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Structural Aspects of *Helicobacter pylori* Antibiotic Resistance

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Abstract

Resistance to antibiotics of *Helicobacter pylori* infections is growing rapidly together with the need for more potent antimicrobials or novel strategies to recover the efficacy of the existing ones. Despite the main mechanisms according to which *H. pylori* acquires resistance are common to other microbial infections affecting humans, *H. pylori* has its own peculiarities, mostly due to the unique conditions experienced by the bacterium in the gastric niche. Possibly the most used of the antibiotics for *H. pylori* are those molecules that bind to the ribosome or to the DNA and RNA machinery, and in doing so they interfere with protein synthesis. Another important class is represented by molecules that binds to some enzyme essential for the bacterium survival, as in the case of enzymes involved in the bacterial wall biosynthesis. The mechanism used by the bacterium to fight antibiotics can be grouped in three classes: (i) mutations of some key residues in the protein that binds the inhibitor, (ii) regulation of the efflux systems or of the membrane permeability in order to reduce the uptake of the antibiotic, and (iii) other more complex indirect effects. Interestingly, the production of enzymes that degrade the antibiotics (as in the case of β-lactamases in many other bacteria) has not been clearly detected in *H. pylori*. The structural aspects of resistance players have not been object of extensive studies yet and the structure of very few *H. pylori* proteins involved in the resistance mechanisms are determined till now. Models of the proteins that play key roles in reducing antimicrobials susceptibility and their implications will be discussed in this chapter.

Keywords

Bacterial resistance · Antibiotics · Resistance mechanism · Mixed resistance · Efflux pump

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1 Introduction

Like *Staphylococcus aureus*, *Campylobacter* spp., *Enterococcus faecium* and few other antibiotic resistant bacteria, *H. pylori* has been categorized as a high-priority target that pose the greatest threat to human health by WHO (http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/). Resistance to feasible antibiotics is growing rapidly worldwide, making *H. pylori* infections more difficult to cure (Alba et al. 2017). The classical therapy used by most physicians consists of a

cocktail of two antibiotics, mostly chlaritromycin (CLR) and metronidazole (MTZ) or amoxicillin (AMX), and a proton pump inhibitor (PPI) or, in the case of eradication failure, of a quadruple therapy, either bismuth including or not (Fallone et al. 2016). The combination of multiple antibiotics is an empirical approach deriving from the assumption that resistance does not frequently affect two antibiotics of different classes at the same time, thus avoiding the usage of antimicrobials susceptibility tests that require 10 days or more to give a response. However, growing resistance incidence makes these approaches no longer capable of achieving high cure rates (Zhang 2015).

More details on the different protocols and novel strategies currently in use are discussed in a separate book chapter (See Chap. 12). While describing the main source of failure in the treatment, often due to bacterial resistance and therapeutic inefficacy, one should keep in mind that infection reoccurrence can also be the result of reinfections, especially in populations of developing countries where inappropriate sanitary conditions could play a significant role in this sense. At the same time, resistance spreading per se is sustained by inadequate hygiene and food handling and consequent recursive reinfections (Hu et al. 2017).

The main mechanisms according to which *H. pylori* acquire antibiotics resistances are common to other bacterial infections affecting humans and can be classified into the following main categories: mutations that impair the capability of antibiotics to interfere with ribosomal activity and protein synthesis; mutations that affect DNA replication, recombination, and transcription; mutations that alter the proper redox-state of bacterial cells altering the activity of oxidoreductases and mutations that modify penicillin binding proteins, involved in peptidoglycan biosynthesis and typical target of β-lactams activity. It should be pointed out in this context that a peculiar feature of *H. pylori* is the absence or very rarely detectable β-lactamases activity within the major features identified in resistant strains. This could be the consequence of the features of the highly variable environment colonized by the bacterium, given the fact that β-lactamases coding genes are acquired by horizontal transfer and, once translated, they are secreted in the periplasmic space to hydrolyze antibiotics.

Another major role in drugs tolerance is played by outer membrane porin and efflux systems. They strongly contribute to keep the toxic agent concentrations inside the bacterial cell lower than expected and make them less able in killing bacteria (Hirata et al. 2010).

Finally, when exploring the sources of reduced susceptibility to antibiotics, other relevant aspects that should be considered in *H. pylori* infections are the capability to oscillate from rod-shaped active bacteria to dormant resting coccoidal state, in response to antimicrobials, and the ability to penetrate and colonize gastric mucosa, forming biofilms on its surface (Yonezawa et al. 2015). Such large aggregates can not only protect the bacteria from the surrounding hostile environment, helping establishing a chronic infection, but also contribute to its reduced susceptibility to antimicrobials agents, if compared to planktonic organization. Indeed, a biofilm organization where bacteria coexist as multiple species, both dead and alive, strongly interconnected by an external matrix of mixed composition, implies a different gene expression profile, the activation of the so-called quorum sensing system for cell-to-cell communication and a signaling pattern of molecules acting as auto-inducers (Attaran et al. 2017). All these components can contribute to an altered response to eradication therapies, as demonstrated by Yonezawa and co-workers (Yonezawa et al. 2015).

The type of mutations responsible for the resistance are in general well known, since several resistant strains have been sequenced. On the opposite, the structural aspects of this resistance have not been object of extensive studies and the structure of very few *H. pylori* proteins involved in the resistance mechanisms have been determined. Fortunately, their amino acid sequences are relatively similar to those of other bacteria whose 3D structures are known and reliable molecular models can be built with mid to high

degree of confidence. Their structures and the implications for the resistance mechanisms will be discussed in this chapter.

