Molecular Mechanisms of Antibiotic Resistance in *Helicobacter pylori*

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Molecular Mechanisms of Antibiotic Resistance in *Helicobacter pylori*

Moleculaire mechanismen van antibioticum resistentie in *Helicobacter pylori*

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CHAPTER 1

Introduction, aims and outline of this thesis

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INTRODUCTION

Helicobacter pylori colonizes the stomach of approximately half the world's population. The bacteria are primarily observed on the mucosa of the gastric antrum [19]. Colonization with *H. pylori* itself is not a disease, but *H. pylori* is an etiologic agent of acute or chronic gastritis, and a predisposing condition to peptic ulcer disease, gastric carcinoma, and B-cell mucosa associated lymphoid tissue (MALT) lymphoma [7, 23, 24]. The clinical outcome of *H. pylori* infection depends on a variety of factors, which are related to the host (e.g. blood group antigens and polymorphisms in interleukin 1 gene cluster), the bacterium (e.g. strain-specific genes, differential gene expression, phase variation and allelic variation), and the environment (e.g. age and smoking) [14].

H. pylori is Gram-negative rod, with a spiral or slightly curved shape (Figure 1). The bacterium is relatively small, 2.5 to 3.5 μm in length and 0.5 to 1.0 μm in width, and contains 4 to 6 unipolar sheathed flagellae [9]. *H. pylori* may change into a coccoid form, especially after antibiotic treatment or prolonged *in vitro* subculturing [17, 22]. Whether the coccoid forms are viable and do have the capacity to return into the spiral shape, is still being discussed [17, 26, 32].

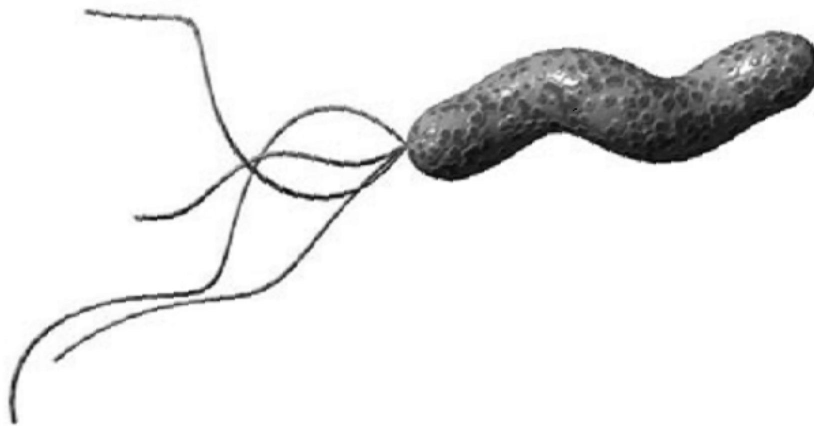

Figure 1. *Helicobacter pylori.* Modified from image L. Marshall, www.hpylori.com.au

Recently, the complete genome sequence of two different *H. pylori* clinical isolates (i.e. 26695 and J99) have been determined and annotated [1, 28]. The genome of these strains are circular and relatively small (1.64 to 1.67 Mb) compared to *Escherichia coli* (4.6 Mb), and have a GC content of 39%. For *H. pylori* strain 26695, 1590 putative open reading frames (ORFs) have been identified, whereas the *H. pylori* genome of strain J99 enclosed 1496 putative ORFs. For approximately 60% of the ORFs a function could be predicted, including several well-known virulence factors such as *cagA*, *vacA* and *iceA*. Roughly 6% of the putative ORFs appear to be strain specific [1, 28]. The high genetic diversity observed among *H. pylori* strains might be explained by naturally competence of *H. pylori* for DNA uptake and/or by the ability of *H. pylori* to rearrange its genome [15, 16].

H. pylori infection can be diagnosed by a variety of invasive and non-invasive tests (Table 1). Histological examination, bacterial culture and rapid urease testing require an gastric biopsy sample [10, 11, 13, 25], whereas serology, polymerase chain reaction (PCR), and the stool antigen test can be performed on alternative clinical samples such as whole blood, serum, saliva, urine or faeces [2, 8, 12, 18, 30]. The choice for use of a specific test depends on several factors including gastric complaints, age, local availability, costs and clinical information sought. As none of the tests to diagnose *H. pylori* infections is 100% reliable, a combination of two tests is advisable [29].

Some clinical conditions warrant endoscopic examination, for instance patients with alarm symptoms such as bleeding and weight loss as well as older patients with newly developed dyspepsia. Endoscopy is also advised for patients who have failed eradication therapy, for culturing and antimicrobial susceptibility testing in order to select an appropriate therapy. When alarm symptoms are absent, the non-invasive 'test and treat' strategy has shown to be safe and cost effective in diagnosis and treatment of *H. pylori* in uninvestigated, young (< 50 years), dyspeptic patients [3].

Table 1. Tests to diagnose *H. pylori* infections.

Test methods	References
Invasive test	
Histological examination	[11]
Bacterial culture	[13]
Rapid urease test	[10, 25]
Polymerase chain reaction (PCR) analysis	[4]
Non-invasive test	
Urea Breath Test	[5]
Serology	[2, 12]
Stool antigen test	[8, 30]
PCR analysis	[18]

H. pylori eradication leads to healing of gastritis and peptic ulcer disease, and probably also has a beneficial effect on regression of atrophic gastritis and prevention of distal gastric cancer [6, 27]. *In vitro, H. pylori* is susceptible to the majority of antibiotics, but *in vivo* only a few antibiotics can be used successfully for eradication of *H. pylori*, i.e. amoxicillin, clarithromycin, metronidazole and tetracycline [20]. As none of these drugs is effective enough to eradicate *H. pylori* in monotherapy, successful treatment of *H. pylori* infection requires combination therapy, consisting of one or two antibiotics, an acid inhibitor and/or a bismuth component. The extensive use and limited choice of the antibiotics have resulted in the development of antibiotic resistance in *H. pylori.* The increased prevalence of antibiotic resistance in *H. pylori* is problematic since it is one of the most important causes of therapy failure.

Antibiotic susceptibility in *H. pylori* is usually assessed by culture-based methods such as E-test, agar dilution and disc diffusion. These tests are slow, as results are usually obtained only after 6 to 10 days. Furthermore, they fail in approximately 5-10% of the cases, and are difficult to compare between different institutes due to lack of standardization [21, 31]. In this light molecular-based methods can offer an attractive alternative. They are independent of cell viability and growth rates of the bacteria, and are easily standardized. In order to develop molecular-based methods for the detection of antibiotic resistance in *H. pylori*, knowledge of the molecular mechanisms underlying antibiotic resistance is mandatory.

AIMS AND OUTLINE OF THIS THESIS

The aims of this thesis were to gain more insight in the molecular mechanisms underlying antibiotic resistance in *H. pylori*. **Chapter 2** described the clinical implications and molecular mechanisms of antibiotic resistance in *H. pylori*, and the molecular detection method developed thus far. **Chapter 3** describes the isolation and characterization of the first Dutch tetracycline-resistant *H. pylori* clinical isolate. As tetracycline resistance in *H. pylori* seems to be related to the presence of the triple-base-pair substitution $AGA_{926\text{-}928}\underline{TTC}$ (underlined letters are used to represent the base pair changes) in both the 16S rRNA genes, the preference for this mutation was studied (**Chapter 4**). Site-directed mutants were created in a similar isogenic background, and growth rates, levels of resistance and stability of the mutations were tested. The data obtained in these two studies was then used for the development and validation of molecular detection methods for tetracycline resistance in *H. pylori*. **Chapter 5** describes a PCR-based restriction fragment length polymorphism (RFLP), and **Chapter 6** describes a real-time PCR assay followed by melting curve analysis using LightCycler technology.

Chapter 7 describes the role of metronidazole reducing enzymes in oxygen-dependent metronidazole resistance in *H. pylori*. Several metronidazole-resistant *H. pylori* isolates, containing null mutation in the *rdxA* and/or *frxA* genes, were exposed to short periods of anaerobiosis, and the effect on cell viability and level of metronidazole resistance was investigated.

Chapter 8 reports the isolation of the first stable amoxicillin-resistant *H. pylori* isolate worldwide, and **Chapter 9** describes the characterization of the molecular mechanism underlying amoxicillin resistance in the *H. pylori* strain isolated in **Chapter 8**. **Chapter 10** describes the molecular mechanism of seven additional amoxicillin-resistant *H. pylori* isolates obtained from dyspeptic patients from Brazil and The Netherlands.

Finally, the research described in this thesis is summarized and discussed in **Chapter 11**.

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CHAPTER 2

Antibiotic resistance in *Helicobacter pylori*: clinical implications and molecular mechanisms

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ABSTRACT

Helicobacter pylori is a Gram-negative rod which colonizes the stomach of approximately half the world's population. First identified in 1983 as a pathogen, it has now been accepted as the causative agent of several gastric disorders ranging from chronic active gastritis and peptic ulcer disease to gastric cancer. The recognition of *H. pylori* as a pathogen has had a significant impact on gastroenterologic practice and has made diagnosis and treatment of *H. pylori* clinically relevant. Although *H. pylori* is susceptible to many antibiotics *in vitro*, only a few antibiotics can be used *in vivo* to cure infected patients. The frequent indication for eradication therapy, and the limited choice of antibiotics have resulted in the development of antibiotic resistance in *H. pylori*, which significantly impairs the treatment of *H. pylori*-associated disorders. The prevalence of antibiotic resistance of *H. pylori* shows regional variation per antibiotic, but can be as high as 95%. In this review the clinical implications, the molecular mechanisms, and recently developed molecular detection methods for antibiotic resistance in *H. pylori* are discussed.

1. INTRODUCTION

In 1983, Warren and Marshall were the first to report the successful cultivation of the human pathogen *Helicobacter pylori* from gastric biopsy samples [159]. They went on to fulfill Koch's postulates in a self-infection experiment, in which they demonstrated that *H. pylori* colonization results in active gastritis, which could be cured by treatment with antibiotics [91]. This important discovery has significantly changed the management of gastroduodenal diseases and has in particular changed peptic ulcer disease from a chronic, relapsing disease of uncertain cause to a curable infectious condition.

Infection with *H. pylori* occurs worldwide. In industrialized countries 20% to 50% of the middle-aged adults is infected with *H. pylori*, compared to 80% or more in many developing countries [36, 120, 125]. In the latter, most people become colonized with *H. pylori* before the age of ten, whereas in industrialized countries only a few children are infected at this age [125, 138]. In industrialized countries, the incidence of *H. pylori* infections has decreased substantially over the recent decades, probably due to improved socio-economic status, sanitation and/or living conditions. Therefore, the increase in prevalence of *H. pylori* with age seen in the industrialized countries is generally explained by a cohort effect [74].

Infection with *H. pylori* is associated with an acute pangastritis. This acute gastritis may be symptomatic with nausea, vomiting and abdominal pain. Although the infection is cleared in some patients, it progresses to chronic active gastritis in most subjects [74]. In a proportion of them, this eventually gives rise to peptic ulcer disease, and atrophic gastritis, and gastric adenocarcinoma [75]. Furthermore, *H. pylori* infections have been associated with gastric B-cell mucosa associated lymphoid tissue (MALT) lymphoma, hypertrophic gastropathy, and hypochlorhydria [55, 115, 131].

2. TREATMENT OF *H. PYLORI* INFECTIONS

Peptic ulcer disease and other *H. pylori*-associated disorders usually regress or heal completely after eradication of *H. pylori* with antibiotics [5, 133]. *In vitro, H. pylori* is susceptible to the majority of antibiotics [94], but *in vivo* most antibiotics are unable to cure infected patients [49]. This is thought to be due to a combination of factors, including (i) the inability of drugs to achieve appropriate levels in the gastric mucus layer [95, 152], (ii) inactivation of drugs at low pH [22, 51], and (iii) the slow growth rate of *H. pylori.* Metronidazole, clarithromycin, amoxicillin and tetracycline are the most widely used antimicrobial drugs for treatment of *H. pylori* [99] (Table 1). Fluoroquinolones, nitrofurans, and rifamycins are occasionally used as alternatives [40, 45, 52, 143, 164]. None of the above-mentioned antibiotics is effective enough to be used as monotherapy. Successful eradication of *H. pylori* therefore requires combination therapy, consisting of one or more antibiotics in combination with an acid-suppressive drug and/or a bismuth component.

Inclusion of an acid-suppressive drug was shown to increase the efficacy of the combination therapy [146]. There is a preference for the use of proton pump inhibitors (PPI), such as omeprazole and lansoprazole rather than H_2-receptor antagonists, even though H_2-receptor antagonists-based regimens, in particular those with ranitidine, were

Table 1. Mode of action, resistance mechanisms and prevalence of antimicrobials used for treatment of *H. pylori* infection.

Antimicrobial compound	Mode of action	Mode of resistance	Prevalence of resistance
Metronidazole	Reduction of prodrug by nitroreductases, leads to formation nitro anion radical and metronidazole-intermediates, and subsequent DNA damage	Absence of metronidazole reduction due to mutations in *rdxA* and *frxA* gene, and reduced expression of other reductases	20-95%
Clarithromycin	Binds 23S rRNA ribosomal subunit, resulting in inhibition of protein synthesis	Point mutations in 23S rRNA	5-30%
Amoxicillin	Binding of β-lactam antibiotic to penicillin-binding proteins (PBP) inhibits cell division	Decreased binding of amoxicillin to PBP1A (resistance) or PBP-D (tolerance), reduced membrane permeability (resistance)	1-2%
Tetracycline	Binding to ribosome prevents association with aminoacyl-tRNA, and subsequent protein synthesis	Point mutations in 16S rRNA gene, *rrnA* and *rrnB*	≤ 1%
Fluoroquinones	Inhibition of DNA gyrase and topoisomerases, interfering with DNA replication	Point mutations in the DNA gyrase gene, *gyrA*	< 1%
Rifamycins	Binding to RNA polymerase, resulting in transcription inhibition	Point mutations in the RNA polymerase gene, *rpoB*	< 1%
Nitrofurans	Reduction of prodrug by nitroreductases, leads to formation nitro-anion radicals, and subsequent DNA damage	Unknown	< 0.1%
Bismuth	Inhibits protein, ATP and cell membrane synthesis	Unknown	Not reported

shown to be equally effective as PPI-based ones [50]. Acid-suppressive drugs primarily increase the intragastric pH, and enhance the activity of the antibiotics. An additional beneficial effect of acid-suppressive drugs is that they decrease the severity of side effects of a given regimen [9], resulting in an increased compliance and chance for successful treatment.

Bismuth salts have already been used in medicine since the 19th century, particularly in the treatment of peptic diseases. Colloidal bismuth subcitrate, bismuth subsalicylate and the newer ranitidine bismuth citrate (acid inhibitor combined with a bismuth compound) are commonly used agents in anti-*H. pylori* therapy [15]. The mode of action of bismuth salt on *H. pylori* is complex and includes inhibition of protein, ATP, and cell wall synthesis [85]. Although bismuth monotherapy effectively suppresses growth of *H. pylori*, the eradication rates with this therapy are low [48]. However, when used in combination with one or two antibiotics, these compounds have synergistic activity. While bismuth-based triple and quadruple therapies seem to be even more effective against *H. pylori* infection than PPI-based therapies, they are usually not given as first-line treatment, because of their more complex dosing schedule and/or frequent side effects [39].

Triple therapies consisting of two of the previous mentioned antibiotics, and a PPI or ranitidine bismuth citrate for 7-10 days are now mostly recommended [19, 43, 90]. There seems to be preference for combination therapy that includes amoxicillin and clarithromycin [90]. Despite good results in many clinical trails, these therapies are not always as successful in daily clinical practice. Failure of first-line therapy is usually related to insufficient patient compliance and/or development of antibiotic resistance. For retreatment, 10 to 14 day treatment courses with higher doses of the antibiotics and/or bismuth component is often advised, usually with inclusion of previously unused antibiotics [40].

Standard third-line 'rescue' therapies are lacking due to a limited choice of antibiotics and the need to individualized treatment depending on which therapies previously failed. Most patients who remain *H. pylori*-positive after two consecutive courses of eradication treatment have been infected with an *H. pylori* strain that is resistant to one or more of the previously used antibiotics [96, 148]. To select an appropriate third-line treatment, endoscopy followed by bacterial culture and antibiotic susceptibility testing is advisable.

An overview of the commonly used dual-, triple- and quadruple therapies is given in Table 2.

3. PREVALENCE OF ANTIMICROBIAL RESISTANCE

As antibiotic resistance in *H. pylori* seems to be the main reason of therapy failure, detection of antibiotic resistance is of importance. Antibiotic susceptibility of *H. pylori* is usually assessed by culture-based methods such as E-test, agar dilution and disc diffusion. These methods offer the opportunity to determine the minimal inhibitory concentration (MIC) of antibiotics, but on the other hand they are time-consuming and results are not always consistent [100, 147]. Factors such as cell viability, inoculation size, incubation conditions, and growth media may affect their outcome [57]. Molecular-based methods are independent of these factors, and thus they offer an attractive alternative. These tests give reproducible results and are easily standardized. Moreover, they are faster than the

Table 2. Current therapies used for treatment of *H. pylori* infections.

Regimen[a]	Dose[b]	Duration (days)
Dual therapy		
RBC + clarithromycin[c]	400 mg b.i.d. / 500 mg b.i.d.	14
PPI + clarithromycin[c]	40 mg q.i.d. / 500 mg t.i.d.	14
PPI + amoxicillin[c]	30 mg t.i.d. / 1g t.i.d.	14
Triple therapy		
Bismuth + metronidazole + tetracycline[c]	120 mg q.i.d. / 250 mg q.i.d. / 500 mg q.i.d.	14
RBC + clarithromycin + amoxicillin	20 mg b.i.d. / 500 b.i.d. / 1 g b.i.d.	7-10
PPI + clarithromycin + amoxicillin [c]	20-30 mg b.i.d. / 500 b.i.d. / 1 g b.i.d.	7-10
PPI + clarithromycin + metronidazole	20 mg b.i.d. / 500 b.i.d. / 500 mg b.i.d.	7-10
PPI + amoxicillin + metronidazole	20 mg b.i.d. / 1 g b.i.d. / 500 mg b.i.d.	7-10
Quadruple therapy		
PPI + bismuth + metronidazole + tetracycline	20 mg b.i.d / 120 mg .q.i.d. / 500 mg t.i.d. / 500 mg q.i.d.	4-7[d]

[a] RBC, ranitidine bismuth citrate; PPI, proton pump inhibitor.
[b] b.i.d., twice daily; t.i.d., three times dialy; q.i.d. four times daily.
[c] Therapies approved by the Food and Drug Administration (FDA).
[d] Antibiotics given for 4 to 7 days; PPI is usually started 3 days earlier.

conventional culture-based assays, and when applied directly on gastric biopsy samples, data can be obtained at the day of endoscopy. Currently available molecular detection methods are summarized in Table 4 and will be discussed in paragraph 6.

