	[114, 116]
ABC	CopA (HP1072)			Copper	[114]
ABC	CopA2 (HP1503)			Metal	[114]
ABC	MsbA (HP1082)			Erythromycin, ethidium bromide, glutaraldehyde, novobiocin, and rifampin; lipopolysaccharide flippase	[27, 105]
H. hepaticus ATCC51449					
RND	HH0174	HP0175			[28, 108]

(continued)

Table 19.3 (continued)

Species/ transporter family	Transporter	Membrane fusion protein	Outer membrane protein	Affected drug susceptibility and functions	References
RND	HH0222 (HefC)	HH0223 (HefB)	HH0224 (HefA)	Amoxicillin, cholic acid, deoxycholic acid, ethidium bromide, ofloxacin, and rifampin; stress response to bile salts	[28, 108]
RND	HH0625 (HefF)	HN0624 (HefE)	HH0623 (HefD)		[28, 108]
RND	HH1859				[108]
MFS	HH1614				[108]
MATE	HH0031				[108]
MATE	HH0167				[108]
SMR	HH0508-0509				[108]
SMR	HH1451-1452				[108]
ABC	HH1857-1858				[108]
H. cinaedi PAGU611					
RND	HCN_0595	HCN_0594	HCN_0593		[106, 110]
RND	HCN_1563	HCN_1564			[106, 110]
MATE	HCN_0708				[106, 110]
MATE	HCN_0807				[106, 110]
MFS	HCN_0741				[106, 110]
SMR	HCN_1599-1600				[106, 110]
SMR	HCN_2016-2017				[106, 110]
ABC	HCN_0962	HCN_0964	HCN_0965		[106, 110]

pumps) [94]; these differences are likely due to the relatively small genome sizes of *Helicobacter* spp. The four RND systems of *H. pylori* are each encoded by a putative three-gene operon [17], which produces the typical three components of RND tripartite efflux complexes; these components include an efflux transporter located in the cytoplasmic membrane, an accessory membrane fusion protein, and an outer membrane channel protein [94]. For two non-*H. pylori* species, putative RND pumps are instead each encoded by a two-gene operon [106] and likely requires an outer membrane channel protein encoded elsewhere in the genome for proper functioning (Table 19.3). Interestingly, unlike in *E. coli* or *P. aeruginosa* [94], no regulatory genes have been identified adjacent to the structural genes of these RND systems. One exception is HefGHI (also known as CrdB-CzcB-CzcA), where the encoded HP1326 (CrdA) is required for induction of HefGHI by copper [114]. CrdA expression is further controlled by a two-component regulatory system CrdRS (HP1364-1365) [124]. A recent study has demonstrated the importance of CrdRS in

nitrosative response of *H. pylori* and its influence on the transcriptional expression of about 100 genes (including the upregulation of *crdA*) [125]. Overall, regulation of *Helicobacter* RND pump expression remains a mystery.

Phylogenetic analysis of the RND pumps of *H. pylori* suggest that HefC is closer to the RND pumps involved in drug efflux while HefF and HefC are related to RND pumps involved in the extrusion of divalent cations [17]. Further studies have been conducted to analyze their expression and functional roles [17, 113]. Despite their expression in wild-type cells, an early study used a genetic inactivation approach to suggest only a minimal role of HefABC, HefDEF, and HefGHI in the antimicrobial resistance in *H. pylori* [17]. Indeed, pretreatment of *H. pylori* cells with CCCP did not result in increased accumulation of either chloramphenicol or tetracycline (on the contrary, reduced drug accumulation was observed), arguing against involvement of a proton motive force-dependent drug efflux pump in intrinsic resistance to chloramphenicol or tetracycline [17].