2 Inhibitors of the Protein Synthesis Through Interaction with the Ribosome Machinery

2.1 Resistance to Macrolide Clarithromycin (CLR)

CLR is a classical bacteriostatic agent adopted as first option in the eradication therapy of symptomatic *H. pylori* infections, in combination with metronidazole or AMX (Gong and Yuan 2018). It belongs to second generation 14-membered-ring macrolides, composed of three structural subgroups: the lactone ring, cladinose, and desosamine sugars. It derives from erythromycin and acts on a large spectrum of infections with good pharmacokinetic properties and relative safety.

CLA and other macrolides interfere with protein synthesis through reversible binding with nanomolar affinity to the peptidyl-transferase region (domain V) of the 23S rRNA, part of the bacterial ribosome subunit 50S. Macrolide-23S rRNA interaction blocks the peptide bond formation and peptidyl tRNA translocation from the A- to P-site. Further consequences described are the premature dissociation of peptidyl tRNA with the accumulation of truncated peptides.

The structural determinants of the inhibitory mechanism have been clarified by crystallographic studies of the complex of CLA and other macrolides with 50S subunit of *Deinococcus radiodurans* ribosome (PDB: 1J5A; Schlünzen et al. 2001). CLA evidenced a common binding mode to all the tested macrolides. Established interactions with specific nucleotides in the peptidyl transferase cavity can be assigned to a multi-branched loop of domain V of the 23S rRNA. No significant conformational changes are induced by macrolides binding.

CLR widespread intensive use, combined with the high frequency of reinfections especially in developing countries, has catalyzed the emergence of resistant strains and more in general reduced susceptibility towards macrolides, with occurrence rates higher than 20% in peculiar contexts (De Francesco et al. 2010).

The most frequently described mechanisms of resistance towards CLR imply the weakening of macrolide interactions, due to mutations occurring at three main positions in 23S-rRNA gene: A2142C, A2142G, and A2143G, the latter accounting for the majority of cases (Chen et al. 2018). Less frequent mutations in the same 23S subunit have been reported to be involved in CLR resistance, such as A2115G, T2117C, G2141A, A2144T, T2182C, G2223A, T2288C and T2711C, despite the roles covered by such alterations remain elusive and sometimes even controversial.

More recently, in an effort to clarify whether other proteins and/or ribosomal subunits could be responsible of higher MIC (minimum inhibitory concentration) values vs. CLR in *H. pylori* isolates, novel candidates have been detected and characterized by low CLR doses exposure of susceptible strains and next generation sequencing (Binh et al. 2014). The authors discovered that mutations in two candidate genes, *infB* and *rpl22*, confer resistance to *H. pylori* and have a synergic effect when coexisting with 23S point mutations. In particular, a point mutation (G160A) has been found to confer higher tolerance towards CLR in translation initiation factor IF-2, also called *infB* (HP1048). IF-2/InfB prevents hydrolysis of formylmethionyl-tRNA, promote the appropriate binding to the ribosomal subunit 30S, formation of Initiation Complex (IC) by joining 30S and 50S subunits to define the 70S IC and initiation of protein synthesis. More in general, IF-2 is one of the three components that assure appropriate velocity of IC formation and translation accuracy in bacteria (Wang et al. 2015).

The structure of IF-2/InfB, a 944 amino acid multidomain protein, can be inferred by homology modeling (Fig. 1b): the core region spanning residues 370–940 shows a high degree of similarity towards classical *Thermus thermopilus* IF-2

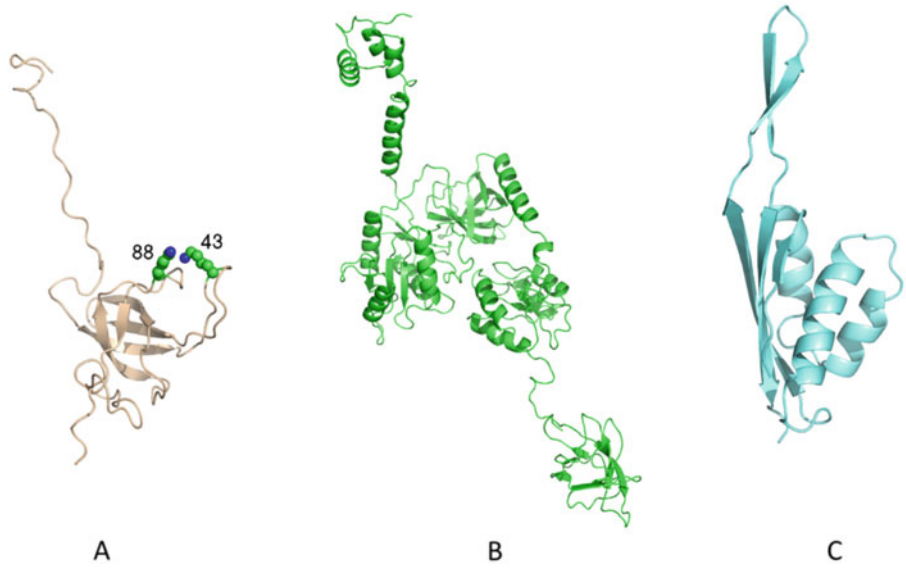

Fig. 1 Inhibitors of the ribosome machinery. (**a**) Cartoon view of Rpsl chain of the 30S ribosome subunit. Two mutations found in *H. pylori* as responsible for resistance to streptomycin are shown as small spheres. Both point mutations correspond to K → R replacements, a substitution that is normally not very significant (the only effect is that a positively charged side chains is replaced by a larger, positively charged one). Nevertheless, the effect is a misplacement of the bound *t*RNA in such a way to induce a codon misreading that impairs protein synthesis. (**b**) Cartoon view of the model of translation initiation factor IF-2 (InfB) from *H. pylori*. The homology modeling has been performed using Phyre 2 software (template structure PDB ID 3J4J). (**c**) Cartoon of 50S ribosomal protein L22. The model was obtained by homology with structure 3jw3 (Phyre2)