The prevalence of antibiotic resistance in *H. pylori* has been assessed worldwide. However, most studies originated from single centers, included only a small number of strains, were often restricted to selected patients, and used different techniques to assess antibiotic susceptibility. Antibiotic resistance rates should preferentially be obtained from large-scale multicenter surveillance programs using standardized detection methods in order to reduce the probability of under- or overestimation of the prevalence of antibiotic resistance in *H. pylori*. However, these surveillance programs are expensive; and only performed in few countries [12, 42, 60, 71, 72, 100, 105, 160], more often investigator driven rather than government induced.

Antibiotic resistance in *H. pylori* is already widespread and increasing. Metronidazole resistance (MIC $\geq$ 8 mg/L) is the most common antimicrobial resistance in *H. pylori*. In industrialized countries approximately 35% of the *H. pylori* strains are metronidazole-resistant [42, 105], whereas in developing countries, the *H. pylori* resistance rates for metronidazole are very high, and in some areas virtually all *H. pylori* strains are metronidazole-resistant [36]. This correlates with the frequent use of metronidazole and related nitroimidazoles for gynaecological-, dental-, and parasitic-related diseases [97].

In comparison with metronidazole resistance, the prevalence of clarithromycin resistance (MIC $\geq$ 2 mg/L) in *H. pylori* is much lower. In industrialized countries, approximately 10% of the *H. pylori* strains is clarithromycin-resistant [33, 42, 105]. In developing countries, resistance rates to clarithromycin are higher and vary between 25 to 50% [102, 141, 153].

Until the end of the 20th century, it was thought that amoxicillin resistance (MIC $\geq$ 0.5 mg/L) and tetracycline resistance (MIC $\geq$ 4 mg/L) were very rare or absent in *H. pylori*. This is in contrast with various other bacteria, where resistance to these antibiotics is widespread. However, the incidence of amoxicillin and tetracycline resistance in *H. pylori* seems to increase [18, 28, 30, 102], especially in certain geographic regions (e.g. Italy, Brazil, Salvador, India, and Lithuania), where these antibiotics can be obtained without prescription. *H. pylori* resistance rates of 72% and 59% for amoxicillin and tetracycline, respectively, have recently been reported in Shanghai, China [161].

4. CLINICAL IMPACT OF ANTIBIOTIC RESISTANCE

Numerous studies have shown that antimicrobial resistance significantly impairs the efficacy of anti-*H. pylori* therapy [63, 84, 149], nevertheless, the clinical relevance of antibiotic resistance in *H. pylori*-associated diseases is still challenged. The extent to which antibiotic resistance reduces *H. pylori* eradication rates depends on a variety of factors, such as the components used in therapy, the dose of the antimicrobial drugs, the duration of therapy, and the level of resistance present in the *H. pylori* strain [148, 162].

Several studies have shown that when a patient is colonized with a metronidazole-resistant *H. pylori* strain, the eradication rates of metronidazole-containing PPI-based triple therapies drops about 20%, from 93% (86-100%) for metronidazole-susceptible strains to 71% (45-94%) for resistant strains [63, 98, 149]. Metronidazole resistance may even have a larger impact on eradication rates obtained with metronidazole-containing PPI-based triple therapies. With these therapies, eradication rates was 91% (56-97%) for susceptible strains versus 63% (17-96%) for resistant strains [63, 98, 149]. Addition of a bismuth component to a PPI-based therapy (quadruple therapy) increased the efficacy of the therapy, from 91% (80-100%) for metronidazole-susceptible strains versus 77% (53-100%) for resistant strains [149]. Although studies on ranitidine bismuth citrate-based triple therapies versus metronidazole resistance are limited, it seems that the eradication rates were almost similar to that of the quadruple therapy, 99% (97-100%) for metronidazole-susceptible strains to 76% (57-95%) for resistant strains [19, 63].

As the prevalence of clarithromycin resistance in *H. pylori* is low, most studies that assessed the effect of clarithromycin resistance on therapy efficacy only included a small number of patients. Despite this limitation, it seems that clarithromycin resistance reduces the efficacy of all clarithromycin-containing regimens dramatically [98]. The eradication rates of clarithromycin-containing dual therapies (PPI or bismuth compounds) decreased approximately with 40%, from 67% (62-74%) for clarithromycin-susceptible strains to 28% (20-40%) for clarithromycin-resistant strains [98]. Although the eradication rates for clarithromycin-containing triple therapies (PPI and amoxicillin or metronidazole) increased (90%) for clarithromycin-susceptible strains, the rates remained low (36%) for clarithromycin-resistant strains [63, 98].

Therapy failure due to the presence of amoxicillin- and tetracycline-resistant *H. pylori* strains has been reported [27, 29, 30, 106, 122], however there is not enough data available yet to measure the impact of these resistances on treatment success.

5. MOLECULAR MECHANISMS OF ANTIBIOTIC RESISTANCE

Antibiotic resistance in *H. pylori* has been described for all of the above-mentioned antibiotics. The molecular mechanisms of antibiotic resistance characterized thus far are all based on point mutations located on chromosome (Table 1). This is in contrast with antibiotic resistance mechanisms observed in other bacteria, where they are frequently located on plasmids, transposons or integrons [44]. In *H. pylori* the majority of antibiotic resistances probably arise *de novo*, although *in vivo* horizontal gene transfer among susceptible and resistant strains has been described [127].

5.1 Metronidazole activity and mechanism of resistance

Metronidazole was initially promoted for the treatment of gynaecological infections caused by *Trichomonas vaginalis*, but soon it became apparent that the drug was also active against anaerobic and some microaerophilic bacteria [35, 103]. As metronidazole is actively released into the gastric juice [152] and its antimicrobial activity is only marginally affected by low pH [22], this drug is highly effective against *H. pylori*.

Metronidazole is administered as a prodrug that needs to be activated within the target cell by one or two electron reduction processes (Figure 1A). This reduction leads to the formation of nitro-anion radicals and metronidazole intermediates that cause lethal damage to subcellular structures and DNA [126]. Theoretically, any protein that possesses a low redox potential can accept electrons from metronidazole, and thus activate the drug. In *H. pylori* several putative electron acceptors have been identified, including ferredoxin (*fdxA*), ferredoxin-like protein (*fdxB*), flavodoxin (*fldA*), NAD(P)H flavin nitroreductase (*frxA*), 2-oxoglutarate oxidoreductase (*oorD*), pyruvate:ferredoxin oxidoreductase (*porD*), and oxygen-insensitive NAD(P)H nitroreductase (*rdxA*) [3, 139].

In *H. pylori*, levels of metronidazole resistance are very diverse, with MICs ranging from 8 to ≥ 256 mg/L [21, 78]. This spread in MIC values suggests that high-level metronidazole resistance can result from mutational changes in one locus, but it is more likely that several pathways are involved. Potential mechanisms of metronidazole resistance studied in *H. pylori* include (i) deficient drug uptake and/or increased drug efflux [83, 108]; (ii) enhanced activity of DNA repair enzymes [14, 137]; (iii) increased oxygen-scavenging capabilities [104, 129]; and (iv) decreased drug activation arising from changes in metronidazole-reducing enzymes [46, 78, 80].

Metronidazole is a typical substrate of resistance-nodulation-division (RDN) multiple-drug efflux mechanism that plays a role in the antibiotic resistance of several bacteria. In the *H. pylori* genome three putative RDN efflux systems have been identified [8]. When these three RDN operons were mutated, it did not affect the *in vitro* susceptibility of *H. pylori* to nineteen antibiotics. Although metronidazole was not included in this study, it was suggested that the expression levels of these proteins were too low to influence antibiotic susceptibility in general [8].

The *recA* gene encodes a protein that plays an essential role in DNA recombination and DNA repair. In *H. pylori*, a *recA* homologue has been identified and functionally characterized. *H. pylori* mutants with an inactivated *recA* gene are severely impaired in their ability to survive UV light treatment and exhibit enhanced susceptibility to metronidazole [14, 137]. Although transfer of the *recA* gene of several metronidazole-resistant *H. pylori* strains to metronidazole-susceptible *H. pylori* strains resulted in an increased MIC of metronidazole, sequence analysis did not established that observed resistance was caused by mutational changes in the *recA* gene [14].

Classic NAD(P)H oxidase activities have been found in several organisms, including *H. pylori* [129]. The exact role of this type of enzymes in metronidazole resistance is unknown, but it has been postulated that they are involved in scavenging of oxygen. NADPH oxidase activity was almost identical for metronidazole-susceptible and metronidazole-resistant *H. pylori* isolates, whereas NADH oxidase activity was three times higher in metronidazole-susceptible *H. pylori* isolates than in metronidazole-resistant *H. pylori* isolates [67, 129]. Low levels of NADH oxidase may compromise intracellular oxygen tension, allowing the redox potential to rise sufficiently to prevent the activation of metronidazole, leading to metronidazole resistance. In *H. pylori*, no genes homologous to NADH oxidase have been found; however, the NADH oxidase activity seems to be associated with the proteins pyruvate oxidoreductase, alkyl hydroperoxide reductase, and glutathione reductase [104, 129, 144].

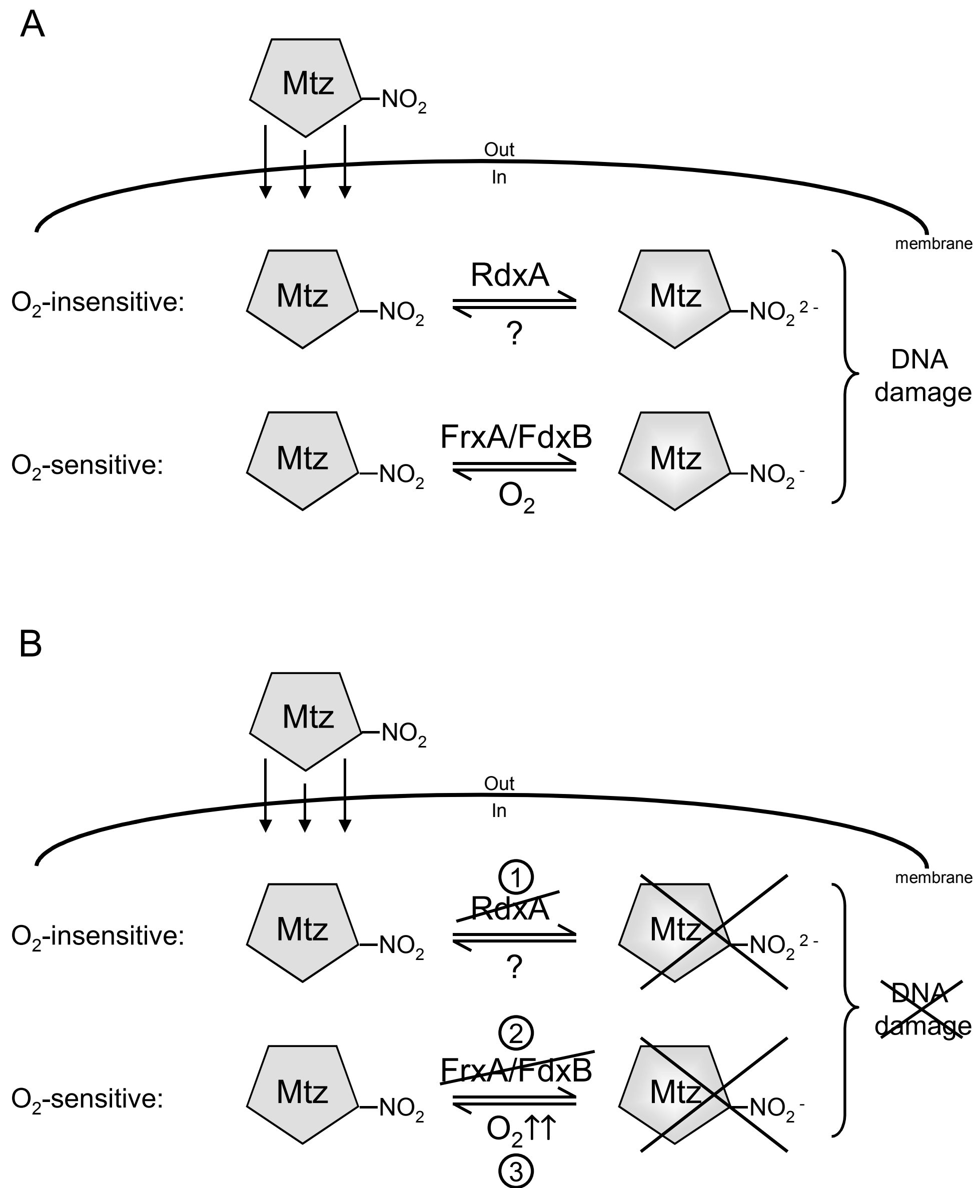

Figure 1. Mode of action of metronidazole and the proposed mechanisms of metronidazole resistance in *H. pylori*. (A) Metronidazole is a prodrug that needs to be activated within the target cell by one (oxygen-sensitive) or two (oxygen-insensitive) electron reduction processes. Reduction of metronidazole leads to the formation of nitro-anion radicals and metronidazole intermediates (NO_2^- and NO_2^{2-}) that cause lethal damage to subcellular structures and DNA. (B) Proposed mechanism of metronidazole resistance in *H. pylori*. Metronidazole resistance in *H. pylori* is mediated by ① mutations in the *rdxA* gene; ② mutations in the *frxA* and *fdxB* genes; and/or ③ decreased levels of NADH oxidase that compromise intracellular oxygen tension.

An important step in the elucidation of the molecular mechanism of metronidazole resistance in *H. pylori* came with the discovery that null mutations in the *rdxA* gene induced metronidazole resistance in a formerly metronidazole-susceptible strain [50]. Many studies have subsequently shown that the majority of metronidazole-resistant *H. pylori* strains contain various mutations within the *rdxA* gene, including frameshifts, premature stop codons, insertion of transposable elements, codon changes resulting in amino acid substitution, and promoter alterations [6, 23, 68, 69, 82, 135]. However, it soon became apparent that the mechanism of metronidazole resistance in *H. pylori* was even more complex, as indications for the involvement of other reducing factors became available [69, 78-80]. Mutational changes of the *frxA* and *fdxB* genes may contribute to metronidazole resistance [69, 78-80].

As metronidazole-resistant *H. pylori* isolates *in vitro* become metronidazole-susceptible after exposure to short periods of anaerobiosis [13, 128], it was suggested that metronidazole susceptibility may be restored at low oxygen conditions through the activation of potential anaerobic reduction pathways that function less, or not all, under microaerophilic conditions. [67, 129]. Using the protein synthesis inhibitor chloramphenicol, the loss of metronidazole resistance under anaerobic conditions still occurred. This suggests that metronidazole is reduced by processes that work under anaerobic conditions and does not require *de novo* protein synthesis (Chapter 7).

A schematic overview of the molecular mechanisms underlying metronidazole resistance in *H. pylori* is given in Figure 1B.

5.2 Clarithromycin activity and mechanism of resistance

Clarithromycin is a bacteriostatic antibiotic that belongs to a group of macrolides that bind to peptidyl transferase loop of domain V the 23S rRNA molecule. This binding interferes with protein elongation, and thus effectively blocks bacterial protein synthesis. The antibacterial activity of clarithromycin is similar to that of other macrolides, but clarithromycin is better absorbed in the gastric mucus layer, more acid-stable, and therefore more effective against *H. pylori* [117].

Resistance to clarithromycin in *H. pylori* is caused by point mutations in two adjacent 23S rRNA nucleotides, namely 2142 and 2143. It can be induced by an adenine (A) to guanine (G) substitution at one of these positions [155] or an adenine (A) to cytosine (C) substitution solely at position 2142 (Figure 2) [132]. In *H. pylori* these substitutions cause decreased affinity of the ribosomes for several macrolides, resulting in increased resistance [111]. The $A_{2142}\underline{G}$ and $A_{2142}\underline{C}$ (underlined letters are used to represent the base pair changes) were significantly more present in isolates with a higher MIC of clarithromycin (> 64 mg/L), whereas the $A_{2143}\underline{G}$ substitution was often found in isolates with a lower MIC (< 64 mg/L) [111, 150, 154, 155]. Occasionally, other 23S rRNA mutations have also been reported for *H. pylori*; some of them are associated with high-level resistance, while others are associated with low-level resistance [34, 54, 64, 65, 70, 124, 140, 157]. To define the apparently high prevalence of the A to G at position 2142 and 2143 (Table 3) among clarithromycin-resistant *H. pylori* clinical isolates, site-directed mutants were created containing either a G, C, or T (thymine) substitution at position 2142 or 2143. In a similar isogenic background it was seen that the preference for A to G substitutions results from higher growth rates, higher MICs and more stable resistance [20, 158].

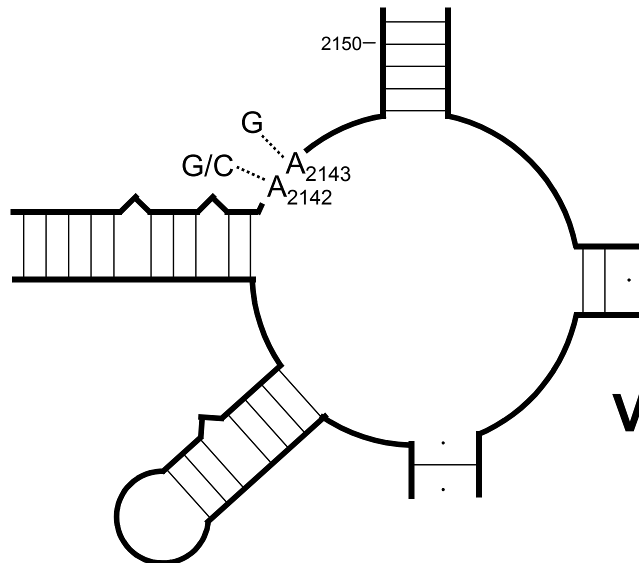

Figure 2. Proposed mechanism of clarithromycine resistance in *H. pylori.* Schematic representation of domain V of the *H. pylori* 23S rRNA, modified from 23S rRNA structure of *Escherichia coli* proposed by Vester *et al.* [156]. The nucleotide changes, i.e. $A_{2142}\underline{G}/\underline{C}$ and $A_{2143}\underline{G}$ (numbering according to the 23S *rrlA* gene of *H. pylori* strain 26695) are associated with clarithromycin resistance in *H. pylori.*

Table 3. Prevalence of 23S rRNA gene mutations conferring clarithromycin resistance in *H. pylori*.

Geographic region	Prevalence 23S rRNA mutation		
	$A_{2142}\underline{G}$	$A_{2142}\underline{C}$	$A_{2143}\underline{G}$
Europe [1, 151]	23-33%	2-10%	44-67%
North America [132, 154]	48-53%	0-7%	39-45%
South America [121, 124]	6-19%	0%	74%-83%
Middle-East [107]	5%	15%	75%
Far-East [32, 88, 92, 163]	0-8%	0%	66-100%

H. pylori contains two 23S rRNA genes and mutations are generally found in both copies, however, heterogeneity has been described [65, 132, 155]. Heterogeneity still results in clarithromycin resistance, but it generally appears to be associated with lower resistance levels than present in homogenic isolates [65, 132]. The higher prevalence of homogeneity over heterogeneity in *H. pylori* may reflect a high efficiency of DNA recombination in this organism. The mutation in one copy of the 23S rRNA may be easily transferred to the other 23S rRNA gene by efficient homologous DNA recombination under selective pressure, conferring higher levels of clarithromycin resistance.