Two other studies indicate a strong contribution of the HefABC efflux system to intrinsic and acquired multidrug resistance [15, 126]. The expression of *hefABC* in one study was generally the strongest among the four RND systems in clarithromycin-resistant (≥1.0 μg/ml clarithromycin) isolates [65]. Inactivation of the HefC pump gene in a wild-type strain rendered the mutant hypersusceptible to β-lactams (aztreonam, cefotaxime, ceftriaxone, penicillin, and piperacillin but not amoxicillin), clindamycin, erythromycin, ethidium bromide, novobiocin, and tetracycline with four- to 330-fold MIC reduction (Table 19.4) [15]. These antimicrobials are known substrates for RND pumps. Furthermore, CCCP treatment of wild-type cells increased the accumulation of ethidium bromide [15]. Chloramphenicol accumulation was increased slightly in CCCP-treated resistant cells [89]. However, susceptibility to quinolones was not affected by genetic disruption of the *hefA* or *hefC* gene [15, 126]. In another study, disruption of *hefA* (but not *hefD*, *hefG*, or *HP1489*) made the cells more susceptible to deoxycholate and novobiocin and the simultaneous inactivation of *hefA* and *hefD* sensitized cells to metronidazole [112]. Together, these results support that the HefABC pump plays an important role in the intrinsic drug resistance of *H. pylori*. This conclusion is further supported by the involvement of HefABC (not HefDEF or HefGHI) in resistance to bile salts and their derivatives, ceragenins [127]. Elevated *hefA* expression was noted in multidrug-resistant chloramphenicol-selected mutants [126] as well as in multidrug-resistant isolates from another study [128].

The contribution of HefC pumps to amoxicillin resistance was noted in certain resistant isolates, and the combination of 1-(1-naphthylmethyl)-piperazine (NMP; an RND pump inhibitor) at 100 μg/ml reduced the amoxicillin MIC by 16-fold in HefC overproducers [61]. In this case, given the overall hydrophilic nature of amoxicillin, one would expect that the efflux process alone may have a limited role in amoxicillin resistance, but this process may still be possible if the influx of amoxicillin is also affected by reduced porin expression (as already reported in the reference [16]; see above in the outer membrane permeability barrier section). Thus, it would be ideal to assess the porins for the isolates of this study [61]. Mutations in multiple genes were analyzed in this study [61], and surprisingly mutations in HefC

Table 19.4 Effect of the *hefABC* inactivation on antimicrobial susceptibility of *H. pylori*

Antimicrobial	Parental strain (MIC in μg/ml)	Δ*hefABC* (MIC in μg/ml)	MIC ratio of parental strain to Δ*hefABC* (fold)
Cefotaxime	0.125	0.015	8
Ceftriaxone	0.125	0.008	16
Penicillin	0.002	0.00006	330
Piperacillin	0.125	0.0008	16
Clarithromycin	0.008	0.002	4
Erythromycin	0.25	0.015	16
Chloramphenicol	4	2	2
Clindamycin	1	0.125	8
Novobiocin	2	0.03	6
Tetracycline	0.125	0.015	8
Ethidium bromide	8	0.5	16
Cefotaxime	Not reported	Not reported	32[a]
Clarithromycin	Not reported	Not reported	8[a]
Chloramphenicol	Not reported	Not reported	16[a]
Gentamicin	Not reported	Not reported	8[a]

Data are from Kutschke and de Jonge [15] (where the tested mutant had **Δ***hefC*) except otherwise noted

[a]Data are from Liu et al. [126] (where the tested mutant had **Δ***hefA*)

(Asp131Glu and Leu378Phe) were identified in several resistant strains; these mutations appeared to yield a gain of function. In another paper, the inclusion of RND pump inhibitor phenylalanine-arginine β-naphthylamide (PAβN) reduced clarithromycin MIC values by four to eightfolds (from 4–32 to 1–8 μg/ml) for 15 clinical clarithromycin-resistant isolates [65] Lastly, HefABC overproduction was revealed to be the first step in the development of acquired resistance [72] to metronidazole. All of these results jointly suggest that HefABC plays a major role in acquired multidrug resistance.

Although the regulation of HefABC expression remains unknown, this efflux system is clearly inducible. The exposure of five clinical isolates to metronidazole at 8 or 16 μg/ml revealed a concentration-dependent increase in *hefA* expression, even though *hefA* was already constitutively expressed in these isolates [129]. The presence of cholesterol also induces *hefABC* expression and thus contributes to resistance to bile salts [127]. It was also found that the expression of all four RND systems was elevated in biofilm cells in comparison with that of planktonic cells [130].