(PDB: 3J4J, Simonetti et al. 2013), *Escherichia coli* (PDB: 1ZO1; Allen et al. 2005) or even mammalian one. Such portion includes a GTPase switch domain and undergoes large conformational changes upon binding to 30S subunit and proper engagement of 50S, as well as in the following steps, where IF-2 should get repositioned and finally released from the ribosome.

The N-terminus, where the resistance-conferring mutation occurs, does not share remarkable homologies with sequences of known structures. However, modeling tools suggest a coiled-coil arrangement of the first 200 amino acids, analogously to the N-terminus region of PCSB protein from *Streptococcus pneumoniae* (PDB: 4cgk; Bartual et al. 2014). PCSB is recruited at the septum by interacting with FtsEX at the N-terminus and is involved in septal cross-wall hydrolysis and cell division.

Multiple types of deletions (three base pairs or nine base pairs deletions) were also reported at the level of ribosomal protein L22 (Rpl22, HP1314), a structural constituent of the large ribosomal subunit interacting with all domains of 23S rRNA and located close to the exit tunnel where new polypeptide chains are assembled during protein synthesis. Remarkably, *rpl22* is known to be involved in the resistance to first generation macrolides in *E. coli*: deletions at positions Met82-Lys83-Arg84 in L22 are reported to cause resistance to erythromycin in *E. coli (*Zaman et al. 2007). A model of the *H. pylori* Rpl22 protein (Fig. 1c) can be easily obtained with high degree of confidence for the full-length sequence, using the same 50S ribosomal protein L22 from *Bacillus subtilis* as a template (PDB:3jw3, with an overall identity of 36%). L22 is a 120 amino acids subdomain with a mixed alpha-beta structure and a protruding beta-hairpin that confers an elongated shape to such subunit.

The reduced strain susceptibility conferred by detected deletions of L22 subdomain can be

explained more by an indirect effect on the affinity of the antibiotic with 50S ribosome subunit than an impact on direct interactions of the macrolide with L22 domain. Indeed, the observed binding and orientation of CLR and other macrolides at the entrance of the tunnel of 50S subunit do not involve direct interactions with L22 residues, with minimal distances never less than 8 Å.

Finally, many studies in the context of macrolides resistance underlined the importance of intrinsic contribution of efflux systems to CLR resistance in *H. pylori*. In particular, the multicomponent HefABC (HP0605/606/607) efflux pump was observed to support the strongest contribution to CLR resistance, given its higher level of expression over the different RND (Resistance Nodulation-Division) transporters observed in CLR-resistant *H. pylori* strains. Being HefABC a multidrug efflux pump, its structural and functional features and their implication in the resistance toward chemotherapeutic agents will be described in details later in this chapter (see Sect. 6).

2.2 Resistance to Tetracyclines

Tetracyclines (TETs) are currently used in second or third-line therapies for *H. pylori* eradication, where either AMX, CLR or MTZ fail to be effective. Extensive usage of TETs in the past decades has severely limited the usefulness of this class of therapeutics nowadays. However, in some developing countries cost-effectiveness considerations still imply the usage of TETs in first-line therapies (Dunn et al. 1997).

Given the limited usage, *H. pylori* TET resistance is less frequently observed in most countries, with reported rates around 2% in several studies, but much higher peaks (>10%) in specific countries and a general tendency to increase through the time (Suzuki et al. 2010).

TETs belong to polyketides, share a four-hydrocarbon-ring structure with hydrophilic functional groups along one side, and behave as reversible bacteriostatic agents. They block *de facto* protein biosynthesis by binding site A of 30S subunit of the ribosome and preventing aminoacyl-tRNA loading during translation. Moreover, TETs prevent binding of both release factors RF-1 and 2 during the termination step, regardless of the stop codon (Brown et al. 1993).

TET binding on the small ribosomal subunit has been structurally characterized. It shows a main high affinity binding site and multiple secondary low affinity sites, whose roles have been poorly characterized so far. Multifactorial resistance mechanisms have been described in the case of TETs, some specific, such as mutations in the ribosomal subunit and ribosomal protection proteins, and others more general, often taking advantage of resistance devices toward other classes of antibiotics.

However, despite more than 60 different classes of genes encoding for TET resistance factors are known both in Gram-positive and Gram-negative bacteria, only a few have been searched and detected in *H. pylori*. The most frequent and better characterized cause of *H. pylori* resistance towards TETs is reported to be due to mutations in the *16S rRNA* gene, occurring at position 926–928 as triple-base changes (AGA to TTC). Double or single-base pair mutations insisting on the same site were also detected, conferring intermediate level of resistance to the corresponding strains as assessed by the Minimum Inhibitory Concentration (MIC) assays (Wu et al. 2005). Other reported mutations occurring at 956–958 site could be implicated in TET resistance, even if with lower frequency.