As expected, clarithromycin resistance coincides with resistance to other macrolides [158]. The $A_{2142}\underline{G}$ and $A_{2142}\underline{C}$ mutations are linked to high-level cross-resistance to all macrolides, whereas the $A_{2143}\underline{G}$ mutation give rise to high-level resistance to erythromycin and intermediate-level resistance to clindamycin and streptogramin [37, 158].

5.3 Amoxicillin activity and mechanism of resistance

Amoxicillin is a bactericidal antibiotic that belongs to group of penicillins. It binds to penicillin-binding proteins (PBPs), and thus interferes with bacterial cell wall synthesis, resulting in the lyses of replicating bacteria. The antibacterial affect of amoxicillin is similar to that of other β-lactam antibiotics, but amoxicillin is released in the gastric juice [110], and thus frequently used in anti-*H. pylori* therapy. Until recently, it was thought that amoxicillin resistance in *H. pylori* did not exist, although resistance to this antimicrobial drug could be induced by continuous exposure of *H. pylori* to amoxicillin *in vitro* [24, 116].

In Gram-negative bacteria, resistance to β-lactam antibiotics is mostly due to the production of β-lactamase, either chromosomally, or plasmid encoded [11]. In *H. pylori*, however, there are no indications that amoxicillin resistance is due to the acquisition or expression of β-lactamase, since no β-lactamase activity has been detected by nitrocephin or cephalosporinase assays (Chapters 8-10, and [26-28, 77, 113]). Other potential mechanisms in *H. pylori* that have been studied in relation to amoxicillin resistance are (i) structural alterations in penicillin-binding proteins (PBPs); (ii) decreased membrane permeability; and (iii) active efflux (Chapter 9, and [8, 24, 77]).

PBPs are enzymes that are involved in the synthesis and maintenance of the peptidoglycan layer of the bacterial cell wall [53]. In *H. pylori* nine putative PBPs have been identified; three high-molecular-weight PBPs and six low-molecular-weight PBPs [26, 56, 66, 73]. Two of these proteins have been associated with amoxicillin resistance in *H. pylori*. At first, the molecular mechanism was described for amoxicillin-resistant *H. pylori* isolates (MIC > 256 mg/L) obtained from dyspeptic patients from Italy and the United States. As these isolates lost their resistance phenotype upon freezing at -80°C [28], they are often referred to amoxicillin-tolerant. PBP binding studies using ^{3}H-benzylpenicillin have shown that amoxicillin resistance in these amoxicillin-tolerant *H. pylori* isolates is mediated by a lack of PBP-D (also called PBP4) [26]. Whether the decreased labeling was caused by reduced expression of the *pbpD* gene or decreased affinity of PBP-D to amoxicillin was not further discussed.

Later, stable amoxicillin-resistant *H. pylori* isolates were obtained with MICs ranging from 8 to 64 mg/L (Chapters 8 and 10, and [27, 77, 113]). Amoxicillin resistance in these isolates was caused by various point mutations in the *pbp1A* gene (Chapter 9-10, and [27, 77, 113]). Additional proof for the role of PBP1A in *H. pylori* amoxicillin resistance was provided by PBP binding studies using biotinylated amoxicillin. Labeling of PBP1A was significantly decreased in amoxicillin-resistant *H. pylori* isolates compared to amoxicillin-susceptible *H. pylori* isolates [24, 113]. This decrease in labeling was caused by reduced affinity of PBP1A for amoxicillin, instead of diminished production of PBP1A molecules [24, 113].

In addition to changes in the *pbp1A* gene, it was postulated that reduced membrane permeability and/or active efflux of amoxicillin in *H. pylori* might also contribute to higher levels of amoxicillin resistance. Both aspects have been tested in a quantitative uptake experiment using the proton translocator CCCP. In this experiment it was seen that amoxicillin-resistant *H. pylori* strains accumulated <60% [^{14}C]-penicillin G compared to amoxicillin-susceptible *H. pylori* strains, both in the presence and absence of CCCP; excluding the role of an active efflux mechanism. This suggests that amoxicillin resistance in *H. pylori* in part is due to an increased diffusional barrier [24, 77], an effect that may be explained by alterations in outer membrane proteins [77].

The molecular mechanisms underlying amoxicillin resistance in *H. pylori* are summarized in Figure 3.

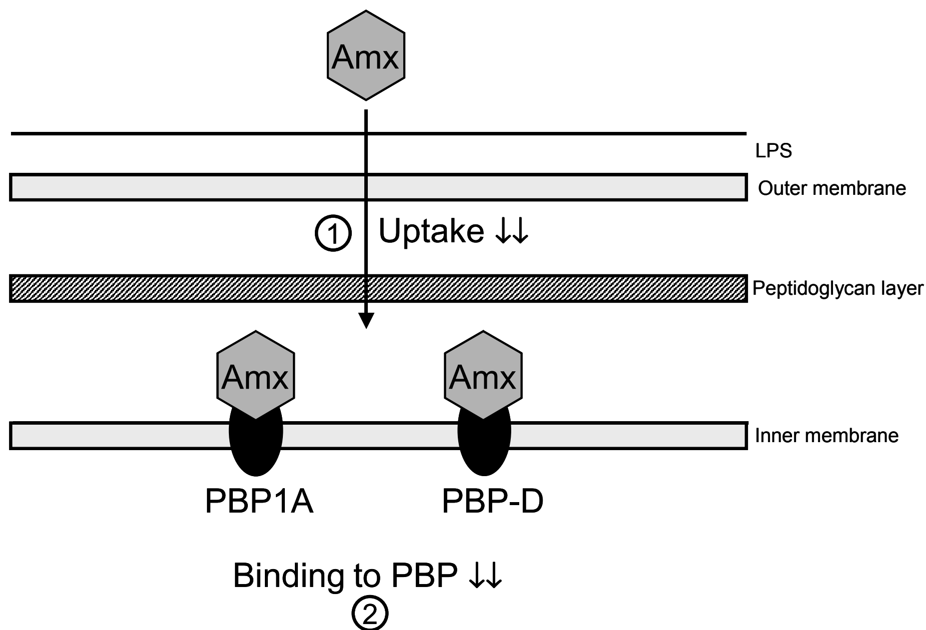

Figure 3. Schematic overview of proposed mechanisms of amoxicillin resistance in *H. pylori.* Amoxicillin resistance in *H. pylori* is mediated by ① decreased membrane permeability; and/or ② reduced affinity of PBP molecules, PBP1A and PBP-D, for amoxicillin.

5.4 Tetracycline activity and mechanism of resistance

Tetracycline is a bacteriostatic antibiotic that is active against several Gram-positive and Gram-negative bacteria, including *H. pylori*. It accumulates in the cytoplasm, where it binds to the 30S ribosomal subunit. Here it interferes with the attachment of aminoacyl-tRNA to the ribosome, and inhibits protein synthesis and bacterial growth [10, 119].

Tetracycline is an antibiotic that is extensively used in many industrialized countries, and as a consequence resistance to this drug has become an emerging problem. In general, four tetracycline resistance mechanisms have been identified: (i) deficient drug uptake and/or increased drug efflux; decreased antibiotic binding by (ii) changes in ribosomal protection proteins, or (iii) mutations 16S rRNA tetracycline binding site; and (iv) enzymatic inactivation of tetracycline [17]. In *H. pylori* only the first three mechanisms have been studied (Chapter 3, and [8, 18, 145]), since orthologs of tetracycline-degrading enzymes are absent in the *H. pylori* genome sequence [139].

In many bacteria, resistance to tetracycline is due to an energy-dependent efflux of tetracycline-cation complexes across the cell membrane by membrane-associated proteins. The exchange of tetracycline-cations by protons reduces the intracellular drug concentration and protects ribosomes against tetracycline. Overexpression of the efflux proteins often confers multidrug resistance, while deletions in these operons increases tetracycline susceptibility [17]. In *H. pylori* the role of several putative efflux proteins in tetracycline resistance has been assessed by mutagenesis [8] and natural transformation (Chapter 3). However, none of the tested efflux systems seems to play a role in tetracycline resistance of *H. pylori* (Chapter 3, and [8]).

The second common mechanism that mediates tetracycline resistance acts by ribosomal protection proteins. These cytoplasmic proteins confer tetracycline resistance either by reduced affinity of ribosomes for tetracycline, or by released binding of the drug to the ribosomes [17]. Although the *H. pylori* genome encodes for several proteins that have homology with the known ribosomal protection proteins, such as elongation factors EF-G, EF-Tu, and initiation factor IF-2, transformation experiments have shown that they probably are not involved in tetracycline resistance of *H. pylori* (Chapter 3).

The most likely mechanism for tetracycline resistance in *H. pylori* is based upon a triple-base-pair substitution in three adjacent 16S rRNA residues, namely $AGA_{926\text{-}928}\underline{TTC}$ (Figure 4) (Chapters 3-5, and [18, 145]). This mutation is located in the primary binding site of tetracycline, and may affect the affinity of the drug-ribosome interaction and thus reduce the efficiency of tetracycline as translational inhibitor. Besides this triple-base-pair substitution, $AGA_{926\text{-}928}\underline{TTC}$ (Chapters 3 and 5, and [145]), several single- and double-base-pair substitutions, i.e. $A_{926}\underline{G}$, $A_{926}\underline{T}$, $A_{928}\underline{C}$, $AG_{926\text{-}927}\underline{GT}$ and $A_{926}\underline{G}/A_{928}\underline{C}$ have been reported to be involved in tetracycline resistance in *H. pylori* [18]. High-level tetracycline seems to be related to the triple-base-pair substitution only (Chapters 3-5, and [145]), whereas low-level tetracycline resistance (MICs below susceptibility breakpoint of 4 mg/L) is associated the single- and double-base-pair substitutions (Chapters 4 and 6, and [18]). Using site-directed mutagenesis, it was demonstrated that the preference for the $AGA_{926\text{-}928}\underline{TTC}$ mutation in high-level tetracycline resistance apparently results from higher growth rates in the presence of tetracycline, higher MICs and more stable resistance (Chapter 4).

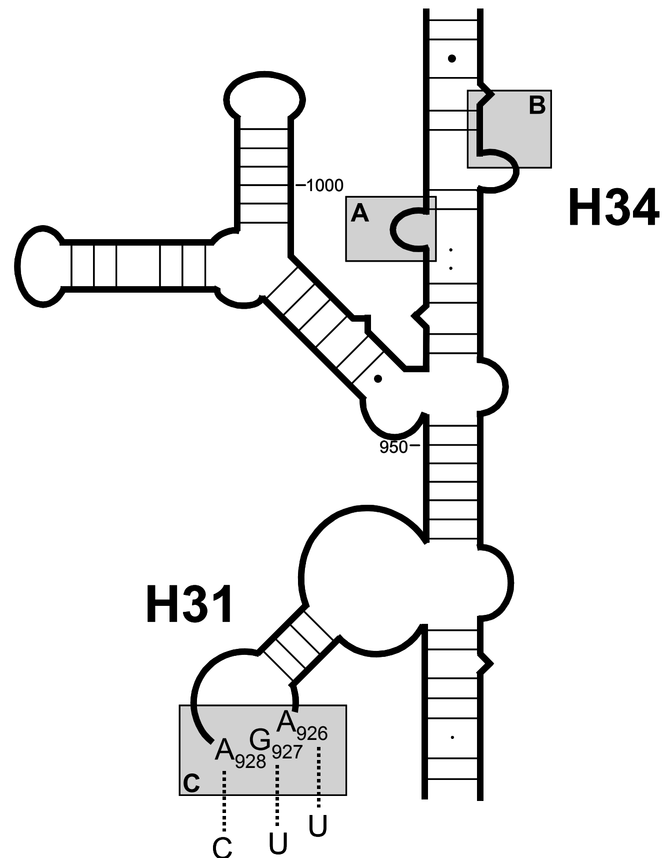

Figure 4. Proposed mechanism of tetracycline resistance in *H. pylori.* Schematic representation of the primary binding site of tetracycline in *H. pylori*, modified from the 16S rRNA structure of *H. pylori* proposed by Trieber *et al*. [145]. In *H. pylori* the primary binding site for tetracycline is formed by the residues by the 16S rRNA residues 1020-1023 (Box A), 1162-1166 (Box B), and 925-928 (Box C) The nucleotide changes $AGA_{926\text{-}928}$TTC (numbering according to the 16S *rrnA* gene of *H. pylori* strain 26695) located in box C is associated with clinical relevant levels of tetracycline resistance in *H. pylori*.

5.5 Activity of to less commonly used antibiotics and their mechanism of resistance

As resistance against the four commonly used antibiotics of the anti-*H. pylori* therapy is increasing, fluoroquinolones, nitrofurans and rifamycins are occasionally being introduced in second- and third-line therapies [40, 45, 143, 164]. Initial results obtained with these antibiotics were promising, but soon the eradication rates dropped as resistance against these drugs developed [21, 62, 81, 102, 109, 136]. The underlying resistance mechanisms have been characterized and will be discussed in the next paragraphs.

5.5.1 Fluoroquinolones

Fluoroquinolones (i.e. ciprofloxacin, moxifloxacin, trovafloxacin and levofloxacin) are bactericidal antibiotics that exert their antimicrobial activity by inhibiting the enzyme DNA gyrase. This enzyme is a tetramer consisting of two A subunits and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively [109]. The main function of this enzyme is to catalyze the negative supercoiling of DNA [123]. Most *H. pylori* isolates are susceptible to fluoroquilones, but the incidence of fluoroquinolone resistance seems to increase [71]. In *H. pylori*, the resistance is caused by point mutations in the so-called quinolone resistance-determining region (QRDR, located between amino acid position 67 and 106) of the *gyrA* gene at amino acids position 87, 88, 91 and 97 [109, 136].

5.5.2 Nitrofurans

Furazolidone and nitrofurantoin are nitroheterocyclic and nitroaromatic compounds that share similarities with metronidazole both in their structures and modes of action. *H. pylori* is usually susceptible to furazolidone and nitrofurantoin, but occasionally strains with an increased MIC have been reported [81, 102]. The resistance mechanism of furazolidone and nitrofurantoin is currently unknown, but it is clear that it differs from that of metronidazole, since inactivation of the *rdxA*, *frxA* and *fdxB* genes, did not result in furazolidone or nitrofurantoin resistance [81].

5.5.3 Rifamycins

Rifabutin and several other derivates of rifampin are bactericidal antibiotics that bind to the β-subunit of DNA-dependent RNA polymerase, resulting in inhibition of transcription [76]. The β-subunit of this complex is encoded by the *rpoB* gene [76]. Until a few years back resistance against rifamycins and rifabutin *in vivo* was very rare, however, the incidence of rifamycin and rifabutin resistance is increasing [71]. In *H. pylori* resistance to these antibiotics is linked to various point mutations in the *rpoB* gene (corresponding to amino acid codon 149, 524-545 and 586) [59, 61, 62].

6. MOLECULAR DETECTION OF ANTIBIOTIC RESISTANCE

Since in *H. pylori* most resistances is due to specific mutations, molecular-based methods offer an attractive alternative to the conventional culture-based methods. Molecular-based methods are independent of cell viability or growth rate of the bacteria, and thus more consistent and reproducible than the culture-based ones. Moreover, molecular-based methods are much faster, and if directly applied to gastric biopsy specimens, results can be obtained at the day of endoscopy. Although DNA sequencing is the gold standard for the detection of mutational changes, this method is not cost effective in a routine setting. Numerous molecular-based methods are now available to assess clarithromycin, tetracycline, and ciprofloxacin resistance in *H. pylori* (Table 4), but lack for the assessment of metronidazole and amoxicillin resistance.

6.1 Molecular detection of metronidazole resistance in *H. pylori*

The development of rapid, genotype-based tests for metronidazole resistance in *H. pylori* is not easy, because the resistance is associated with various unrelated mutations within the *rdxA* gene and other reductase-encoding genes. Despite these barriers, there is new evidence that indicates that a system could be developed based on detection of the RdxA protein. Using immunoblot with specific anti-RdxA antibody [87], a 24 kDa immunoreactive band was observed in all metronidazole-susceptible isolates but was absent in most (90%) metronidazole-resistant isolates [86].

6.2 Molecular detection of clarithromycin resistance in *H. pylori*

Numerous techniques have been developed for the detection of clarithromycin resistance in *H. pylori.* Most assays are polymerase chain reaction (PCR)-based using different methods to study the amplicons. Restriction fragment length polymorphism (RFLP) is an simple method that is based on the occurrence of restriction site within the amplicon. This assay allows for the detection of the previously mentioned 23S rRNA mutations using the restriction endonucleases, *Mbo*II ($A_{2142}\underline{G}$), *Bbs*I ($A_{2142}\underline{G}$), *Bsa*I ($A_{2143}\underline{G}$) and *Bce*AI ($A_{2142}\underline{C}$) [101, 111, 134, 154]. As the PCR-RFLP was initially not able to detect the $A_{2142}\underline{C}$ mutation, a 3'-mismatch reverse primer PCR method (3M-PCR) was developed [2].

Other methods, such as PCR-DNA enzyme immunoassay (DEIA), PCR oligonucleotide ligation assay (OLA), preferential homoduplex formation (PHFA) and PCR-line probe assay (LipA), include an additional hybridization step after the PCR. The PCR products were hybridized with labeled oligonucleotide probes under highly stringent conditions and hybrids were subsequently detected with specific antibodies or streptavidin-alkaline phosphatase [89, 118, 132, 150].

Recently, several real-time PCR hybridization assays have been developed. In these assays a 23S rDNA fragment is amplified in the presence of a fluorescent-labeled mutation and anchor probe. When these probes hybridize with the PCR product, a fluorescence signal is emitted. After completion of the PCR, the temperature is increased to determine the melting point of the mutation probe. The temperature at which the fluorescent signal drops indicates the point at which the mutation probe dissociates (melting point). When there are mismatches present in the target sequence, lower melting temperatures are obtained compared to the matched hybrid. This technique is simple and quick, and if applied directly on gastric tissue, results can be obtained within 3 hours [16, 38, 93, 114].

There is also a possibility to detect clarithromycin resistance without performing PCR, by using fluorescence in situ hybridization (FISH). In this assay intact *H. pylori* are hybridized with fluorescent-labeled *H. pylori*-specific 16S and 23S rRNA probes. The labeled bacteria were subsequently visualized by fluorescence microscopy. This assay allows detection of *H. pylori* and clarithromycin resistance simultaneously. Moreover, this assay does not require DNA preparation and can directly be applied to gastric biopsy samples [142].

6.3 Molecular detection of other antibiotics resistances in *H. pylori*

As the molecular mechanism underlying tetracycline resistance in *H. pylori* has only recently been identified, data on molecular detection techniques are still limited. Thus far, two PCR-based methods have been developed; (i) a PCR-RFLP (Chapter 5), and (ii) a real-time PCR assay (Chapter 6). In addition to the molecular detection methods for clarithromycin and tetracycline a real-time PCR assay has been develop for ciprofloxacin resistance based on the same principle [41].

Table 4. Molecular methods for detection of clarithromycin, metronidazole and tetracycline resistance in *H. pylori*.