Another RND pump of *H. pylori*, CznABC (i.e., HefDEF), is a metal efflux pump involved in cadmium, nickel, and zinc resistance. Only minimal growth occurred for pump-deficient mutants in the presence of cadmium (10 μM), nickel (1.2 mM), and zinc (0.8 mM) [113]. Furthermore, CznABC is also critical for gastric colonization and modulation of urease activity [113], providing a possible physiological role of RND pumps in *H. pylori*. (Urease activity plays an essential role in acid tolerance of *H. pylori* [131].) The third RND pump, HefGHI, confers resistance

to copper, and its inactivation renders cells more susceptible to copper – with only minimal growth in the presence of 0.1 mM copper [114]. Based on the CrdRS system's involvement in regulation of *crdA* (located immediately upstream of the *hefGHI* genes in the same transcriptional direction) [125], CrdRS could also influence *hefGHI* expression, and thus HefGHI may possibly contribute to nitrosative stress response. Indeed, the role of RND pumps in nitrosative stress response has been demonstrated in *E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* [132–134]. Additionally, in contrast to the HefC pump, both HefF and HefH are not involved in cholesterol-dependent resistance to bile salts [127]. Inactivation of either the HefF or HefI pump did not alter the drug susceptibility of cells (with 20 tested agents) [15].

Several RND systems exist in *H. hepaticus* (Table 19.3). Intriguingly, *H. hepaticus* strains appear to be much less susceptible to amoxicillin than *H. pylori*, and they do not have PBP alterations nor produce β-lactamases [28]. Inactivation of *hefA* rendered a mutant strain hypersusceptible to amoxicillin (256-fold MIC reduction but not to another tested β-lactam tested, cefotaxime), rifampicin (ninefold MIC reduction), ofloxacin (fourfold MIC reduction), ethidium bromide (>fourfold MIC reduction), and bile salts (2.5- to 10-fold MIC decreases) [28]. Thus, HefABC likely contributes to intrinsic resistance in *H. hepaticus* to multiple agents including amoxicillin. Moreover, the expression of *hefA* is inducible by bile salts (but not by amoxicillin) [28]. This fact suggests that HefABC may be involved in the survival of *H. hepaticus* in the gastrointestinal tract where it would be exposed to high bile salt concentrations, similar to the role of the *E. coli* AcrAB-TolC system and the *Campylobacter jejuni* CmeABC system [135, 136].

19.4.3 Non-RND Pumps

This group includes ABC, MFS, MATE, and SMR pumps that remain to be characterized (Table 19.3) [105, 112, 115]. Inactivation of the ABC-type MsbA transporter rendered the mutant strain more susceptible to several agents including erythromycin and glutaraldehyde. The impact of CCCP treatment on the accumulation of ethidium bromide supported an efflux process contributed by MsbA [27]. This pump also cooperates synergistically with another lipopolysaccharide biogenesis protein OstA to enhance hydrophobic drug resistance [27]. However, since MsbA is a lipopolysaccharide flippase [107], mutants with MsbA deficiency (and/or with OstA defect) have a reduced lipopolysaccharide production [27]. A few ABC transporters such as CadA and CopA are involved in heavy metal resistance [114, 116].

The HP1165 protein is a homolog of the TetA(P) efflux pump of *Clostridium perfringens* belonging to the MFS family [83]. Its gene is constitutively expressed in any growth phase of a wild-type tetracycline-susceptible strain and its inactivation renders the mutant strain more susceptible to tetracycline (tenfold MIC reduction). While the overproduction of HP1165 is has been linked to tetracycline

resistance following tetracycline exposure, its absence abolishes the ability of tetracycline to induce tetracycline resistance [83]. Additionally, the HP1181 protein is another putative MFS exporter, a homolog of the NorA pump of *Staphylococcus aureus*, but its functional properties remain to be characterized [115].