Several studies have demonstrated that TETs resistance can occur in the absence of mutations in 16S rRNA, implying other escape strategies through the accumulation of changes that may affect TET-ribosome affinity and other functions (Dailidiene et al. 2002). Within the ribosome

protecting proteins (RPPs), TET (O) and TET (M) are the most extensively studied, but never characterized in *H. pylori* according to our knowledge.

tet genes products are mainly composed of two subgroups, the RPPs, that dislodge TETs by binding ribosomes and enable the protein synthesis to go ahead, and efflux pumps, that promote extrusion of toxic agents such as antibiotics. Indeed, a protein homologue of the well-known TET efflux gene *tetA* (P), HP1165, has been proved to be involved in tetracycline resistance in *H. pylori* 26,695 strain (Li and Dannelly 2006). The occurrence, role and structural properties in the context of antimicrobial resistance are discussed later in a separate paragraph (see Efflux pumps, Section 6).

2.3 Resistance to Aminoglycoside

Streptomycin (STR), an antibiotic belonging to the aminoglycosides family, is mostly used against tuberculosis and is quite effective also against *H. pylori* resistant strains (Hu et al. 2016). STR binds to 30S subunit of the bacterial ribosome, altering the correct binding of formyl-methionyl-tRNA to the ribosome and, consequently, impairing the protein synthesis. The mechanism of resistance in *H. pylori* has not been extensively studied, but it appears similar to that in *E. coli* and *Mycobacterium tuberculosis*: point mutations in the *rpsL* gene, coding for the ribosomal protein S12 (HP1197), are responsible for mismatch binding of the tRNA to the ribosome (Sharma et al. 2007; Ulger et al. 2009). Mutations in *H. pylori* have been observed at positions 43 or 88, two positions close in space. They both correspond to a lysine mutated to arginine. A molecular model of the protein has been built by homology modeling based on the structure of the orthologous protein of chloroplast ribosome from spinach (PDB ID 5X8R), that bears a surprisingly high sequence identity with our protein, 74.8%. Interestingly, both point mutations are conservative, i.e. a potentially positively charged residue, lysine, is replaced by the similar, positively charged residue arginine (Fig. 1a). This mutation does not prevent the correct functioning of the ribosome, nevertheless it prevents the binding of the antibiotic. More in depth investigations are probably necessary to better clarify this kind of resistance mechanism.

3 Inhibitors that Interfere with DNA or RNA Machinery

3.1 Resistance to Fluoroquinolones

Levofloxacin (LVFX) is used, eventually in combination with other antibiotics, to treat several bacterial infections, from pneumonia to urinary tract infections, to tuberculosis, meningitis and others. LVFX functions as a bactericide by inhibiting DNA gyrases and/or topoisomerase IV. In *H. pylori,* DNA gyrase is encoded by two genes, *gyrA* and *gyrB*, and topoisomerase IV by genes *parC* and *parE*. Mutations in the last two genes have not been observed in the bacterium, and mutations in *gyrA* gene seem to be the main reason of the resistance. Point mutations conferring resistance have been found at positions 87, 88 and 91 (Barnard and Maxwell 2001; Moore et al. 1995; Miyachi et al. 2006; Lee et al. 2008; Murakami et al. 2009, Hanafi et al. 2016; Miftahussurur et al. 2016). The structure of GyrA is not available, so the molecular model (Fig. 2a) of the product of *gyrA* gene was obtained by homology modelling from the structure of topoisomerase from *S. pneumoniae* (PDB ID 4Z2C) that presents 56% identity with the protein from *H. pylori*. The mutated residues belong to the initial part of helix 86–97 that is close to one of the regions of binding of the DNA double helix. In the complex of gyrase from *S. pneumoniae* with moxiflavicin, the inhibitor is bound to this area, suggesting that LVFX binds preventing the binding of the double-stranded DNA. Nevertheless, the mutations do not influence the functionality of the protein and do not prevent the binding of DNA. The region of the protein that binds DNA is quite extended, and to overcome the resistance a drug that binds to a different area of the protein should be tried.