Antibiotic	Molecular method	References
Metronidazole	Immunoblotting with specific antibodies	[86, 87]
Clarithromycin	PCR-restriction fragment length polymorphism (RFLP)	[101, 111, 134, 154]
	PCR-oligonucleotide ligation assay (OLA)	[132]
	PCR-DNA enzyme immunoassay (DEIA)	[118]
	PCR-line probe assay (LipA)	[150]
	PCR-preferential homoduplex formation assay (PHFA)	[89]
	3'-mismatched reverse primer PCR (3M-PCR)	[2, 31]
	Real time PCR hybridization assay	[16, 38, 93, 114]
	Fluorescent in situ hybridization (FISH)	[142]
Tetracycline	PCR-restriction fragment length polymorphism (RFLP)	Chapter 5
	Real time PCR hybridization assay	Chapter 6
Ciprofloxacin	Real time PCR hybridization assay	[41]

7. CONCLUSIONS

Antibiotic resistance in *H. pylori* is widespread and further increasing. This constitutes a considerable clinical problem, as antibiotic resistance negatively affects the efficacy of anti-*H. pylori* therapy. For this reason antibiotic resistance in *H. pylori* should be monitored. Data obtained in such a surveillance program are of great value to guide anti-*H. pylori* therapy and help to gain a better understanding of the effect of resistance on therapy outcome. However, in most countries, including The Netherlands, these surveillance programs are lacking, and therapy recommendations may be based on insufficient data.

Conventional culture-based susceptibility testing of *H. pylori* is relatively slow and cumbersome. Thus molecular-based methods offer an attractive alternative. Using the knowledge on molecular mechanisms underlying antibiotic resistance in *H. pylori*, as described in this review, several molecular-based method have been developed (Table 4). It is likely that real-time PCR will have an expanding role in the rapid detection of antibiotic resistance in *H. pylori*, particularly if results are obtained directly from gastric

biopsy samples without the need for cultivation. Since for amoxicillin and metronidazole resistance in *H. pylori* no appropriate tests are available yet, research on the elucidation of molecular mechanisms and development of molecular detection methods should be continued.

As novel antibiotics are not rapidly forthcoming the search for new antimicrobial agents and co-therapies should be intensified. Antimicrobial peptides [4, 25, 58], porphyrines [130], essential oils [7, 112], but also probiotics [47] may prove to be helpful. Although initial studies using these components are promising, there is still a long way to go before they can and will be used in clinical practice.

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CHAPTER 3

16S rRNA mutation-mediated tetracycline resistance in *Helicobacter pylori*

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ABSTRACT

Most *Helicobacter pylori* strains are susceptible to tetracycline, an antibiotic commonly used for the eradication of *H. pylori*. However, an increase in incidence of tetracycline resistance in *H. pylori* has recently been reported. Here the mechanism of tetracycline resistance of the first Dutch tetracycline-resistant (Tet^R) *H. pylori* isolate (strain 181) is investigated. Twelve genes were selected from the genome sequences of *H. pylori* strains 26695 and J99 as potential candidate genes, based on their homology with tetracycline resistance genes in other bacteria. With the exception of the two 16S rRNA genes, none of the other putative tetracycline resistance genes was able to transfer tetracycline resistance. Genetic transformation of the tetracycline-susceptible (Tet^S) strain 26695 with smaller overlapping PCR fragments of the 16S rRNA genes of strain 181, revealed that a 361-bp fragment that spanned nucleotides 711 to 1071 was sufficient to transfer resistance. Sequence analysis of the 16S rRNA genes of the Tet^R strain 181, the Tet^S strain 26695, and four Tet^R 26695 transformants showed that a single triple-base-pair substitution, $AGA_{926\text{-}928}\underline{TTC}$ (underlined letters are used to represent the base pair changes), was present within this 361-bp fragment. This triple-base-pair substitution, present in both copies of the 16S rRNA gene of all our Tet^R *H. pylori* transformants, resulted in an increased minimal inhibitory concentration for tetracycline that was identical to that for the Tet^R strain 181.

INTRODUCTION

Helicobacter pylori is a spiral-shaped, Gram-negative bacterium that causes chronic infections in the gastric musoca [6]. This infection will persist for life, unless treated with antibiotics. Cure of *H. pylori* infection results in ulcer healing and may reduce the risk of gastric cancer and gastric lymphoma [23, 29]. The highest cure rates have been obtained with antimicrobial treatments that include two or more antimicrobial drugs, a bismuth component and/or a proton pump inhibitor [14, 26]. For the treatment of *H. pylori* infections, tetracycline-based triple or quadruple therapies are often used as a second-line treatment [7, 9, 18]. Until the end of the last century only a few reports were published on spontaneous tetracycline resistance [16, 19], and it was generally accepted that tetracycline resistance (MIC ≥ 4 µg/ml) in *H. pylori* is very rare [5, 12]. However, in the last 2 years an increase in the incidence of tetracycline resistance in *H. pylori* has been reported [2, 11, 13, 21, 31].

Tetracycline inhibits the protein synthesis by binding to the 30S ribosomal subunit [3, 20]. In most bacteria resistance to tetracycline is due to an energy-dependent efflux of tetracycline-cation complexes across the cell membrane by membrane-associated efflux proteins. Export of tetracycline-complexes out of the cell reduces the intracellular drug concentration and protects the ribosomes from tetracycline [4]. Overexpression of the efflux genes confers tetracycline resistance, while the sensitivity to tetracycline increases by deletions in these genes. The second common mechanism of resistance is mediated through ribosomal protection proteins. These cytoplasmic proteins confer tetracycline resistance either by a reduction of the affinity of ribosomes for tetracycline or by releasing the bound antibiotic from the ribosome. The ribosomal protection proteins, such as TetM, TetO and TetS show homology with the elongation factors, EF-G and EF-TU (Table 1) [4]. Beside these two most common tetracycline resistance mechanisms two other mechanisms have been described. One is based on enzymatic inactivation of tetracycline by the product of TetX in the presence of oxygen and NADPH, and the other originates from mutations in the 16S rRNA genes that affect the binding site of tetracycline [4, 22, 25].

We recently isolated a tetracycline-resistant (Tet^R) *H. pylori* isolate (strain 181), from a 72-year-old male dyspeptic patient. Here we describe the molecular mechanism of tetracycline-resistance in this strain. To achieve this, twelve genes were selected from the published *H. pylori* genomes [1, 24] as potential candidates, based on their homology with tetracycline resistance genes in other bacteria (Table 1). These putative tetracycline resistance genes were amplified from the genome of the Tet^R strain 181 and used for genetic transformation of the tetracycline-susceptible (Tet^S) strain 26695 in order to identify the changes responsible for tetracycline resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions

H. pylori strains used in this study were the Tet^R strain 181, and the Tet^S *H. pylori* strains 26695 [24], J99 [1], SS1 [15], and ATCC 43504 (American Type Culture Collection). Bacteria were routinely grown on Columbia agar plates (Becton Dickonson, Cockeysville, MD, USA) supplemented with 7% lysed horse blood (BioTrading, Mijdrecht, the Netherlands) and *H. pylori* Dent selective supplement (Oxoid, Basingstoke, UK), referred

Table 1. *Helicobacter pylori* genes potentially involved in tetracycline resistance.

Putative function of selected gene (gene name) [a]	Gene no. (amino acids in ORF)		Primer sequence (5'-3') [b]		Expected product size (bp) [c]
	26695	J99	Forward	Reverse	
Membrane proteins					
GTP-binding membrane protein (*lepA*)	HP0355 (602)	JHP0329 (604)	AGAGTTTGACTGACGCTATT	TTTGCCATAGAAGCTAAACG	1874
GTP-binding membrane protein (*yihK*)	HP0480 (599)	JHP0432 (599)	CGCCATTTGGGGCTATTAT	CCTACAGCTAAAGACTTGCC	2018
α-ketoglutarate permease (*kgtP*)	HP1091 (426)	JHP0334 (437)	TCCCTTTTAGCCGCTAGTTC	ATGACATAGCCCACAAACCC	1181
Tetracycline resistance protein (*tetA*)	HP1165 (386)	JHP1092 (386)	GCAGTCATTCGCTAATTCAA	AACGGCTTAGCCTTATACAA	1418
Multidrug-efflux transporter	HP1181 (443)	JHP1107 (443)	TTTCCATTAGCGTTAGTGTC	CTAAAGTTTTGCGCTAAGTG	1310
Cons. hypoth. integral membrane protein	HP1185 (391)	JHP1111 (391)	CCAAAAGAGCGCCAACAAAC	CTTGCGTGTGGTAGTAATGC	1601
Protein export membrane protein (*secD*)	HP1550 (503)	JHP1449 (526)	CACCCCATAATTGGAATAAC	CTAGAAACTAAAGGCCCTAA	1568
Cytoplasmic proteins					
Translation initiation factor IF-2 (*infB*)	HP1048 (944)	JHP0377 (949)	CGCTAAAGCCTCTTGCAGTA	TGATTGGCAAAGGCCGTAGTT	3041
Translation elongation factor EF-G (*fusA*)	HP1195 (692)	JHP1118 (692)	TTGCTAGGCACTTCGCCATA	ATGGATGCGGCTAGCGATAA	2152
Translation elongation factor EF-Tu (*tufB*)	HP1205 (399)	JHP1128 (399)	TCAGAACACTTCAACCCTA	GTTTCCCGCTCCATTTTTA	1511
Ribosomal RNA genes					
16S ribosomal RNA (*rrnA* and *rrnB*) [d]			TTTATGGAGAGTTTGATCCT	AGGAGGTGATCCAACCGCA	1494

[a] Genes were selected from the published *H. pylori* genomes as potential Tet^R candidate genes, based on their homology with tetracycline resistance genes in other bacteria.

[b] Primers used for amplification were based on the published genome sequences of *H. pylori* strains 26695 (24) and J99 (1).

[c] Fragment length is based on the genome sequence of *H. pylori* strain 26695 (24).

[d] The primers used for amplification of the 16S rRNA genes did not distinguish between the two copies present on the chromosome.

to as Dent plates. Bacteria were inoculated on these plates and incubated for 48 to 72 hours at 37ºC in a microaerophilic atmosphere of 5% O_2, 10% CO_2, 85% N_2. Bacterial stocks were prepared by suspending bacteria, harvested from culture plates with a sterile cotton swab in Brain Heart Infusion (BHI) with 20% glycerol and stored at -80ºC.

Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) was routinely determined with the E-test (AB Biodisk, Solna, Sweden) [8]. Inocula were prepared from a fresh *H. pylori* culture grown routinely for 2 days on Dent plates. Columbia agar plates containing 7% lysed horse blood but no Dent supplement were inoculated with approximately 2×10^8 CFU (colony forming units) in 20 µL of 0.9% NaCl, the plates were dried for 3 to 4 minutes, and then the E-test strips were applied to the agar surface. The plates were incubated at 37ºC under microaerophilic conditions, and three days later the MIC was determined by the intercept of the zone of inhibition with the graded E-test strip. By this method the susceptibility was determined for tetracycline, doxycycline, minocycline, amoxicillin, clarithromycin and metronidazole (AB Biodisk). The isolates were considered resistant when the MICs of tetracycline, doxycycline, and minocycline were $\geq$ 4 mg/L, and of amoxicillin, clarithromycin and metronidazole were $\geq$ 8 mg/L, $\geq$ 2 mg/L and $\geq$ 8 mg/L, respectively [5, 13].

Natural transformation of *H. pylori*.

Bacteria were transformed with ~1 µg genomic DNA, or ~250 ng PCR amplified gene products from strain 181, as described previously [28]. Tet^R transformants were selected on Dent plates containing 2 mg/L tetracycline (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands). As controls, bacteria were transformed with either genomic DNA of the Tet^R strain, TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), or DNA from the Tet^S strains 26695, J99, SS1, and ATCC43504. Individual bacterial colonies present on tetracycline containing plates (2 mg/L) were selected and their MIC of tetracycline was determined.

PCR

Oligonucleotide primers (Isogen, Maarsen, The Netherlands) used for PCR amplification were based on the genome sequences of *H. pylori* strains 26695 and J99 (Table 1, Figure 1 and Figure 2) [1, 24]. PCR was performed in an automated thermal cycler (I-Cycler, Biorad), in a final volume of 50 µL using the PCR-core system I (Promega, Madison, WI, USA), with approximately 25 pg of template genomic DNA and 25 pmol of each primer.

Sequence analysis

Direct sequencing of the obtained PCR products was performed by Baseclear Inc. (Leiden, The Netherlands). Sequence data were analyzed with help of Lasergene (DNAstar, Madison, WI, USA), and Sci Ed Central (Scientific & Educational Software, Durham, USA) software.

Nucleotide sequence accession number.

The 16S rRNA gene sequence of the Tet^R *H. pylori* strain 181 has been deposited into to the Genbank sequence database, under accession no. AF512997.

RESULTS

Determination of the MIC against various antibiotics

MICs of the four commonly used antibiotics in the anti-*H. pylori* therapy, as well as two antibiotics that belong to the tetracycline family, were determined by E-test for the Tet^R *H. pylori* strain 181 and the *H. pylori* reference strain 26695 (Table 2). The MIC of tetracycline for strain 181 was 8 mg/L (susceptibility breakpoint ≥ 4 mg/L), while the MIC for strain 26695 was 0.19 mg/L. The MIC values of the two other tetracyclines, doxycycline and minocycline, were also significantly higher for strain 181 than for strain 26695. For the three other routinely used antibiotics; amoxicillin, clarithromycin, and metronidazole the MICs varied between < 0.016 mg/L and 0.064 mg/L and did not differ significantly between strains 181 and 26695.

Table 2. MIC values of Tet^R *H. pylori* strain 181, Tet^S reference strain 26695, and Tet^R 26695 transformants as determined by E-test.

Antibiotic	MIC (mg/L)[a]		
	Tet^R strain 181	Reference strain 26695	Tet^R 26695 transformant
Tetracycline	8	0.19	8
Doxycycline	12	0.19	12
Minocycline	8	0.125	6
Amoxicillin	< 0.016	< 0.016	< 0.016
Clarithromycin	< 0.016	< 0.016	< 0.016
Metronidazole	0.19	0.016	0.064

[a] Data shown are the averages of three independent experiments. The isolates were considered resistant when the MICs of the tetracyclines (tetracycline, doxycycline, and minocycline), amoxicillin, clarithromycin and metronidazole were ≥ 4 mg/L, ≥ 8 mg/L, ≥ 2 mg/L and ≥ 8 mg/L, respectively [5, 13].

Transfer of tetracycline resistance by natural transformation

Transformation of *H. pylori* strain 26695 (MIC, 0.19 mg/L) with genomic DNA of strain 181 (MIC, 8 mg/L) resulted in Tet^R colonies with a transformation frequency of 6×10^{-5} CFU/µg DNA. The MIC of tetracycline of the ten randomly selected Tet^R transformants (obtained from three independent transformation experiments), determined by E-test was 8 mg/L (Table 2), which is identical to that of the Tet^R *H. pylori* strain 181. The Tet^R transformants also displayed an increase of MIC of the tetracycline derivatives, doxycycline and minocycline (Table 2).

Transformation with PCR products of putative tetracycline resistance genes

Based on their homology with tetracycline resistance genes in other bacteria, twelve genes were selected from the published genome sequences of *H. pylori* strains 26695 [24] and J99 [1] (Table 1). The Tet^S *H. pylori* strain 26695 was transformed with the PCR products of the selected tetracycline resistance genes, which were amplified from genomic DNA of the Tet^R strain 181. Only transformation with the PCR product of the 16S rRNA genes resulted in Tet^R transformants with a transformation frequency of 4×10^{-5} CFU/µg DNA. No Tet^R transformants were found after transformation with one of the other selected

genes, TE or DNA from the Tet^S strain 26695. Similar results were found for the Tet^S strains J99 (MIC 0.5 mg/L), SS1 (MIC 0.19 mg/L), and ATCC43504 (MIC 0.125 mg/L). For all strains the MIC of tetracycline of ten randomly selected Tet^R transformants (obtained from three independent transformation experiments) determined by E-test was 8 mg/L, which is identical to that of the Tet^R donor strain 181.

Comparison of the 16S rRNA gene sequences of the Tet^R strain 181, the Tet^S strain 26695 and four randomly Tet^R 26695 transformants (obtained after transformation with genomic DNA of strain 181) revealed several base pair differences in the Tet^R strain 181 as well as in the Tet^R transformants that did not occur in the Tet^S strain 26695 (Figure 1). Three Tet^R 26695 transformants had incorporated the complete 16S rRNA gene of strain 181, while the fourth transformant contained the first part of the 16S rRNA gene of strain 26695 and the second part of strain 181. The DNA crossover in this transformant occurred between nucleotide 93 and 128 (numbering according to 16S *rrnA* of *H. pylori* strain 26695). For each strain or transformant, only one sequence was obtained for the 16S rRNA genes, indicating that these 16S rRNA genes were identical in these strains.

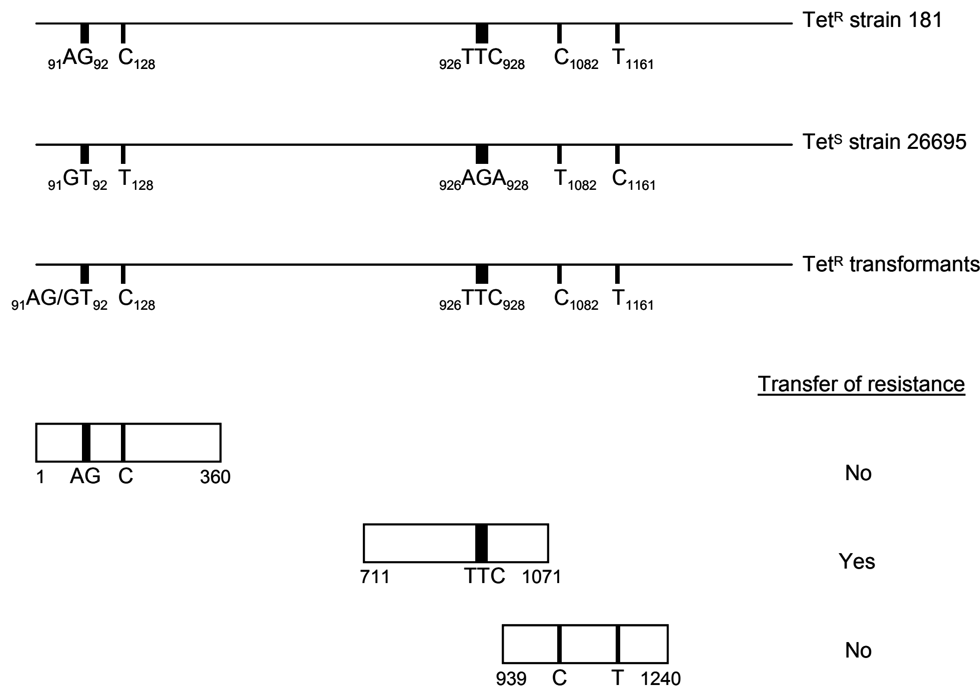

Figure 1. Schematic representation of the 16S rRNA genes of *H. pylori*. Alignment of the 16S rRNA genes (*rrnA* and *rrnB*) from the Tet^R strain 181, the Tet^S strain 26695 and four Tet^R 26695 transformants. Sequence analysis of the 16S rRNA genes revealed only a few base pair substitutions (numbering according to 16S *rrnA* of *H. pylori* strain 26695) in the Tet^R strain 181 that did not occur in the Tet^S strain 26695. For the identification of the 16S rRNA region required for tetracycline resistance, the Tet^S strain 26695 was transformed with smaller overlapping PCR fragments of the 16S rRNA gene of the Tet^R strain 181. The transformants were selected on tetracycline (2 mg/L) containing Dent plates. Primers used for the amplification of the smaller overlapping PCR fragments started at the outside of the fragment and each had a length of 20 bp.