19.4.4 Effect of Efflux Pump Inhibitors and Methodological Considerations

As described above, many studies have employed efflux pump inhibitors in characterizing drug efflux contribution to resistance in *Helicobacter*. PAβN and NMP are known inhibitors of RND pumps [94]. We have been unable to find data on the activities of these two inhibitors alone against *Helicobacter* spp. (such as MIC values), but these values can be informative in assessing the effect of these agents themselves on *Helicobacter* [94]. In one study [65], PAβN was used at 10, 20, 40, 60, and 120 μg/ml, and it appeared that this agent alone at levels of up to 40 μg/ml did not affect the growth of a particular *H. pylori* strain [65]. Thus, the observations with effect of 40 μg/ml PAβN on the reduction of the MIC values of clarithromycin [65] and metronidazole [72] are interpreted as the involvement of an efflux mechanism. Similarly, NMP can be used at 100 μg/ml without detectable adverse impact on *Helicobacter*, and thus the effect of NMP on HefC is also considered to be related to efflux inhibition [61]. In this regard, it is worth mentioning that the inclusion of a plant extract (baicalin, berberine, emodin, or schizandrin) enhanced antibacterial activity of amoxicillin and tetracycline [128].

Multiple studies have used the proton conductor CCCP, which abolishes proton motive force across the cytoplasmic membrane and therefore is not an efflux pump inhibitor per se. In two independent studies, CCCP at 40 and 100 μM reduced (instead of increased) the accumulation of chloramphenicol and tetracycline [17, 122], suggesting an impact on uptake processes [17]. However, two other studies showed an increase in accumulation of ethidium bromide or chloramphenicol in the presence of 10 or 100 μM CCCP [27, 89]. No impact of 40 or 100 μM CCCP on penicillin and tetracycline resistance was also reported [61, 89]. CCCP at 100 μM produced more effects in drug-CCCP combination susceptibility testing on chloramphenicol-selected multidrug-resistant isolates than the parental strains for multiple drugs [137]. There are also studies that used CCCP at a high level of 200 μM, which increased accumulation of ethidium bromide and tetracycline [84, 138]. Given the apparently inconsistent results on the effect of CCCP on drug accumulation in *H. pylori*, additional investigations are needed to carefully reassess the use of CCCP including its appropriate concentrations.

H. pylori infection also requires the treatment with the proton pump inhibitor acid-inhibitory drugs as part of a drug combination regimen. Proton pump inhibitors themselves exhibit anti-*H. pylori* activities at the levels which are not achievable *in vivo* [13]. Interestingly, studies have examined the effect of proton pump inhibitors on the multidrug resistance phenotype of either bacterial isolates or

mammalian tumor cells [139–141]. Specific to *H. pylori*, esomeprazole, lansoprazole, omeprazole, pantoprazole, and rabeprazole (each at 10 μg/ml) were found to exhibit certain reduction of MICs of amoxicillin and metronidazole (particularly with pantoprazole and rabeprazole) [137]. Yet, clinical relevance of this observation remains unknown.

19.5 Concluding Remarks

The treatment of *Helicobacter* infections is adversely affected by the increasing emergence of acquired resistance in *H. pylori*. Even though drug-specific mechanisms are predominantly responsible for the clinically relevant resistance phenotypes that compromise *H. pylori* infection therapy effectiveness, the contribution of drug efflux systems (particularly the RND-type pumps) to intrinsic and acquired multidrug resistance in *Helicobacter* spp. is being recognized. Furthermore, as already observed in other bacteria of the same class as *Helicobacter* spp., these efflux systems likely also function beyond drug resistance and are involved in its pathogenesis. However, the data regarding the role of the HefABC pump and its substrate profile vary within the literature. These discrepancies may be due to methodological challenges in conducting antimicrobial susceptibility testing with *H. pylori* as well as the high susceptibility of the wild-type *H. pylori* to many agents *in vitro*. A better understanding of *Helicobacter* drug efflux pumps should be pursued to characterize better strategies for therapeutic interventions. Agents that inhibit the efflux pumps could serve as antimicrobial adjuvants to improve the activities of the existing anti-*Helicobacter* drugs. Furthermore, an open-ended question remains on how expression of these efflux systems is regulated. Physiological roles of the *Helicobacter* drug efflux systems are also likely linked to stress response or colonization [113, 125, 127]. The current knowledge clearly warrants further investigations of *Helicobacter* drug efflux pumps and, in particular, their regulation and functional roles.

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