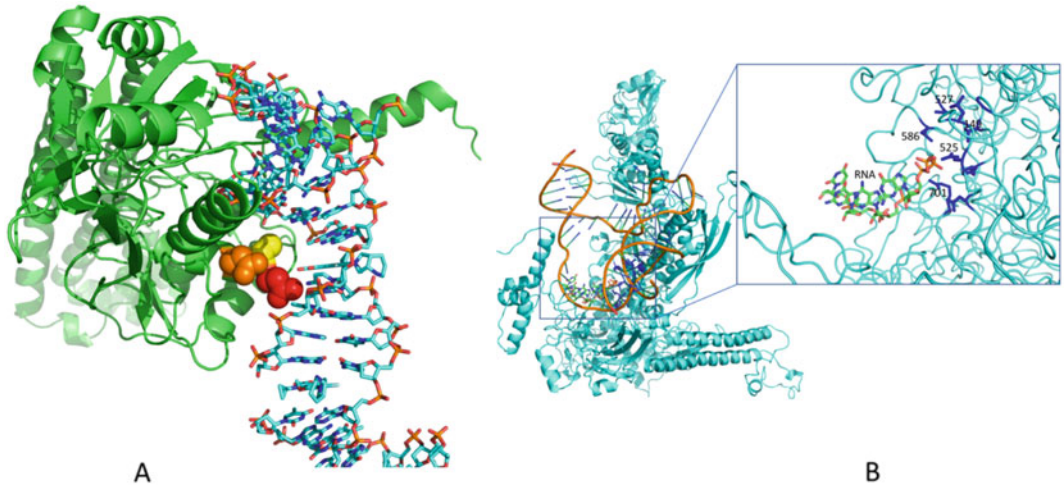

Fig. 2 Inhibitors that interfere with DNA or RNA machinery. (**a**) Cartoon view of one monomer of DNA gyrase (GyrA) model (green) with side chains of the mutated residues (87, red, 88, yellow and 91, orange) in the resistant strains of *H. pylori*. For clarity of the picture, only one monomer is shown; the second monomer is related to the first one by a twofold axis. A portion of a bound double-helix DNA is shown in cyan and brown. (**b**) Cartoon view of the model of *H. pylori* RpoB subunit (cyan). The traces of DNA promoter and RNA transcript are shown in orange, as bound to *E. coli* RopB (PDB ID 5IPM). A detail region of the protein where mutations responsible for the resistance in *H. pylori* are located is shown in the insect, where side chains of the most frequent mutated residues are shown in blue

3.2 Resistance to Rifamycinoid Antibiotics

Rifabutin (RBU), a derivative of rifamacyn S, is very effective towards Gram-positive and Gram-negative bacteria, since it inhibits transcription by binding to the β-subunit of the DNA-dependent RNA polymerase RpoB (Jin and Gross 1988). Its high efficiency at low concentrations makes it a component of the second-line therapy in drug-resistant infections (Malfertheiner et al. 2012).

The crystal structure of RpoB (HP1198) is not known, but a model can be built by homology modelling using as template 5ipl.1.C, its ortholog from *E. coli* with 47.30% of similarity (Liu et al. 2016). The entire protein complex in bacteria includes at least six polypeptide chains, in addition to the promoter DNA and the nascent RNA. In the model in Fig. 2b, only the *H. pylori* model of the β–subunit is shown, along with the DNA and RNA chains as bound to the *E. coli* complex (PDB ID 5IPL).

Mutations in RpoB observed in some resistant strains (Heep et al. 2000) are essentially confined to an area close to the active site, where the nascent RNA chain is forming. This indicates that the antibiotic binds there and that single-point mutations hinder the binding of the antibiotics, without altering or preventing the enzymatic activity of the complex. The positions of the mutations detected are illustrated in Fig. 2b. Moreover, some other resistant strains do not show mutations in RpoB, suggesting that these mutations are not the sole cause of resistance.

4 Mixed Resistances

4.1 Resistance to β-Lactams

AMX is a crucial component of the triple therapy in the attempt to eradicate the *H. pylori* infection. The general mechanism of β-lactams, a group of antibiotics containing a four-membered ring cyclic amide, is the binding to penicillin-binding proteins (PBPs); the latter are involved in peptidoglycan synthesis and its inhibition blocks the bacterial wall biosynthesis (Cho et al. 2014). The

resistance to this class of antibiotics in bacteria is mostly due to the presence of β-lactamases, enzymes able to catalyze the breaking of the β-lactam ring, or eventually to a reduced membrane permeability that reduces the uptake of the antibiotic (Livermore 1995). *H. pylori* seems to differ in this contest from other bacteria, since a significant β-lactamase activity has not been very rarely detected in AMX-resistant strains. On the contrary, resistance has been associated to point-mutations to the PBPs. There are nine different PBPs, three of high molecular weight and six of low molecular weight (labelled from PBP1 to PBP9).

The crystal structure of PBP2 of *H. pylori* is available (PDB ID 5LP5, Contreras-Martel et al. 2017) and models of PBP1 and PBP3 can be quite confidently built by homology modelling (PBP1 shares 45% sequence identity with structure 2OQO; PBP3 28.1% with structure 5DF9). The overall folds of PBP2 and PBP3 proteins are quite similar (the overall root mean square deviation of the Cα atoms for the entire structure is 2.2 Å): the protein is organized in anchor, head, linker e transpeptidase domains (Fig. 3a). In the isolated PBP3 protein, the anchor is clasped against the head, whilst when PBP forms a complex with the MreC core elongation factor the anchor region shifts away from the head, exposing a hydrophobic surface that allows the protein-protein interaction. The binding of MreC to PBP represents a key event in the peptidoglycan biosynthesis and consequently in the cell wall elongation.

PBP1 is the most different among the three proteins (Fig. 3b): whilst the transpeptidase domain is quite similar (the root mean square deviation of the equivalent Cα atoms is 1.9 Å), the other domains present a different fold and a different orientation.

H. pylori resistance is primarily due to mutations in PBP1 and are localized mostly in two areas, characterized by conserved residues 402–404 and 555–557. Both are relatively close to the binding site of cephalosporin, ampicillin, aztreonam and others (Fig. 3b). The model of *H. pylori* PBP1 with superimposed the inhibitor acyl-ampicillin as bound to *E. coli* PBP1 (PDB ID 5hl9; King et al. 2017) clearly explains why the mutated residues that confer the major resistance against β-lactams are T556S, N562Y and T593A (in red in the figure). The latter are in fact located very close to the binding site of the drug and a mutation in one of these residues hamper the binding of the compound. The other residues (in blue in Fig. 3b) are more distant and possibly they have some influence on the binding, but not at a level to confer a strong resistance.