Identification of 16S rRNA mutations involved in tetracycline resistance

To determine which residues of the 16S rRNA genes were responsible for tetracycline resistance in strain 181, the Tet^S strain 26695 was genetically transformed with smaller overlapping PCR products of the 16S rRNA gene fragments, amplified from genomic DNA of the Tet^R strain 181. Tet^R 26695 transformants were only observed after transformation with a 361-bp DNA fragment that spanned nucleotides 711 to 1071 (numbering according to 16S *rrnA* of *H. pylori* strain 26695), with a transformation frequency of 5×10^{-6} CFU/µg DNA. Transformation with the other DNA fragments did not result in transfer of tetracycline resistance. The MIC of tetracycline of ten randomly selected Tet^R transformants determined by E-test was 8 mg/L, which was identical to that of the Tet^R donor strain 181. The only difference found between these Tet^R 26695 transformants and Tet^S strain was the triple-base-pair substitution $AGA_{926\text{-}928}$ <u>TTC</u> (Figure 1).

Both copies of 16S rRNA genes are involved in tetracycline resistance

The primers that were originally used for amplification of the 16S rRNA genes did not distinguish between the two copies present on the *H. pylori* chromosome [1, 24]. To assess the involvement of each copy of the 16S rRNA genes in tetracycline resistance, specific oligonucleotide primers were developed (Figure 2). These specific primers are based on sequences which are located approximately 350 to 600 base pairs outside the both 16S rRNA genes, *rrnA* and *rrnB*. This allowed amplification of *rrnA* and *rrnB* specific sequences. *rrnA* and *rrnB* containing PCR-fragments were obtained both for the Tet^R strain 181, the eight 26695 transformants, and Tet^S strain 26695, and their DNA sequences were determined. As expected, the *rrnA* and *rrnB* sequences were identical, whereas the sequences outside the 16S rRNA genes were different. While the Tet^S strain-derived fragments contained the AGA sequence in both genes, both for strain 181 and the eight 26695 transformants, the triple-base-pair substitution $AGA_{926\text{-}928}$<u>TTC</u> was found in both copies of the 16S rRNA genes.

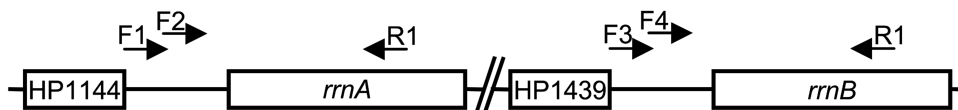

Figure 2. Both 16S rRNA genes are mutated in *H. pylori* tetracycline resistance. *rrnA* and *rrnB* specific sequences were amplified using specific primers based on sequences which are found outside the two 16S rRNA genes. The *rrnA*-specific primers, F1 and F2, are located at position 1207020 and 1207242 (numbers corresponding to the *H. pylori* 26695 sequence [24]), respectively, and the *rrnB*-specific primers, F3 and F4, are located at position 1510569 and 1510809, respectively. For amplification, primer R1 (located at position 1208293 and 1511828) was used in combination with one of the other primers. All primers had a length of 20 bp.

DISCUSSION

Until recently tetracycline resistance in *H. pylori* was rare [5, 12], but in the last two years several Tet^R *H. pylori* strains have been isolated [2, 13, 16, 31]. These Tet^R clinical isolates showed, besides tetracycline resistance, cross-resistance to metronidazole [2, 13, 16, 31] . The tetracycline resistance present in these strains was always transferred together with metronidazole resistance to a Tet^S strain [13]. In these Tet^R strains it is not clear whether the tetracycline resistance is caused by a known metronidazole resistance mechanism, a multidrug resistance mechanism, or an unknown tetracycline resistance mechanism [13]. In our Tet^R *H. pylori* strain 181, no cross-resistance was found against metronidazole, which indicated that the molecular mechanism of tetracycline resistance in strain 181 could be different from that of these earlier-described Tet^R *H. pylori* strains.

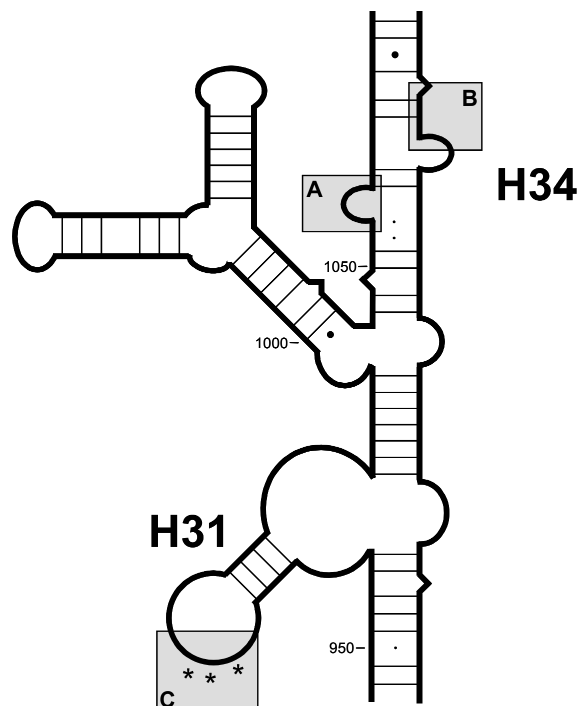

Figure 3. Schematic representation of the primary binding site of tetracycline, based on the 16S rRNA sequence of *Thermus thermophilus* proposed by Wimberly et al. [30]. The primary binding pocket for tetracycline is formed by the 16S rRNA residues 1054-1056 (box A) and 1196-1200 (box B) of helix 34, and 964-967 of helix 31 (box C). The interaction between tetracycline and this pocket are formed by hydrophobic interactions, hydrogen bonds, and salt bridges (3). The triple-base-pair substitution AGA$_{926-928}$TTC (corresponding to base pair 965-967 of *E. coli* 16S rRNA) is located in box C, and is indicated by asterisks.

In *H. pylori* strain 181 resistance to tetracycline is mediated by a single triple-base-pair substitution, $AGA_{926-928}\underline{TTC}$ (corresponding to basepair 965-967 of *Escherichia coli* 16S rRNA) present in both copies of the 16S rRNA gene. Tetracycline has one primary and multiple secondary binding sites within the 30S ribosomal subunit (3, 20). In the primary binding site, tetracycline binds exclusively to the 3'-major domain of the 16S rRNA. The primary binding pocket for tetracycline is formed by the 16S rRNA residues 1054-1056 and 1196-1200 of helix 34, and 964-967 of helix 31 (numbers corresponding to *E. coli* 16S rRNA) [3]. The residues 1054 and 1196 interact primarily with tetracycline through hydrophobic interactions, but the majority of the interaction with the drug is made through hydrogen bonds and salt bridges between tetracycline and the 16S rRNA residues (Figure 3, [3]). In the Tet^R *H. pylori* strain 181, the triple-base-pair substitution $AGA_{926-928}\underline{TTC}$ is located right in the primary binding site of tetracycline. Mutations in this primary binding site are likely to affect the affinity of the drug-ribosome interaction and thus the efficacy of tetracycline as translational inhibitor.

In *E. coli,* the nucleotides G_{966} and C_{967} are not only located in the primary binding site of tetracycline but also in a functional region of the ribosome, the P-site [17, 27]. Mutations in this region may affect protein synthesis [10], either by a change in binding of tRNA to the P-site itself or by blocking the conformational change needed for the tRNA binding to the A-site. In *H. pylori* strain 181 and the Tet^R transformants of strain 26695, the triple-base-pair substitution $AGA_{926-928}\underline{TTC}$ had no effect on the growth rate of the bacterium in the presence or absence of tetracycline (data not shown). Similar observations were found in *E. coli* after the substitution of the nucleotides G_{966} and C_{967} [10]. This suggests that the triple-base-pair substitution $AGA_{926-928}\underline{TTC}$ present in *H. pylori* strain 181 does not seem to affect protein synthesis of *H. pylori*.

During revision of this work, Trieber and Taylor [25] reported that the identical $AGA_{926-928}\underline{TTC}$ substitution mediates tetracycline resistance in an unrelated *H. pylori* strain [16]. None of the other mutations found in their isolates ($G_{332}\underline{A}$, and the deletions G_{733} and G_{903}, numbering according to 16S *rrnA* of *H. pylori* strain 26695) play a role in tetracycline resistance of the Tet^R strain 181, since these mutations were not present in our Tet^R isolate. The differences found in MIC of tetracycline for the triple-base-pair substitution between the study of Trieber and ours are only due to the methods used for the determination of the MIC (data not shown). The finding that in two unrelated *H. pylori* strains the exact same mutation is responsible for tetracycline resistance opens possibilities for the development of molecular screening tests for tetracycline resistance in *H. pylori.*

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CHAPTER 4

Effects of 16S rRNA gene mutations on tetracycline resistance in *Helicobacter pylori*

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ABSTRACT

The triple-base-pair 16S rDNA mutation $AGA_{926-928}$TTC mediates high-level tetracycline resistance in *Helicobacter pylori*. In contrast, single- and double-base-pair mutations only mediated low-level tetracycline resistance and decreased growth rates in the presence of tetracycline, explaining the preference for the TTC mutation in tetracycline-resistant *H. pylori* isolates.

Tetracycline is a cheap and effective antibiotic for the treatment on *Helicobacter pylori* infections [7, 8], but in the past few years the incidence of tetracycline resistance has significantly increased [1, 5, 6, 9, 13]. The only known mechanism mediating tetracycline resistance in *H. pylori* involves mutations at positions 926 to 928 in both the 16S rRNA genes [2, 4, 12]. *H. pylori* isolates with high-level tetracycline resistance carried the triple-base-pair mutation AGA$_{926\text{-}928}$TTC in both copies of the 16S rRNA genes [4, 12], whereas low-level tetracycline-resistant (Tet^R) strains only contained single- and double-base-pair mutations in the exact same region [2]. As the different mutations were present in unrelated strains, it is still unclear whether high-level tetracycline resistance requires the AGA$_{926\text{-}928}$TTC mutation or whether single- or double-base-pair mutations at these positions may suffice for high-level tetracycline resistance. Therefore, we have created all possible combinations of single-, double- and triple-base-pair mutations of the AGA$_{926\text{-}928}$TTC mutation in tetracycline-susceptible (Tet^S) *H. pylori* strain 26695, and have determined the effect of the mutations on levels of tetracycline resistance, stability and growth rate.

Site-directed mutants at position 926 to 928 was carried out by using a three-step PCR approach [3, 10] with primers listed in Table 1, followed by natural transformation to Tet^S *H. pylori* reference strain 26695 [4]. Tet^R *H. pylori* colonies were selected on plates containing tetracycline (1 mg/L). For each possible base pair mutation or combination thereof, eight Tet^R transformants from at least two independent transformation experiments were selected. Both alleles of the 16S rRNA genes were amplified by PCR [4] to confirm the presence of the desired base pair mutations. With the exception of the AGC mutants, all mutants contained the desired mutations in both alleles of the 16S rRNA genes. All AGC mutants were heterozygous; and contained the AGC mutation in the *rrnA* gene and a TTC mutation in the *rrnB* gene.

The effects of the 16S rRNA mutations on both the stability and the level of tetracycline resistance were determined by subculturing two mutants of each type for twenty passages on Columbia agar plates supplemented with 7% lysed horse blood (BioTrading, Mijdrecht, The Netherlands) in either the presence or absence of tetracycline (1 mg/L). After each five rounds of subculturing the minimal inhibitory concentration (MIC) of tetracycline was determined by E-test (AB Biodisk, Solna, Sweden) [4]. In addition, for all mutants at time point zero (t_0) and after passages 20 (t_{20}) the 16S rRNA genes were sequenced. The stability of the various types of mutations and their effects on the levels of resistance are summarized in Table 2.

At t_0, the single- and double-base-pair mutations did not result in tetracycline resistance with levels of clinical relevance, as the MIC of tetracycline was below 4 mg/L. With the exception of the AGC mutants, the MIC of tetracycline was similar at t_{20} when compared to t_0 (Table 2). The MIC of tetracycline of the AGC mutants increased from 1.5 to 6.0 mg/L. Subsequent analysis of the 16S rRNA gene sequences of all mutants at t_0 and t_{20} revealed that the TGA and AGC mutations were unstable. Already during the two subculturing steps needed for propagation of the colonies to obtain sufficient material for storage at -80ºC, the sequence of both 16S rRNA genes of the TGA mutants had changed to TGC, and while stable during the initial propagation steps, after 20 rounds of subculturing both AGC mutants contained a TTC sequence at position 926 to 928 within both 16S rRNA genes instead of only in the *rrnB* gene. Apart from these TGC and AGC mutations, all other mutations were stable as the complete 16S rRNA genes sequences did not reveal any other sequence changes except for the desired mutations.

Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')[a]	Position 16S rRNA gene[b]	16S rRNA mutation[c]
Sequencing, cloning and PCR			
Hp16S-F1	CTGACGCTGATTGCGCGAAA	711 to 730, forward	N.A.
Hp16S-F2	CCTGCTGGAACATTACTGAC	696 to 715, forward	N.A.
Hp16S-F3	TTTATGGAGAGTTTGATCCT	1 to 20, forward	N.A.
Hp16S-R1	TCGTTGCGGGACTTAACCCA	1071 to 1052, reverse	N.A.
Hp16S-R2	TGGCTCCACTTCGCAGTATT	1245 to 1226, reverse	N.A.
Hp16S-R3	AGGAGGTGATCCAACCGCA	1499 to 1480, reverse	N.A.
Hp16S-*rrnA*	CCCAAATCCTTGAGCGTTTA	1840 to 1821, reverse[d]	N.A.
Hp16S-*rrnB*	CGCATTCATAATCAGCTCAG	1826 to 1807, reverse[e]	N.A.
Mutagenesis			
Hp16S-mutF1	TAATTCGATGATACACGAAG	918 to 937, forward	TGA
Hp16S-mutF2	TAATTCGAATATACACGAAG	918 to 937, forward	ATA
Hp16S-mutF3	TAATTCGAAGCTACACGAAG	918 to 937, forward	AGC
Hp16S-mutF4	TAATTCGATTATACACGAAG	918 to 937, forward	TTA
Hp16S-mutF5	TAATTCGATGCTACACGAAG	918 to 937, forward	TGC
Hp16S-mutF6	TAATTCGAATCTACACGAAG	918 to 937, forward	ATC
Hp16S-mutF7	TAATTCGATTCTACACGAAG	918 to 937, forward	TTC
Hp16S-mutR1	CTTCGTGTATCATCGAATTA	937 to 918, reverse	TGA
Hp16S-mutR2	CTTCGTGTATATTCGAATTA	937 to 918, reverse	ATA
Hp16S-mutR3	CTTCGTGTAGCTTCGAATTA	937 to 918, reverse	AGC
Hp16S-mutR4	CTTCGTGTATAATCGAATTA	937 to 918, reverse	TTA
Hp16S-mutR5	CTTCGTGTAGCATCGAATTA	937 to 918, reverse	TGC
Hp16S-mutR6	CTTCGTGTAGATTCGAATTA	937 to 918, reverse	ATC
Hp16S-mutR7	CTTCGTGTAGAATCGAATTA	937 to 918, reverse	TTC

[a] Primers used for amplification were based on the published genome sequence of *H. pylori* strain 26695 [11]. The substituted residues are underlined.
[b] Position of oligonucleotides correspond to relative start point of the 16S *rrnA* residues of *H. pylori* strain 26695 [11].
[c] 16S rRNA mutations at position 926 to 928 (numbering according to 16S *rrnA* of *H. pylori* strain 26695 [11]. The substituted residues are underlined. N.A., not applicable.
[d] The *rrnA*-specific oligonucleotide is located outside 16S rRNA gene at position 1207242 to 1207261 of the *H. pylori* 26695 genome sequence [4, 11].
[e] The *rrnB*-specific oligonucleotide is located outside 16S rRNA gene at position 1510809 to 1510828 of the *H. pylori* 26695 genome sequence [4, 11].

To determine whether the 16S rRNA mutations affected the growth rates of the mutants in the presence or absence of tetracycline, all mutants from t_0 were cultured in duplicate in Brucella broth supplemented with 3% newborn calf serum (Life Technologies Ltd., Auckland, New Zealand). One culture of each mutant was supplemented with tetracycline (1 mg/L), whereas the other culture was kept unsupplemented. Growth was monitored by measuring the optical density at 600 nm each 24 hours for a period of 72 hours. In the absence of tetracycline, the growth of the mutants did not differ from that

of the *H. pylori* wild-type strain 26695 (Table 2). However, in the presence of tetracycline the growth of the wild-type strain and the single- and double-base-pair mutants was clearly reduced compared to that of the TTC mutant. The reduction was most pronounced in the wild-type strain and the AGC, ATA and TGC mutants (Table 2).

Table 2. Characterization of site-directed mutants.

Strain or mutants[a]	MIC of tetracycline (mg/L)[b]			Growth rate	
	t_0[c]	t_{20}			
		On Tet-free medium	On Tet (1 mg/L)	On Tet-free medium[d]	On Tet (1 mg/L)[e]
26695	0.19	0.19	0.19	+++	No growth
TGA[f]	ND	ND	ND	ND	ND
ATA	1.5	1.5	2.0	+++	+
AGC[g]	1.5	6.0	6.0	+++	+
TTA	1.5	2.0	2.0	+++	++
TGC	1.0	1.5	2.0	+++	+
ATC	2.0	2.0	3.0	+++	++
TTC	6.0	6.0	8.0	+++	+++

[a] The substituted residues are underlined.
[b] MIC were determined by E-test for two independent mutants. MIC shown are means of results from four to six independent experiments. Tet, tetracycline, ND not determined.
[c] At t_0 all mutants were already subcultured twice in order to obtain sufficient bacteria required for storage at -80ºC.
[d] Growth rates of the isogenic mutants relative to the growth rate of the WT 26695 (100%), +++, 95 to 100%; ++, 70 to 95%; +, 50 to 70%.
[e] Growth rates of the isogenic mutants containing single- and double-base-pair mutations relative to the growth rate of the isogenic mutants containing the triple-base-pair mutation TTC (100%), +++, 95 to 100%; ++, 70 to 95%; +, 50 to 70%.
[f] The TGA sequence had already changed into the TGC sequence during subculturing before storage.
[g] Each of the tested mutants contained an AGC mutation in the *rrnA* gene and a TTC mutation in the *rrnB* gene.

From this study it is apparent that for high-level tetracycline resistance, *H. pylori* requires the triple-base-pair mutation $AGA_{926\text{-}928}TTC$ in both copies of the 16S rRNA genes. Such high-level tetracycline resistance is most likely generated by a stepwise process that is driven by a selection that depends on both the duration and the dose of exposure to tetracycline. For this reason, it was not surprising that the previously described low-level Tet^R *H. pylori* strains contained only single- or double-base-pair mutations in the exact same region as the TTC mutation [2]. In this report [2] it was also suggested that mutant *H. pylori* strains with even a small increase in tetracycline resistance have an advantage whenever inhibitory concentrations of tetracycline are encountered. However, it could not be excluded that the observed differences were due to strain differences or secondary mutations. The data presented here have been obtained from mutants with an identical genetic background, thus excluding the effect of strain differences. Taken together, the preference in *H. pylori* for particular 16S rRNA gene mutations mediating tetracycline resistance not only results from the differences in MICs but also from the differences in growth rates in the presence of tetracycline and from the stability of the mutations.

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CHAPTER 5

Detection of high-level tetracycline resistance in clinical isolates of *Helicobacter pylori* using PCR-RFLP

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ABSTRACT

Tetracycline is one of four antibiotics commonly used for the treatment of *Helicobacter pylori* infection, but its effectiveness is decreasing as the incidence of tetracycline resistance is increasing. In five Brazilian tetracycline-resistant (Tet^R) *H. pylori* isolates, high-level tetracycline resistance is mediated by the triple-base-pair substitution $AGA_{926-928}$<u>TTC</u> in both 16S rRNA genes, as was previously observed in two independent high-level Tet^R *H. pylori* strains. A polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) assay was developed for the detection of the $AGA_{926-928}$<u>TTC</u> substitution, and confirmed the presence of the aforementioned triple-base-pair substitution in all five Brazilian Tet^R *H. pylori* isolates. This PCR-RFLP based approach distinguishes the high-level Tet^R *H. pylori* isolates from the low-level Tet^R and tetracycline-susceptible *H. pylori* strains and thus allows the direct detection of Tet^R *H. pylori* isolates.

INTRODUCTION

Helicobacter pylori is a spiral-shaped, Gram-negative bacterium that persistently colonizes the human stomach. It is the main causative agent of chronic superficial gastritis and peptic ulcer disease, and infection with *H. pylori* is strongly associated with the development of gastric cancer and gastric lymphomas [6]. Successful treatment of *H. pylori* infection not only results in the eradication of the pathogen, but often also cures and prevents the development of the associated diseases [13]. Although most anti-*H. pylori* regimens are still highly effective, the eradication rates of these therapies are negatively affected by the increasing incidence of antibiotic resistance [10]. *In vitro*, *H. pylori* is susceptible to a wide range of antibiotics, but for effective treatment a combination of drugs is required [5]. Tetracycline-based combination regimens are often used after first-line treatment with amoxicillin, clarithromycin and/or metronidazole fails [9, 16], or when reduction of treatment costs are important [12].

Tetracycline is an antibiotic that binds to the 30S ribosomal subunit, and thus blocks protein synthesis, inhibiting the growth of the bacteria [2, 3]. Most *H. pylori* isolates are still susceptible to tetracycline (MIC < 4 mg/L), but in the past few years the incidence of tetracycline resistance has increased, in particular in certain geographical regions [17, 18, 22]. In *H. pylori* the molecular mechanism underlying this resistance is attributed to mutations in the primary binding site of tetracycline [4, 7, 21]. In the two Tet^R *H. pylori* isolates previously described, high-level tetracycline resistance is mediated by the triple-base-pair substitution $AGA_{926-928}\underline{TTC}$, within both copies of the 16S rRNA genes [7, 21]. In this study, we show that this identical triple-base-pair substitution also mediates high-level tetracycline resistance in five Brazilian *H. pylori* isolates. We have subsequently developed a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) assay allowing rapid and reproducible identification of the mutations mediating high-level tetracycline resistance in *H. pylori*.

MATERIALS AND METHODS

Bacterial isolates, growth conditions and resistance testing

H. pylori isolates used in this study and their origins are listed in Table 1. Five unrelated Brazilian Tet^R isolates (BZ series) were obtained as previously described [11]. All bacteria were routinely grown on Brain Heart Infusion (BHI) agar plates (Difco, Detriot, MI, USA) supplemented with 2.5 g/L yeast extract (Difco), 10% lysed sheep blood (BBV, Campinas, Brazil), and *H. pylori* selective supplements for 48 to 72 hours at 37ºC in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 [17]. The minimal inhibitory concentration (MIC) of tetracycline was routinely determined by E-test (AB Biodisk, Solna, Sweden) [7]. The isolates were considered to be resistant when the MIC of tetracycline was ≥ 4 mg/L [5, 7]. For each experiment, MIC determinations of the tetracycline-susceptible (Tet^S) *H. pylori* strain 26695 and Tet^R *H. pylori* strain 181 were included as controls [7].

Table 1. *H. pylori* isolates used in this study and their MICs of tetracycline determined by E-test.

Isolates/reference strains[a]	Origin/Reference	Disease	MIC (mg/L)
Clinical isolates			
BZ002	[11]	Gastric ulcer	6
BZ197	[11]	Gastritis	6
BZ261	[11]	Gastric ulcer	6
BZ288	[11]	Gastric ulcer	4
BZ291	[11]	Gastric ulcer/ Duodenal ulcer	4
181	[7]	Gastritis	8
Reference strains			
26695	[20]		0.19
J99	[1]		0.5
ATCC 43504	ATCC		0.125
NCTC 11638	NCTC		0.38
SS1	[14]		0.19

[a] Tet^R isolates were obtained from individual dyspeptic patients [11]. ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures.

[b] MIC shown are means of results from three independent experiments. The isolates were considered to be resistant when the MIC of tetracycline was ≥ 4 mg/L [5, 7].

Natural transformation of *H. pylori*

The Tet^S *H. pylori* reference strain 26695 was transformed [7] with ~1 µg of genomic DNA from a Tet^R strain (Table 1), or ~250 ng of 16S rRNA gene products amplified from this genomic DNA. Tet^R transformants were selected on selective *H. pylori* media supplemented with 2 mg/L tetracycline (Sigma Aldrich Corp.), and their MICs of tetracycline were determined. As controls, bacteria were transformed with either DNA from strain 181 (Tet^R), DNA from strain 26695 (Tet^S), or TE (1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA).

General DNA techniques

Oligonucleotide primers (Isogen, Maarsen, The Netherlands) used in this study are listed in Table 2. PCR was performed in an automated thermal cycler (I-cycler, Biorad Laboratories BV, Veenendaal, The Netherlands) using the PCR-core system I (Promega, Madison, WI, USA). Direct sequencing of the obtained PCR products was performed by Baseclear Inc. (Leiden, The Netherlands) and the Hemocentro/UNICAMP genome center (Campinas, Brazil).

RFLP analysis

Genomic DNA of the Tet^R isolates was amplified with the primers Hp16S-F3 and Hp16S-R2, and digested with the restriction enzyme *Hin*fI (Promega). The restriction products were analyzed by electrophoresis on a 2% agarose gel, and visualized using ethidium-bromide. As controls, 16S rDNA fragments of the Tet^R *H. pylori* strain 181 and Tet^S *H. pylori* reference strains 26695, J99, ATCC 43504, NCTC 11638 and SS1 were included (Table 1).

Nucleotide sequence accession numbers

The nucleotide sequences of the primary binding site of the five Brazilian Tet^R *H. pylori* isolates (BZ002, BZ197, BZ261, BZ288 and BZ291) analyzed in this study have been deposited in GenBank under accession numbers AY278526 to AY278530, respectively.

Table 2. Oligonucleotides used in this study.

Primer name	Primer sequence (5'-3')[a]	Position 16S rRNA gene[b]
Hp16S-F1	TTTATGGAGAGTTTGATCCT	1 to 20, forward
Hp16S-F2	CCTGCTGGAACATTACTGAC	696 to 715, forward
Hp16S-F3	CTGACGCTGATTGCGCGAAA	711 to 730, forward
Hp16S-F4	TAGTTGTTGGAGGGCTTAGT	789 to 808, forward
Hp16S-R1	TCGTTGCGGGACTTAACCCA	1071 to 1052, reverse
Hp16S-R2	TGGCTCCACTTCGCAGTATT	1245 to 1226, reverse
Hp16S-R3	AGGAGGTGATCCAACCGCA	1499 to 1480, reverse
Hp16S-*rrnA*	CCCAAATCCTTGAGCGTTTA	1840 to 1821, reverse
Hp16S-*rrnB*	CGCATTCATAATCAGCTCAG	1826 to 1807, reverse

[a] Oligonucleotides used for amplification were based on the published genome sequence of *H. pylori* strain 26695 [20].

[b] Position of oligonucleotides are given to the relative start point of *rrnA* gene of *H. pylori* strain 26695 [20].

RESULTS

MICs of tetracycline for Tet^R *H. pylori* isolates

The MICs of tetracycline were determined by E-test for the five Brazilian Tet^R *H. pylori* isolates (BZ series), the Dutch Tet^R *H. pylori* strain 181, and the Tet^S reference strains 26695, J99, ATCC 43504, NCTC 11638 and SS1 (Table 1). For the five Brazilian Tet^R *H. pylori* isolates the MICs of tetracycline ranged between 4 and 6 mg/L (susceptibility breakpoint ≥ 4 mg/L), while the MIC of the Tet^S strains ranged between 0.125 and 0.5 mg/L. The MICs of tetracycline for the control strains Tet^R strain 181 and Tet^S reference strain 26695, were 8 mg/L and 0.19 mg/L, respectively.

Identification of 16S rDNA sequence changes in the primary binding site of tetracycline

The 16S rDNA fragments containing the primary tetracycline binding sites of the five Brazilian Tet^R *H. pylori* isolates were amplified and sequenced. Comparison of the sequences of the PCR products that spanned nucleotides 696 to 1245 (numbering according to the *rrnA* gene of *H. pylori* strain 26695) from the five Tet^R isolates, and Tet^S *H. pylori* strain 26695 revealed several base pair differences in the five Brazilian Tet^R isolates that did not occur in the Tet^S strain 26695 (data not shown). However, all the sequence changes found in this part of the 16S rDNA fragment were identical to those of the previously reported high-level Tet^R *H. pylori* strain 181, including the $AGA_{926\text{-}928}\underline{TTC}$ mutation that mediates tetracycline resistance in *H. pylori* strain 181 [7].

Transfer of the $AGA_{926-928}$TTC substitution mediates tetracycline resistance

To confirm that only the $AGA_{926-928}$TTC triple-base-pair substitution was responsible for the observed high-level tetracycline resistance, the Tet^S *H. pylori* strain 26695 was transformed with either genomic DNA of the five Tet^R Brazilian isolates, or with specific PCR products amplified from these isolates. For the latter, the whole 16S rRNA gene was amplified using the oligonucleotides Hp16S-1F and Hp16S-3R, while the oligonucleotides Hp16S-3F and Hp16S-1R (Table 2) were used to create and sequence a 361-bp fragment that only contained the triple-base-pair substitution $AGA_{926-928}$TTC. For all Brazilian Tet^R isolates, Tet^R transformants were readily obtained after transformation with either genomic DNA, the 1499-bp DNA fragment (spanning the complete 16S rRNA gene), or the smaller 361-bp PCR product. No Tet^R transformants were found after transformation with DNA from the Tet^S strain 26695 or with TE. The MICs of tetracycline of the Tet^R transformants were identical to those of the corresponding Tet^R donor strains. Sequence analysis of the 16S rDNA fragment encompassing nucleotides 696 to 1245 of all independent Tet^R transformants confirmed that the transformants had incorporated the triple-base-pair substitutions $AGA_{926-928}$TTC within both copies of the 16S rRNA genes. Apart from this triple-base-pair substitution, no other sequence changes were present in this fragment.

Detection of *H. pylori* 16S rDNA allele-specific mutations

To analyze whether one or both 16S rRNA genes of these *H. pylori* strains carried the substitution, the two 16S rRNA gene copies were amplified separately [7] and digested with the restriction enzyme *Hin*fI (5'-$G^{\downarrow}$ANTC-3'). This enzyme digests the $AGA_{926-928}$TTC substitution mediating high-level tetracycline resistance ($G^{\downarrow}$ATTC, from position 924 to 928 within both copies of the 16S rRNA genes *rrnA* and *rrnB*), but not that of the low-level Tet^R ($AGA_{926-928}$GGA, AGC, GTA or GGC) or Tet^S ($AGA_{926-928}$) isolates. For both 16S rDNA allele-specific PCR fragments, the other conserved *Hin*fI sites (i.e. not affected by the Tet^R inducing $AGA_{926-928}$TTC substitution) were used as internal digestion controls. All 16S rDNA allele-specific fragments derived from the five Brazilian high-level Tet^R isolates were completely digested with *Hin*fI at position 926 to 928, while this did not occur for the Tet^S *H. pylori* reference strain 26695 (data not shown). Thus all five Brazilian Tet^R isolates contain the triple-base-pair substitution $AGA_{926-928}$TTC within both copies of the 16S rRNA genes.

Detection of high-level tetracycline resistance in *H. pylori* by PCR-RFLP

In order to set up a molecular screening approach for clinical purposes, the allele-specific PCR-RFLP was simplified. Both 16S rRNA genes were amplified within a single PCR reaction with the primers Hp16S-F3 and Hp16S-R2, and digested with *Hin*fI. For the five Tet^S *H. pylori* reference strains one cleavage site was present within the 16S rDNA fragment, resulting in a 281- and 254-bp digestion product. This site served as control for the correct digestion of the fragments. For the five Brazilian Tet^R isolates and strain 181, the $AGA_{926-928}$TTC mutation that mediates high-level tetracycline resistance generates an additional *Hin*fI cleavage site, resulting in digestion of the 254-bp fragment into a of 214- and 40-bp fragment (Figure 1).

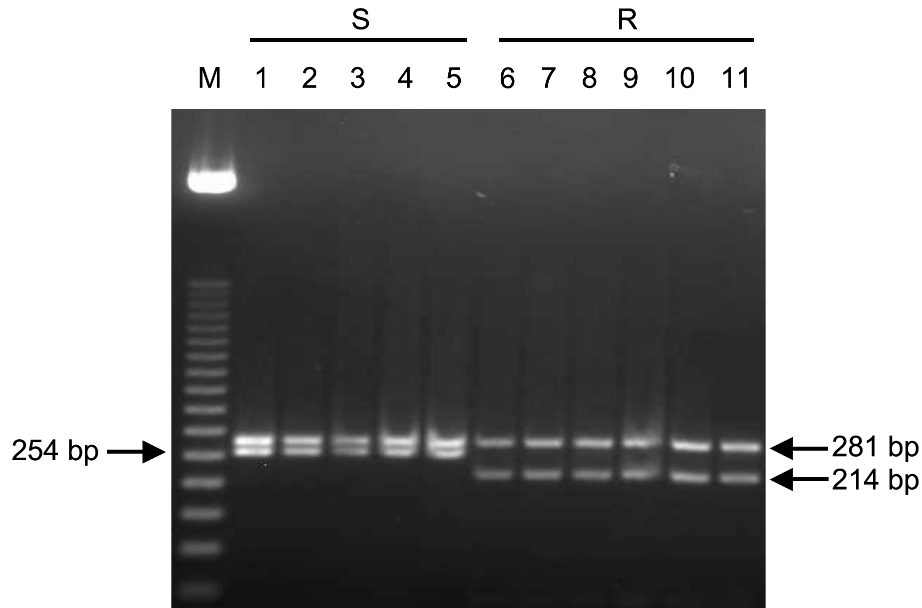

Figure 1. Detection of the 16S rDNA $AGA_{926-928}$<u>TTC</u> substitution by *Hin*fI mediated PCR-RFLP. The presence of the $AGA_{926-928}$<u>TTC</u> substitution creates an additional *Hin*fI restriction site (wildtype GAAGA, mutant G↓ATTC). The 16S rDNA fragments derived from the Tet^S reference strains 26695, J99, ATCC 43504, NCTC 11638 and SS1 (lane 1-5, respectively) were cleaved into two fragments, 281-bp and 254-bp, while the digestion of the PCR fragments of the five high-level Brazilian Tet^R isolates BZ002, BZ197, BZ261, BZ228 and BZ291 (lane 6-10, respectively), and strain 181 (lane 11) results in three fragments, 281-bp, 214-bp, and 40-bp. The 281-bp, 254-bp and 214-bp fragments are marked by arrows. Due to its small size the 40-bp fragment is not visible on this reproduction. R, tetracycline-resistant; S, tetracycline-susceptible; M, marker, 50-bp DNA step ladder (Promega).

DISCUSSION

Tetracycline is an antibiotic which is frequently used in first- and second-line regimens for the treatment of *H. pylori* [9, 12, 16]. This antimicrobial drug is not only widely available and cheap, but until recently had the additional advantage that resistance against it was rare in *H. pylori*. Unfortunately, the incidence of tetracycline resistance appears to be increasing, especially in countries where tetracycline can be obtained without prescription [17, 18, 22]. This increased incidence of high-level tetracycline resistance in *H. pylori* is a serious concern as it negatively affects the efficacy of tetracycline-containing regimens [9, 19].

It was previously demonstrated that in two unrelated high-level Tet^R *H. pylori* strains, tetracycline resistance is conferred by an identical triple-base-pair substitution ($AGA_{926-928}$<u>TTC</u>) within both copies of the 16S rRNA genes [7, 21]. Subsequently, several low-level Tet^R *H. pylori* isolates were described, and in all these strains the resistance was based on single- and double-base-pair mutations in the exact same region [4]. However, the MIC values of tetracycline for these low-level Tet^R isolates were below the susceptibility

breakpoint of 4 mg/L and thus commonly considered as tetracycline-susceptible. Our current findings in the five Brazilian high-level Tet^R clinical isolates confirm and extend the conclusions of previous studies in the sense that high-level tetracycline resistance in *H. pylori* is conferred by the same triple-base-pair substitution $AGA_{926-928}$<u>TTC</u> as observed in the two other high-level Tet^R *H. pylori* isolates [7]. It is striking that all characterized Tet^R *H pylori* isolates contain mutations in the exact same 16S rRNA region, especially because these isolates were obtained from dyspeptic patients living in different geographic regions (Asia, Australia, Eastern- and Western Europe, and Central- and South-America) [4, 7, and this study]. This observation suggests that *H. pylori* requires mutations within the 16S rRNA primary binding site for tetracycline resistance. Probably this resistance arises by *de novo* mutations, although the acquisition of mutant 16S rRNA alleles through horizontal gene transfer cannot be excluded.

Conventional methods to assess levels of tetracycline resistance of *H. pylori* are based on culture in combination with agar dilution or E-test [15]. Both methods are not only time-consuming, but also difficult to standardize. Since tetracycline resistance seems to be restricted to the occurrence of specific mutations in a small region of the 16S rRNA molecule [4, 7, 21], molecular methods offer an attractive alternative. In the present study a PCR-based RFLP was used to detect the presence of the triple-base-pair substitution $AGA_{926-928}$<u>TTC</u> in the 16S rRNA genes. This PCR-RFLP assay distinguishes the high-level Tet^R isolates from the low-level Tet^R and Tet^S *H. pylori* strains. Since all high-level Tet^R *H. pylori* (MIC $\geq$ 4 mg/L) isolates characterized thus far contain the $AGA_{926-928}$<u>TTC</u> substitution [4, 7, 8, 21], and to our knowledge low-level tetracycline resistance is not linked to therapy failure, this PCR-RFLP approach is useful for the detection of clinically relevant levels tetracycline resistance in *H. pylori.* In conclusion, our PCR-RFLP assay allows rapid detection of high-level tetracycline resistance in *H. pylori*.