Mutations in PBP2 or PBP3 also seem to facilitate the effect of the primary mutations. Probably a suite of mutations on the three proteins altogether increase the effect and contribute to a stronger resistance (Rimbara et al. 2008).

Finally, it seems that a decreased permeability of the membrane to AMX can also contribute to the resistance, through mutations of *hopB* and *hopC* genes (Co and Schiller 2006), indicating that the resistance to AMX is a multifactorial event, not simple to contrast.

5 Resistance Due to Indirect Effects

5.1 Resistance to Nitroimidazoles

5-nitroimidazole, including MTZ, is a prototype of nitro-imidazoles used for several infections, from anaerobic bacteria to protozoa, and is also useful against *H. pylori*. It is a prodrug that must be activated, giving rise to a complex mechanism of action: a bacterial nitroreductase enzyme reduces nitroso and hydroxylamine derivatives to inhibit acid synthesis. In the presence of molecular oxygen, the latter brings to the formation of superoxides that damage bacterial DNA. Since the action of MTZ is not direct, the resistance mechanism is also complex and four possible mechanisms have been proposed: (i) increased activity of oxygen scavengers, (ii) increased activity of the enzymes, (iii) reduced activity of nitroreductases and (iv) reduced uptake of the compound. About point (i), a mutant of the ferric-uptake regulator Fur that overexpresses SodB affects the resistance to MTZ, despite the fact that there is no evidence of differences in the superoxide dismutase (SOD) activity in resistant

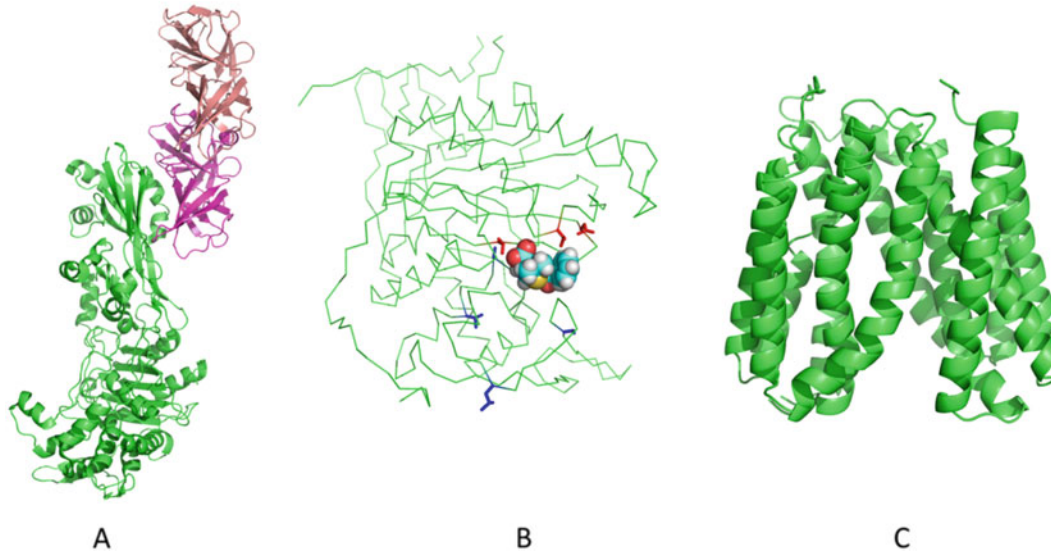

Fig. 3 Resistance due to other effects. (**a**) Cartoon structure of protein PBP2 from *H. pylori*, (coordinates from PDB ID 5LP5, Contreras-Martel et al. 2017). (**b**) Ribbon view of the C-terminal domain of the model of PBP1 of *H. pylori*, built by homology modelling with the *E. coli* protein (PDB ID 5hlb, 29% identity, King et al. 2017). The acyl-ampicillin (represented as van der Waals spheres) was positioned by superimposing the structure of the complex of the *E. coli* protein (PDB ID 5u2g) to our model and assuming that the binding position was conserved. Side chains of residues in red are those that confer stronger resistance, the blue ones are less effective. (**c**) Ribbon view of the model of HP1165, a protein homologue of the tetracycline efflux gene *tetA*, demonstrated to be involved in tetracycline resistance

strains. In addition, mechanism (ii) has not been proven, since the lack of *recA* gene, coding for a repair enzyme, does not seem to show a significant decreased resistance to MTZ. The diminished presence of an efflux pump has not been proven too. The predominant effect could be the absence of a low enough redox potential due to mutations that inactivate oxidoreductases, as RdxA (oxygen insensitive NADPH nitroreductase), FrxA (NADP:Flavin oxidoreductase) and FdxB (ferrodoxin-like enzyme), since they reduce the amount of nitroreductase present, necessary for the activation of MTZ.

6 Resistance Due to Efflux Pumps

6.1 Outer Membrane Proteins and Efflux Pumps

Together with escape mutations and drug inactivation, drugs import inside the bacterial cells and their efflux define the major mechanisms of treatment reduced susceptibility (Nikaido 1998). Import and export balance is part of the intrinsic response to antibiotics exposure, since it occurs in the absence of any genetic alterations. Moreover, efflux systems expression can be constitutive and/or induced by antibiotics acting at the transcriptional level by interacting with regulatory mechanisms (Roberts 1996; Ryan et al. 2001).

H. pylori genome codes for 32 outer membrane proteins (OMPs) and 27 drug transporters, some of which only annotated by homology searches, other better characterized and experimentally proved to act as drug exporters. The most relevant efflux systems play a significant role in multidrugs resistance by transporting a wide spectrum of structurally diverse compounds.

Efflux systems can be classified according to five main classes: (i) major facilitator superfamily (MFS), (ii) Resistance-Nodulation-Division family of transporters (RND), (iii) multi drug and toxic extrusion family (MATE), (iv) small

multidrug resistance members (SMR) and (v) the ancient ATP-dependent ATP-binding cassette transporters (ABC transporters, members of a transport system superfamily). The energy costs for drugs ejection are sustained either by ATP hydrolysis, as in the case of ABC transporters, or proton gradient.

The role of specific members of any efflux-mediator families in *H. pylori* has been investigated in multiple studies, sometimes with contradictory results. What became clear is that they definitely play a major role in antibiotics resistance. Bina and co-workers first identified the presence of transporters potentially associated with drugs tolerance; they described active members belonging to at least four classes of efflux systems (Bina et al. 2000). Proofs of their significant contribution to antibiotics efflux and the consequent reduced accumulation of toxic agents in the bacterial cytoplasm came later (Dailidiene et al. 2002; van Amsterdam et al. 2005).

Together with RND transporters, the YajR homologue TetA (HP1165) has been discovered to contribute to multidrugs exposure tolerance in *H. pylori*, while ATP dependent efflux systems have never been found to be over-expressed in stressed conditions as well as in clinical isolates.

In this context, efflux pumps inhibitors received increasing attention, given their potential both as tools and therapeutic agents, as they should restore the activity of standard eradication treatments. Their roles on multidrug resistance have been investigated selecting a panel of inhibitors targeting different transporters families. A synergic effect on five of the nine tested antibiotics (chloramphenicol, TET, erythromycin, cefotaxime and ceftriaxone) was observed using carbonyl cyanide m-chlorophenyl hydrazine (CCCP) inhibitor, with reduced MIC values ranging from 19- to 4-fold, respectively. CCCP is an energy blocker which inhibits efflux pumps driven by proton gradient, and not those that are ATP dependent. These results confirmed that most likely the kind of efflux systems involved in *H. pylori* resistance do not act in a ATP hydrolysis dependent manner.

Finally, the same PPIs that are currently proposed in the first-line eradication regimen are small molecules acting as proton motive force uncoupling agents. They have been demonstrated to contribute to eradication therapy and, in particular, rabeprazole and pantoprazole positively impact on MICs of antibiotics in multidrug-resistant *H. pylori* strains. However, the mechanism of synergy and actual targets of such PPIs are far from being understood and will require further studies to be clarified (Liu et al. 2008).

6.2 RND Efflux Systems

RND efflux systems represent a relevant mechanism of resistance for multiple classes of antibiotics. In *H. pylori* cultures, they have been proven to be implicated in the resistance toward highly diverse treatments, ranging from macrolides (CLR, erythromycin and others) to penicillin G, MTZ, cefotaxime, clindamycin, novobiocin, and ethidium bromide.

The mRNA expression profile of at least four RND efflux mediators have been detected in *H. pylori,* that is HP0605–HP0607, HP0971–HP0969, HP1327–HP1329, HP1489–HP1487 (Kutsche and de Jonge 2005). Like the classical three-components RND transporters present in many Gram-negative bacteria, *H. pylori* members are characterized by a translocase, a TolC homolog and an accessory component spanning the membrane and connecting the other two components.

The best characterized in this context is the HP0605–HP0607 transporter, elsewhere identified as HefABC or acrA/B/TolC system, where *hp0605* codes for a TolC homologue (HefA), *hp0606* corresponds to the membrane fusion mediator AcrA/HefB and *hp0607* codes for the pump component on the cytoplasmic side (AcrB/HefC) (Hirata et al. 2010).

Homology modeling of the HP0605/HP0606/HP0607 members of RND transporter allows to describe the main features of such apparatus and compare it with other extensively characterized HefABC transporters such as the *E. coli*

prototype. Best template for building the model of HP0607 has been identified in the AcrA protein from *E. coli a*nd the AcrA homologue ZneB from *Cupriavidus metallidurans,* a membrane fusion protein (Mfp) implicated in heavy metal cation efflux (De Angelis et al. 2010). A model with high confidence can be obtained (Phyre2, $\geq$ 99% confidence) despite the limited sequence identity, which do not exceed 16% over the entire protein sequence.

It conserves an elongated multidomain shape, where three tertiary structure motifs can be identified: a beta-barrel domain, predicted to localize close to the inner membrane, composed of six antiparallel beta-strands, hosting a key cysteine residue for lipid acylation and anchoring; a central lipoyl domain, composed of seven beta-strands arranged in a beta-sandwich; a coiled-coil alpha-helical hairpin that includes two helices composed of hepta-peptides repeats, deriving from the interruption of the two sides of the sandwich in the primary sequence. The beta-hairpin helices of *H. pylori* HP0606 and Zneb from *C. metallidurans* are slightly shorter that the *E. coli* ones but, analogously to any of the AcrA homologues studied till know, is connected to the central lipoyl domain by a hinge region that confers large flexibility to such coiled-coil structure and could explain its capability to induce conformational changes implied in the channel opening at the outer membrane face. Intriguingly, ZneB can bind zinc ions and such feature seems to be involved in conformational dynamics. However, the residues involved in the metal coordination at the N-terminus include an extended fragment that is not conserved in *H. pylori*.