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CHAPTER 6

Rapid detection of 16S rRNA gene mutations in *Helicobacter pylori* associated with resistance to tetracycline by real-time PCR

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ABSTRACT

Recommended first-line therapy of *Helicobacter pylori* usually consists of a proton pump inhibitor, combined with two antimicrobials. However, the effectiveness of this triple therapy is decreasing due to resistances against metronidazole and clarithromycin. Quadruple therapies usually contain tetracycline and/or a bismuth component, and are often used as second-line treatment. Nevertheless, resistances against tetracycline, caused by mutations in the 16S rRNA genes (*rrnA* and *rrnB*), are emerging and can result in an unsuccessful eradication treatment of *H. pylori*. In order to recognize possible resistances against tetracycline, we developed a real-time PCR-based on the fluorescence resonance energy transfer (FRET)-method. The assay was validated using seven tetracycline-resistant (Tet^R) *H. pylori* isolates exhibiting the $AGA_{926\text{-}928}\underline{TTC}$ triple-base-pair mutation, eight different artificially created mutants showing various combinations of mutations affecting the positions 926 to 928 in the 16S rRNA genes and 150 *H. pylori* clinical isolates. Our real-time PCR recognized all Tet^R isolates and artificially created mutants, and distinguished them from the tetracycline-susceptible (Tet^S) isolates. Moreover, this method was applicable to DNA extracted from cultured bacteria and gastric biopsy samples. Thus, our real-time PCR is an excellent tool to detect tetracycline resistance in *H. pylori*, particularly in cases when conventional susceptibility testing fails.

INTRODUCTION

Helicobacter pylori infection is chronic in nature and has been shown to cause gastritis, and increases the risk of peptic ulcer disease, MALT-lymphoma and gastric cancer development [16, 21, 23]. Increasing resistances against first-line antibiotics like clarithromycin are compromising the eradication of *H. pylori* resulting in therapy failures [8]. Thus, alternative treatment trials including tetracyclines are recommended [1, 17].

Tetracylines are bacteriostatic drugs with a broad spectrum activity. They exert their antimicrobial effects by affecting the 30S ribosomal subunit and blocks the binding of aminoacyl-tRNA resulting in an impaired protein biosynthesis [3, 25]. Resistance of *H. pylori* against tetracyclines was reported to be caused by mutations in the 16S rRNA. *H. pylori* isolates showing $AGA_{926\text{-}928}\underline{TTC}$ triple-base-pair substitutions [4, 5, 25] are classified as high-level tetracycline-resistant, whereas single- or double-base-pair mutations in the exact same region were rather associated with low-level tetracycline resistance [3, 4].

In routine practice, susceptibility of *H. pylori* against both tetracycline and other antibiotics is examined by culturing *H. pylori* and subsequent agar diffusion (E-test) or agar dilution tests, which are accepted to be the reference methods [11]. These methods are not only time-consuming but also vulnerable to contaminations. In addition, *H. pylori*-positive patients are often on long term anti-acids and as a consequence gastric biopsy samples contain many bacterial species, making it difficult - if possible at all - to obtain a pure culture of the fastidious *H. pylori*. Finally, inappropriate transport conditions, for example unsuitable transport media or extended transport times are common. In such cases, culture followed by antimicrobial sensitivity testing might be impossible. In the case of failure, molecular-based methods seem to be a promising way to solve those problems in routine laboratories. Well established methods like PCR and sequencing are more robust, but they are often complex, time-consuming, and thus expensive. Fluorescence resonance energy transfer (FRET)-based real-time PCR methods, using fluorescent labeled hybridization probes, allows a rapid mutation screening of a PCR product by melting curve analysis. In *H. pylori*, it is shown, that FRET-based real-time PCR assays are an accurate method for the detection of clarithromycin [18, 19] and ciprofloxacin resistance [7].

In the present study, we developed a rapid and reliable real-time-PCR for the detection of 16S rRNA mutations associated with tetracycline resistance in *H. pylori* using a LightCycler®. To validate the assay seven high-level tetracycline-resistant (Tet^R) *H. pylori* isolates from The Netherlands, South-America and Canada, and eight artificially created Tet^R *H. pylori* 16S rRNA mutants and 150 *H. pylori* clinical isolates and their according gastric biopsy samples were tested.

MATERIAL AND METHODS

Bacterial strains and culture conditions

H. pylori strains used in this study were the Tet^R strain 181 from the Netherlands, one Tet^R Canadian isolate (KC617), the tetracycline-susceptible (Tet^S) reference strain 26695 [24], 150 *H. pylori* clinical isolates from our routine laboratory as well as eight artificially 16S rRNA mutants created in the *H. pylori* reference strain 26695 as previously described [4]. All bacteria were cultured under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C for 48 hours and identified as *H. pylori* by standard criteria [13, 22].

The Canadian isolate KC 617 was kindly provided by Dr. G. Cooper-Lesins from Halifax, Canada.

Determination of the minimal inhibitory concentration of tetracycline by E-test

For all *H. pylori* isolates, the E-test-method (AB Biodisk, Sweden) was used to determine the minimal inhibitory concentration (MIC) of tetracycline. The method was performed according to a protocol previously described [11]. Strains showing MICs $\geq$ 4 mg/L were classified as high-level tetracycline-resistant, whereas strains with MICs between > 0.5 mg/L and < 4 mg/L, and MICs $\leq$ 0.5 mg/L were considered low-level resistant and susceptible, respectively.

DNA extraction and 16S rRNA gene amplification

DNA of *H. pylori* isolates and DNA of the corresponding gastric biopsy samples were extracted using the QIAmp DNA mini kit (Qiagen, Hilden, Germany). DNA extracts from gastric samples were checked for the presence of *H. pylori* by amplifying the *vacA* signal sequence as described earlier [22]. Amplification of a 361-bp fragment of the 16S rRNA genes was performed using the primers Hp16S-F1 (5'-CTGACGCTGATTGCGCGAAA-3') and Hp16S-R1 (5'-TCGTTGCGGGACTTAACCCA-3') as described before [4]. Amplicons were examined by applying 7 µL on a 1.2% agarose gel (Peqlab, Erlangen, Germany) and then purified using the QIAquick PCR puri-fication kit (Qiagen).

DNA-extracts of five Brazilian Tet^R strains (BZ002, BZ197, BZ261, BZ288 and BZ291) exhibiting the TTC triple-base-pair mutation were kindly provided by Dr. M.L. Ribeiro and Prof.dr. J. Pedrazzoli Jr. from São Francisco University Medical School, Brazil.

DNA Sequencing

Purified PCR products were sequenced with the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Warrington, U.K.) using the PCR-primers as sequencing primers. Sequencing was accomplished with an ABI 310 DNA-Sequencer (Applied Biosystems).

Detection of 16S rRNA gene mutations of *H. pylori* by a FRET-based real-time-PCR

FRET-based real-time PCR was performed on bacterial DNA extracted from isolates and DNA extracted from gastric biopsy samples. The method included the amplification of the 16S rDNA gene fragment of *H. pylori* using the primers mentioned above and the simul-taneous detection of the PCR-product by hybridization probes.

For the detection of the 16S rDNA gene mutations, two hybridization probes, an anchor-probe Tet-A (5'-TCTAGCGGATTCTCTCAATGTCAAGCCTAG-3'; 3'-labeled

with fluorescein) and a mutation-probe Tet-M (5'-AAGGTTCTTCGTGTATCTTCG- 3'; 5'-labeled with LC-Red®640 and 3'-phosphorylated) were used. Probes were delivered by TIB Molbiol (Berlin, Germany), primers by Hermann GmbH (Freiburg, Germany).

Real-time PCRs were performed in 20 µL-volumes in glass capillaries (Roche Diagnostics, Mannheim, Germany) using a LightCycler® (Roche Diagnostics). Twenty microliters of PCR mixture contained 10 µL of SYBR-Green Master Mix (Qiagen), 0.4 µL of each of the primers Hp16S-F1 and Hp16S-R1 (25 µM each), 2 µL of the anchor-probe and 2 µL of the mutation-probe (2 µM each), 3.2 µL of H_2O and 2 µL of template DNA.

Cycling conditions consisted of an initial denaturation step of 95°C for 15 min, followed by 15 cycles with denaturation at 95°C for 20 sec, annealing at 65°C for 20 sec and an elongation step at 72°C for 30 sec. Then, the annealing temperature was stepwise decreased for 1°C per cycle to a final annealing temperature of 57°C, and the PCR was continued for 27 cycles at 57°C (50 cycles in total). After amplification, samples were denatured at 95°C for 0 sec and cooled down to 30°C, where the temperature was hold for 30 sec. Then, samples were slowly heated to 85°C at a ramp rate of 0.1°C/sec with continuous acquisition of fluorescence decline. Melting curves were plotted automatically and analyzed with the LightCycler®-software.

RESULTS

Design of hybridization probes

For the detection of the 16S rRNA mutations, we designed a pair of hybridization probes, consisting of an anchor- and a mutation-probe. The anchor-probe is covering the nucleotides 975-946, the mutation-probe the nucleotides 943-923 (Figure 1).

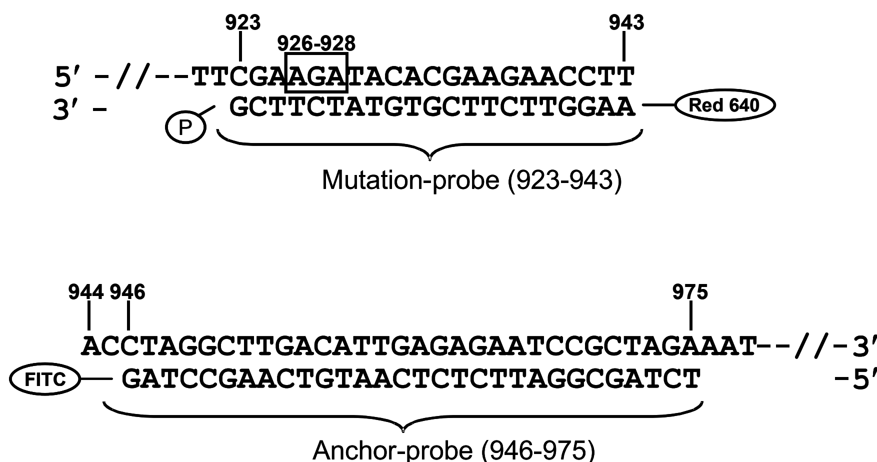

Figure 1. Locations and sequences of the hybridization probes used. The mutation-probes is 3'-phosphorylated and 5'-labeled with LC-Red640, the anchor probes are each 3'-labeled with fluorescein. The nucleotides are numbered according to the GenBank accession no. AF512997 (*rrnA)*.

In order to prove the specificity of the hybridization probes, various bacterial species were tested, i.e. *H. pylori* reference strains 26695 [24], *Escherichia coli* (clinical isolate), *Campylobacter fetus* (clinical isolate), *Campylobacter coli* (clinical isolate), *Campylobacter jejuni* (clinical isolate) and *Lactobacillus* species (clinical isolates). For all bacteria, the convential PCR using the Hp16S-F1 and Hp16S-R1 primers rendered a PCR product of similar size, but the hybridization probes Tet-A and Tet-M were only *H. pylori*-specific. No melting curve was constructed when applying DNA of other bacteria.

MICs of tetracycline

The MICs of tetracycline were determined by E-test for the Dutch Tet^R isolate 181, the five Brazilian Tet^R isolates, the Canadian Tet^R isolate KC617, eight artificially created Tet^R *H. pylori* mutants of strain 26695, Tet^S *H. pylori* strain 26695 and 150 *H. pylori* clinical isolates. The Dutch, Brazilian and Canadian isolates as well as the artificially generated TTC mutant showed MICs between 6 mg/L and 16 mg/L and were classified high-level tetracycline-resistant (Table 1). For the five mutants containing double-base-pair mutations (i.e. TGC, ATC, GGC, TTA, and GTA), the MICs of tetracycline ranged between 1 and 2 mg/L (Table 1), while the MICs for the single-base-pair mutants ATA and GGA were 1.5 mg/L and 0.75 mg/L, respectively (Table 1). All artificial mutants were classified as low-level resistant. For the 150 *H. pylori* clinical isolates, 142 isolates had MICs < 0.5 mg/L and were classified as susceptible, whereas eight clinical isolates had a MIC of 0.75 mg/L and were classified as low-level resistant.

Table 1. MICs of tetracycline, phenotype of the 16S rRNA mutants and melting temperatures (Tm) in the real-time PCR assay.

Strain or mutant[a]	MIC of tetracycline (mg/L)[b]	Phenotype	Tm (°C)
26695 (AGA)	0.19	susceptible	57.4
GGA mutant	0.75	low-level resistant	53.8
ATA mutant	1.5	low-level resistant	49.6
TGC mutant	1.0	low-level resistant	46.9
ATC mutant	2.0	low-level resistant	46.3
TTA mutant	1.5	low-level resistant	47.9
GTA mutant	1.5	low-level resistant	49.1
GGC mutant	1.0	low-level resistant	47.4
TTC mutant	6.0	high-level resistant	46.0
Strain 181 (TTC)	8.0	high-level resistant	46.0
BZ002 (TTC)	6.0[c]	high-level resistant	46.0
BZ197 (TTC)	6.0[c]	high-level resistant	46.0
BZ261 (TTC)	6.0[c]	high-level resistant	46.0
BZ288 (TTC)	4.0[c]	high-level resistant	46.0
BZ291 (TTC)	4.0[c]	high-level resistant	46.0
KC617 (TTC)	16.0	high-level resistant	46.0

[a] Genotype at positions 926-928. The substituted residues are underlined.

[b] MICs of tetracycline were determined by E-test. The isolates were considered high-level resistant when the MIC of tetracycline was ≥ 4 mg/L [5]; low-level resistant when the MIC > 0.5 mg/L and < 4 mg/L; and susceptible when the MIC ≤ 0.5 mg/L.

[c] The MICs of tetracycline were determined as described previously [20].

Detection of 16S rDNA mutations by real-time PCR
The Tet^S *H. pylori* reference strain 26695 and the 142 Tet^S *H. pylori* isolates had melting temperatures of 57.4°C (Figure 2, Table 1), while the eight low-level Tet^R *H. pylori* clinical isolates had melting temperatures of 53.8°C. Sequence analysis revealed that the first 142 isolates contained an AGA wild-type sequence, while for the latter eight isolates a GGA sequence was found. The Dutch, Brazilian and Canadian high-level Tet^R isolate (containing the TTC triple-base-pair mutation), as well as the DNA extract of the gastric biopsy sample of strain 181, showed in comparison with the Tet^S *H. pylori* wild-type strain a clearly decreased melting temperature of 46.0°C (Table 1). As expected, the artificially generated high-level resistant TTC-mutant of strain 26695 had the same melting temperature as the naturally occurring Tet^R clinical isolates (Table 1).

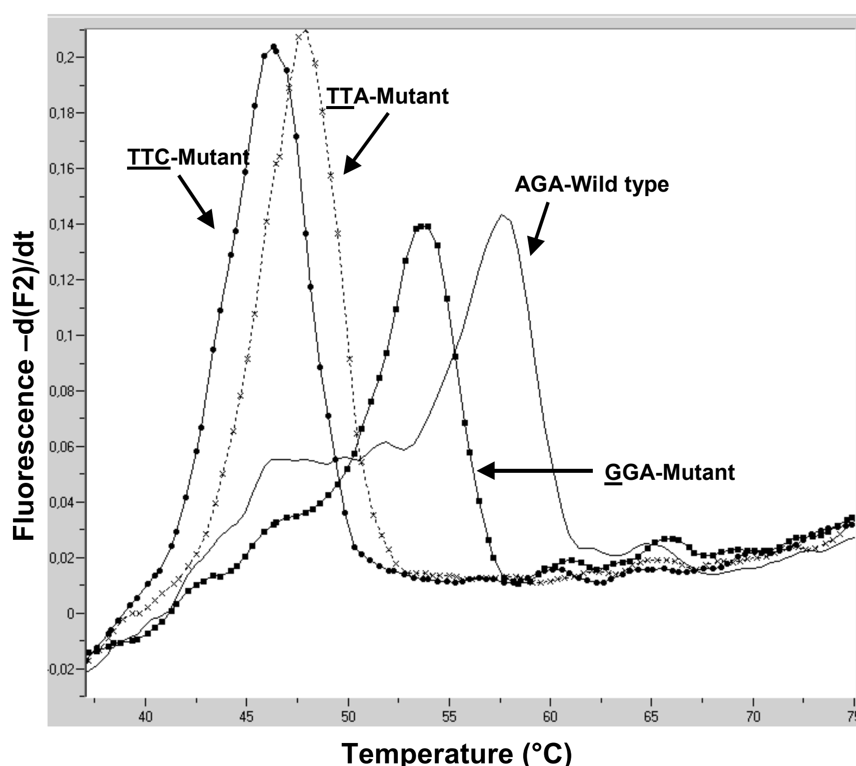

Figure 2. Melting curve analysis and detection of the AGA-wild-type, the high-resistant TTC-mutant and the low-level resistant GGA-mutant (single mutation) and TTA-mutant (double mutation). Compared to the AGA-wild-type (57.4°C), the TTC-mutant had the lowest melting temperature (46.0°C). Melting points of the GGA- (53.8°C) and the TTA-mutant (47.9°C) are ranging between the AGA wild-type and TTC-mutant.

All mutants harboring double-base-pair mutations were also mismatched to the mutation-probe reflected by a decrease of the melting temperature when compared to the Tet^S wild-type 26695. The GTA mutant had a melting temperature of 49.1°C followed by the TTA mutant with 47.9°C, the GGC mutant with 47.4°C, the TGC mutant with 46.9°C and the ATC mutant with 46.3°C (Figure 2, Table 1). The artificial single-base-pair mutants ATA and GGA had melting temperatures of 49.6°C and 53.8°C, respectively (Figure 2, Table 1).

All isolates tested, including the artificial mutants, showed a homozygous genotype (same mutation in the *rrnA* and *rrnB* gene), as only one peak was visible in the melting-curve analysis.