HP0605 protein can be modeled with a relatively high degree of confidence using the crystal structure of the *C. jejuni* CmeC outer membrane channel or the multidrug efflux outer membrane protein OprM from *Pseudomonas aeruginosa*, again despite a limited sequence identity (15%; 4MT4; Su et al. 2014).

CmeC is part of a CmeABC tripartite multidrug efflux pump, homologous to HefABC apparatus. It belongs to TolC/OprM family with the typical trimeric assembly (about 500 aa per protomer) forming a long tunnel devoted to antimicrobial export, heavily charged by acidic residues on the internal side of the cavity. Each protomer has an elongated α/β-structure that contributes to form a basal membrane spanning β-barrel (4 β-strands per protomer) and a helical periplasmic domain composed of six α-helices per protomer. CmeC from *C. jejuni* and HP0606/TolC model from *H. pylori* share an extra domain decorating the channel at the mid-section. Such domain is present also in OprM from *P. aeruginosa,* while in *E. coli* and other family members it is smaller, less ordered and less pronounced.

Analogously to HP0606/AcrA, a reliable model for HP0607/AcrB protein can be built, based on the CmeB component of *C. jejuni* drugs efflux system as a template (5LQ3). A homotrimer assembles according to a typical cytoplasmic RND-type pump, with each monomer contributing with 12 transmembrane helices to the core of the structure and an elongated periplasmic protrusion defined by the association of six mixed α/β subdomains, two per monomer.

The overall trimer undergoes asymmetrical changes, depicted by the structures obtained for many AcrB homologues, since each protomer can alternatively explore different conformational states often called “access”, “binding” and “extrusion” states, where the clefts present both in the transmembrane and periplasmic subdomains can alternatively switch from closed to open arrangements. This implies that such RND pump must synchronize each protomer state to go through the different steps in the drugs export mechanism (Su et al. 2017).

6.3 Tetracycline Efflux Protein

Tetracycline efflux protein (TetA), HP1165, has been demonstrated to be involved in TET resistance in *H. pylori* strain 26,695 (Li and Dannelly 2006). Structural homology searches against Protein Data Bank (PDB) evidenced 25.2% identity with the drug efflux protein YajR from *E. coli* (PDB ID 3WDO; Jiang et al. 2013).

YajR is a 49-kDa secondary active transporter, part of the highly conserved major facilitator

superfamily (MFS), playing a role in substrate sensing, signaling and active export of antibiotics. MFS members follow a mechanism supported by the electrochemical potential across the cell membrane and share a canonical 12 transmembrane helices core, generated by four three-helix repeats and divided in two subdomains of six helices, each of them related by intra and inter pseudo twofold symmetries.

The two subdomains (H 1-6 and H 7-12) undergo about 40° rotation between outward and inward states during transport process, where outward conformation represent the ground state and inward the exited and protonated one. The negative inside rule drives the inward shift of YajR in the excited state while a patch of basic amino acids, enriched on the cytoplasmic side of the transporter, participates in the transport mechanism providing an energy contribution which facilitates the inward-to-outward conformational recycling.

Motif A, a highly-conserved sequence present in multiple insertion loops between TM-helices of different MSF, contribute to outward state stabilization through interactions centered at a charge-helix dipole interaction. The model (Fig. 3c) evidences a strict homology of HP1165 with YajR and other representative *E. coli* members of MSF family such as lactose permease (LacY proton/sugar symporter, PDB ID 1PV7) or multidrug transporter MdfA (PDB ID 4ZP0).

The transmembrane core is highly conserved (27% sequence similarity over 95% of the sequence, 17% identity with YajR) and all the members show a heavily charged state of cytoplasmic and periplasmic sides of the molecule, but only *E. coli* YajR and not *H. pylori* homologue HP1165 presents an extra C-terminal negative charged ferredoxin-like domain protruding as a flexible and independent appendage on the cytoplasmic side.

Evidence of antimicrobial drugs binding and export properties have been strikingly demonstrated by Heng and co-workers (Heng et al. 2015), who were able to trap *E. coli* MdfA in complex with chloramphenicol in the inward facing conformation. The aspartic acid residue buried inside the structure and located in close proximity to antibiotic binding site (Asp34) has been proved to respond to chloramphenicol binding by changing its protonation state. However, such acidic residue is not conserved in HP1165, which exposes a histidine residue (His24) in the corresponding position (same transmembrane helix and internal orientation). His24 could eventually play an analogous role given its pH responsive nature.

7 Conclusions

The mechanisms used by the Gram-negative bacterium *H. pylori* to counteract the effects of antibiotics are partially known and they share several properties in common with many other pathogenic bacteria. They present also specific features, mostly due to the peculiar niche where *H. pylori* is living, the human stomach, and possibly to the quite limited contacts the bacterium has with other bacterial species. For example, only very few examples of β-lactamases activity have been detected in *H. pylori*, and bacterium has developed its own resistance mechanism to β-lactam antibiotics.

Finally, it is important to consider that about 30% of the proteins coded by the *H. pylori* genome are essential for the survival and colonization, many of which being putative targets for the design of novel antimicrobials. Despite the efforts of many research groups all over the world, the discovery and introduction of new drugs against *H. pylori* have not been achieved till now and new approaches are required.

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