DISCUSSION

Conventional *H. pylori* eradication regimens are normally based on a combination of proton pump inhibitors, amoxicillin and clarithromycin [17]. Increasing resistances against these antibiotics, mainly clarithromycin [8], jeopardize the successful treatment of *H. pylori* infections, so other strategies are suggested like second-line therapies including tetracyclines, fluoroquinolones or RNA-polymerase inhibitors [10, 17, 27]. Recent publications provide data about successful treatment trials using proton pump inhibitors, metronidazole, a bismuth salt and tetracycline [1, 2, 6, 17]. Before using antibiotics in anti-*H. pylori* therapy, testing their susceptibility is desirable, particularly in patients that have already been treated unsuccessfully. Antimicrobial susceptibility testing is routinely performed by E-test after successful culture of the bacterium, but sometimes these conventional methods are unsuccessful because of heavily contaminated biopsy samples or growth failure e.g. due to retarded transport times. In these cases, molecular-based methods like real-time PCR can be applied as a diagnostic rescue technique for detection of antibiotic resistance in *H. pylori.* Such real-time PCR assays have recently been published for the detection of clarithromycin and ciprofloxacin resistance in *H. pylori* [7, 18, 19], and in the present study a real-time PCR method was developed and validated for the detection of tetracycline resistance in *H. pylori.*

Tetracycline resistance of *H. pylori* is reported to be mediated by mutations in the 16S rRNA [3-5, 25], for example an $AGA_{926\text{-}928}$TTC triple-base-pair mutation or an $AGA_{926\text{-}928}$GTA double-base-pair mutation [3]. Those mutations lead to alterations of the 16S rRNA primary binding site of tetracycline resulting in increased MICs of tetracycline [3, 25]. The $AGA_{926\text{-}928}$TTC mutation was reported to be associated with clinically relevant high-level tetracycline resistance (MICs ≥ 4 mg/L [4, 5]). To our knowledge, strain 181 is the only high-level Tet^R *H. pylori* isolate reported in Northern Europe [5]. In contrast to Northern Europe, about 5% of *H. pylori* isolates in Japan and Korea [12, 15], 10% in Brazil [9], and about 60% of isolates from Shanghai, China [26], were reported to be resistant against tetracyclines. Due to the increased double resistances of *H. pylori* against clarithromycin and metronidazole in Germany [14], second-line strategies based on tetracycline are more often used, and probably this will lead to an increased incidence of Tet^R *H. pylori* isolates. In anticipation of this, we decided to establish a FRET-based real-time PCR for a rapid screening in order to detect tetracycline resistance in *H. pylori*. Due to the lack of Tet^R *H. pylori* isolates in Northern Europe, our method was validated using the high-level Tet^R *H. pylori* clinical isolate 181 [5], one high-level Tet^R *H. pylori* isolate

from Canada and five high-level Tet^R *H. pylori* isolates from Brazil [20]. Those strains all exhibit the $AGA_{926\text{-}928}$TTC triple-base-pair mutation. Additionally, we applied eight artificially created 16S rRNA gene mutants of strain 26695 harboring different mutations in the 926 to 928 triplet and 150 *H. pylori* clinical isolates to verify our method

The majority (142 isolates) of the *H. pylori* clinical isolates were tetracycline susceptible and harbored the $AGA_{926\text{-}928}$ triplet resulting in the highest melting temperature 57.4°C. Eight *H. pylori* clinical isolates contained a GGA triplet resulting in a decreased melting temperature of 53.8°C. Due to the triple-base-pair exchange, the melting temperatures of the naturally occurring high-level Tet^R *H. pylori* isolates and the high-level Tet^R TTC mutant were clearly decreased (46.0°C). The other mutants showed melting temperatures between 49.6°C and 46.3°C and were discriminable among each other.

In conclusion, we have shown that our assay is able to detect 16S rRNA mutations which are associated with a clinically relevant high-level tetracycline resistance in *H. pylori* [3-5, 25]. The method was developed and validated on 150 *H. pylori* clinical isolates from our routine laboratory, seven high Tet^R clinical isolates and eight artificial Tet^R mutants exhibiting alterations in the $AGA_{926\text{-}928}$ triplet of the 16S rRNA. In contrast to the PCR-RFLP [20], our real-time PCR is not only able to differentiate between wild-type strains and high-level resistant strains harboring the $AGA_{926\text{-}928}$TTC triple-base-pair substitution, but also between mutants exhibiting single- or double-base-pair substitutions, allowing a more accurate classification to Tet^S or Tet^R strains. Since our assay is also applicable on DNA extracted from gastric biopsy samples and cultured *H. pylori* isolates, it is an excellent tool to detect tetracycline resistance in *H. pylori*.

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CHAPTER 7

The role of the *rdxA* and *frxA* genes in oxygen-dependent metronidazole resistance of *Helicobacter pylori*

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ABSTRACT

Almost 50% of all *Helicobacter pylori* isolates are resistant to metronidazole, which reduces the efficacy of metronidazole-containing regimens, but it does not make them completely ineffective. This discrepancy between *in vitro* metronidazole resistance and treatment outcome may partially be explained by changes in oxygen pressure in the gastric environment, as metronidazole-resistant (Mtz^R) *H. pylori* isolates become metronidazole-susceptible (Mtz^S) under low oxygen conditions *in vitro*. In *H. pylori* the *rdxA* and *frxA* genes encode reductases are required for the activation of metronidazole, and inactivation of these genes results in metronidazole resistance. Here the role of inactivating mutations in these genes on the reversibility of metronidazole resistance under low oxygen conditions is established. Clinical *H. pylori* isolates containing mutations resulting in a truncated RdxA and/or FrxA protein were selected and incubated under anaerobic conditions, and the effect of these conditions on minimal inhibitory concentrations of metronidazole, amoxicillin, clarithromycin, and tetracycline, and cell viability were determined. While anaerobiosis had no effect on amoxicillin, clarithromycin and tetracycline resistance, all isolates lost their metronidazole resistance when cultured under anaerobic conditions. This loss of metronidazole resistance also occurred in the presence of the protein synthesis inhibitor chloramphenicol. Thus, factor(s) that activate metronidazole under low oxygen tension are not specifically induced by low oxygen conditions, but are already present under microaerophilic conditions. As there were no significant differences in cell viability between the clinical isolates, it is unlikely that the *rdxA* and the *frxA* gene participate in the reversibility of metronidazole resistance.

INTRODUCTION

Helicobacter pylori is a spiral-shaped, Gram-negative bacterium that colonizes the stomach of approximately half the world's population [3]. Colonization with *H. pylori* is the most common cause of chronic active gastritis and peptic ulcer disease, and is strongly associated with the development of gastric cancer and gastric lymphoma. Unless treated with antibiotics, *H. pylori* colonization tends to persist for life. Cure of *H. pylori* infection results in ulcer healing and may reduce the risk of gastric cancer and gastric lymphoma development [33, 38]. *In vitro, H. pylori* is susceptible to the majority of antibiotics, but for effective treatment a combination of drugs is required [7]. Currently used anti-*H. pylori* therapies often consist of two antibiotics with a proton pump inhibitor and/or a bismuth component [27]. Metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is a key component of such combination therapies [27, 36].

In Western Europe it has been estimated that 20 to 45% of the *H. pylori* isolates are metronidazole-resistant (Mtz^R) [13, 26]. This percentage is even higher in developing countries and immigrant populations [10, 25]. Although there are conflicting reports concerning the clinical relevance of metronidazole resistance in *H. pylori*, metronidazole resistance reduces the efficacy of metronidazole-containing regimens significantly [17, 37], but surprisingly does not render them inactive.

Metronidazole is a prodrug that needs to be activated by a reduction of the nitro group that is attached to the imidazole ring. This reduction step leads to the production of DNA-damaging nitroso- and hydroxylamine-compounds. Exposure to these toxic compounds causes DNA damage, and subsequently results in the death of the bacterium. In *H. pylori*, it is believed that reduction of metronidazole is mainly mediated by an oxygen-insensitive NADPH nitroreductase (RdxA) [14, 29], but recently it has been shown that the NADPH-flavin-oxidoreductase (FrxA) also participates in the reduction of metronidazole [18].

In *H. pylori*, metronidazole resistance is primarily associated with mutational inactivation of *rdxA* gene [8, 14, 16]. However, recently it has been demonstrated that inactivation of the *frxA* gene also confers metronidazole resistance, either alone or in association with the *rdxA* gene [18, 21-23]. Whether mutational inactivation of these two enzymes accounts for metronidazole resistance in all clinical isolates is still being debated [2, 5, 21], but they most likely reflect the two major contributing factors.

The discrepancy between the *in vitro* resistance to metronidazole and treatment outcome may be explained by the antimicrobial activity of other components in the regimens and/or duration and doses of the therapy [37]. Apart from these factors, it is likely that low oxygen tension in the gastric environment may also be involved [31], since low oxygen conditions affect the activity of metronidazole reducing enzymes [15]. As *in vitro* Mtz^R *H. pylori* isolates become susceptible to metronidazole after a short period of anaerobic incubation [4, 31], it has been suggested that the FrxA protein and/or other ferredoxin and flavin reductases may contribute to the activation of metronidazole under these conditions [14, 19].

In this study the role of null mutations in the *rdxA* and *frxA* genes on the reversibility of metronidazole resistance under low oxygen conditions was determined.

METHODS

Strains and growth conditions

H. pylori isolates used in this study and their respective *rdxA* and *frxA* gene status inferred from DNA sequences are listed in Table 1. The *H. pylori* isolates were routinely grown on Dent plates as described previously [11]. Broth cultures were grown in Brucella broth supplemented with 3% newborn calf serum (BBN). All cultures were incubated either under microaerophilic (5% O_2, 10% CO_2 and 85% N_2) or anaerobic conditions (10% H_2, 5% CO_2 and 85% N_2) at 37°C. The anaerobic culture condition was created using the Anoxomat (Mart, Lichtenvoorde, The Netherlands) in combination with a catalyst. *Escherichia coli* strain DH5α MCR (Life Technologies BV) was grown on Luria-Bertani agar plates [28] for 24 hours at 37ºC in an aerobic environment. Selection of *E. coli* transformed with pGEM-T Easy clones was performed on LB-agar plates containing ampicillin to a final concentration 100 mg/L (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands).

Table 1. Mtz^R *H. pylori* strains and their *rdxA* and *frxA* gene status

H. pylori isolate	MIC	Accession number[b]	
	(mg/L)[a]	*rdxA* (HP0954)	*frxA* (HP0642)
Truncated rdxA gene			
BH9711-176	24	AY568322	AY568330
DM9735-58	> 256	AY568328	AY568336
Truncated frxA gene			
BH9713-141	> 256	AY568323	AY568331
DM9642-108	> 256	AY568325	AY568333
DM9716-140	32	AY568326	AY568334
Truncated rdxA and frxA genes			
BH9714-19	128	AY568324	AY568332
DM9727-179	192	AY568327	AY568335
ATCC 43504[c]	> 256	[8]	[23]

[a] MICs shown are means of results from three independent experiments. The isolates were considered to be resistant when the MIC of metronidazole was ≥ 8 mg/L [11].
[b] *rdxA* and *frxA* gene sequences have been deposited into the Genbank database.
[c] ATCC, American Type Culture Collection.

DNA manipulation

DNA manipulations were performed according to standard protocols [28]. Oligonucleotides (Table 2, Isogen, Maarsen, The Netherlands), PCR-core system I (Promega, Madison, WI, USA) and pGEM-T Easy vector (Promega) were used according to the manufacturer's recommendations. Plasmid DNA was isolated with Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Sequencing of the obtained plasmid and PCR products was performed by Baseclear (Leiden, The Netherlands).

Random amplified polymorpic DNA (RAPD) fingerprinting

RAPD analysis of chromosomal DNA was performed with three independent primers, 1254, D11344 and D9355 as described before [1]. RAPD products were separated in 2% agarose gels containing 0.5 mg/L ethidium-bromide (Promega).

Table 2. Oligonucleotides used in this study

Gene/Primer name	Nucleotide sequence (5'→3')[a]
rdxA (HP0954)	
RdxA-F1	GCTGATTGTGGTTTATGGTTTGG
RdxA-F2	TTGGATCAAGAAAAAAGAAGACAATTATTAA
RdxA-F3	GCTGATTGTGGTTTATGGTTTGG
RdxA-F4	GAGAGCCGGACAGCCAAATG
RdxA-R1	CACCCCTAAAAGAGCGATTAAAACC
RdxA-R2	GCAAGAATGGCGCTCGTT
RdxA-R3	CCCACAGCGATATAGCATTG
frxA (HP0642)	
FrxA-F1	GGATATGGCAGCCGTTTATCATT
FrxA-R1	GAATAGGCATCATTTAAGAGATTA
FrxA-R2	TGGTTCAAGCCCGATTGAAG

[a] Oligonucleotides used for amplification were based on the published genome sequence of *H. pylori* strain 26695 [34].

The influence of the length of anaerobic incubation on antibiotic resistance

Minimal inhibitory concentrations (MICs) were routinely determined by E-test (AB Biodisk, Solna, Sweden) [11] or agar dilution [35]. The plates were incubated under anaerobic conditions for 0, 0.25, 0.5, 1, 2, 4, and 8 hours, and subsequently incubated for 3 days at 37 °C under microaerophilic conditions. The isolates were considered resistant when the MICs of amoxicillin ≥ 8 mg/L, clarithromycin ≥ 2 mg/L, metronidazole ≥ 8 mg/L and tetracycline ≥ 4 mg/L [11]. As controls, resistant strains were included for each tested antibiotic [6, 11, 12]. All MIC determinations were performed in triplicate.

The influence of chloramphenicol on metronidazole resistance during anaerobiosis

Bacteria freshly grown on Dent plates were harvested and inoculated in BBN to a cell density of ~1×10^7 colony forming units per mL (CFU/mL), and incubated overnight with gentle agitation under microaerophilic conditions. Fumaric acid (Sigma Aldrich Chemie, final concentration 0.1%) was then added to the overnight culture to facilitate the generation of anaerobic conditions [16]. The culture was split then into 10 mL portions and when indicated either supplemented with metronidazole (Sigma Aldrich Chemie, final concentration 16 mg/L) and/or chloramphenicol (Sigma Aldrich Chemie, final concentration 10 mg/L). Subsequently the cultures were incubated under microaerophilic or anaerobic conditions for 0, 2, 4, 6 and 8 hours. At each time point 1 mL of the broth was taken and washed twice with phosphate buffered saline to remove the antibiotics. In order to determine the number of viable bacteria, 50 µL of undiluted suspension and 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions were plated on Columbia agar plates containing 7% lysed horse blood, and incubated under microaerophilic conditions. Present colonies were counted and data was expressed as CFU/mL.

Nucleotide sequence accession numbers
The *rdxA* and *frxA* gene sequences of the seven Mtz^R *H. pylori* isolates have been deposited into the GenBank database (Table 1)

RESULTS AND DISCUSSION

Effect of anaerobic incubation on minimal inhibitory concentration
To evaluate the effect of anaerobic incubation on the MIC of metronidazole, seven Mtz^R *H. pylori* clinical isolates and the Mtz^R *H. pylori* reference strain ATCC 43504 were selected for this study. To ensure that these strains represented different isolates, RAPD fingerprinting was performed with primers D11344 (Figure 1), 1254 and D9355 (data not shown). All strains gave different profiles with each of the three primers, indicating they represent unrelated isolates.

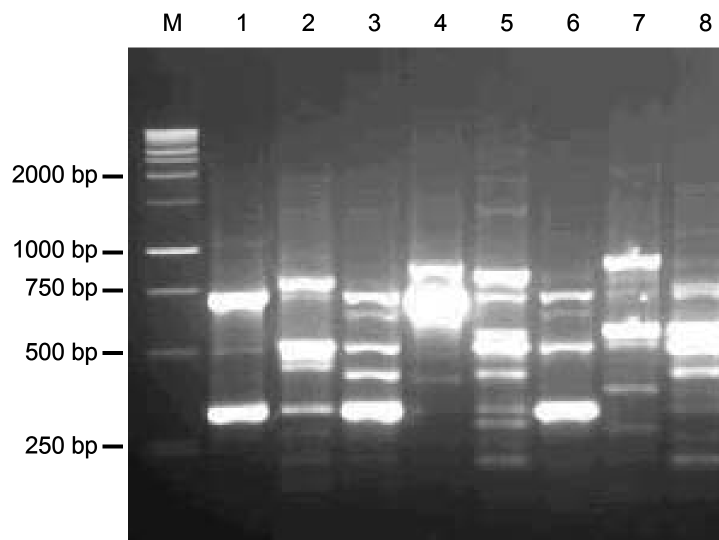

Figure 1. The *H. pylori* isolates used in this study represent unrelated isolates, as shown by RAPD-PCR. DNA isolated from the Mtz^R isolates were amplified with primer D11344 according to standard procedures [1]. The RAPD-PCR products were separated on a 2.0% agarose gel and stained with ethidium-bromide. Lane 1, represents *H. pylori* reference strain ATCC 43504, Lane 2 to 8, represent BH9711-176, BH9713-141, BH9714-19, DM9735-58, DM9727-179, DM9642-108, and DM9716-140, respectively. M, 1 kb marker (Promega).

The seven Mtz^R isolates and reference strain ATCC 43504 were incubated in microaerophilic and anaerobic conditions, and the MIC of metronidazole was determined by the E-test and agar dilution. Under standard microaerophilic culture conditions, the MIC of metronidazole for the eight isolates, as determined by E-test, ranged from 24 to > 256 mg/L, (Table 3). These MICs of metronidazole decreased under anaerobic conditions. After 4 hours of anaerobic incubation, the MIC values of metronidazole dropped

below the breakpoint of metronidazole (8 mg/L) for three of the eight isolates, and after 8 hours, all Mtz^R isolates had become metronidazole-susceptible (Table 3). In contrast to metronidazole, the MICs of amoxicillin, clarithromycin and tetracycline were stable during anaerobic incubation (data not shown). There were no clear differences found in the MIC values between the E-test and agar dilution.

Table 3. The effect of anaerobic incubation on metronidazole resistance.

H. pylori isolates	MIC (mg/L)[a]			
	T_0	T_2	T_4	T_8
Truncated rdxA *gene*				
BH9711-176	24	16	12	3
DM9735-58	> 256	32	4	0.75
Truncated frxA *gene*				
BH9713-141	> 256	48	24	3
DM9642-108	> 256	64	24	2
DM9716-140	32	8	4	0.75
Truncated rdxA *and* frxA *genes*				
BH9714-19	128	48	12	4
DM9727-179	192	96	16	0.5
ATCC 43504	> 256	48	6	0.25

[a] MICs shown are means of results from three independent experiments. The isolates were considered to be resistant when the MIC of metronidazole was ≥ 8 mg/L [11]. T_0, time point zero, start point; T_2, T_4 and T_8, after 2, 4 and 8 hours of anaerobic incubation, respectively.

Effect of metronidazole and anaerobic incubation and on cell viability

To determine the effect of metronidazole and anaerobic incubation on cell viability, all seven Mtz^R *H. pylori* isolates and the Mtz^R reference strain ATCC 43504 were cultured in broth under microaerophilic and anaerobic conditions either in the presence or absence of 16 mg/L metronidazole, and at different time intervals the amount of viable bacteria (CFU/mL) were determined. Under standard microaerophilic conditions, the amount of viable cells for all tested Mtz^R isolates varied between 10^6-10^7 CFU/mL, and there were no significant differences observed in CFU/mL between the cultures with and without metronidazole (Figure 2). Similar data were obtained for the cultures without metronidazole that were incubated anaerobically (Figure 2). This suggests that neither the incubation with metronidazole nor anaerobic growth conditions alone affect the cell viability of the Mtz^R isolates.

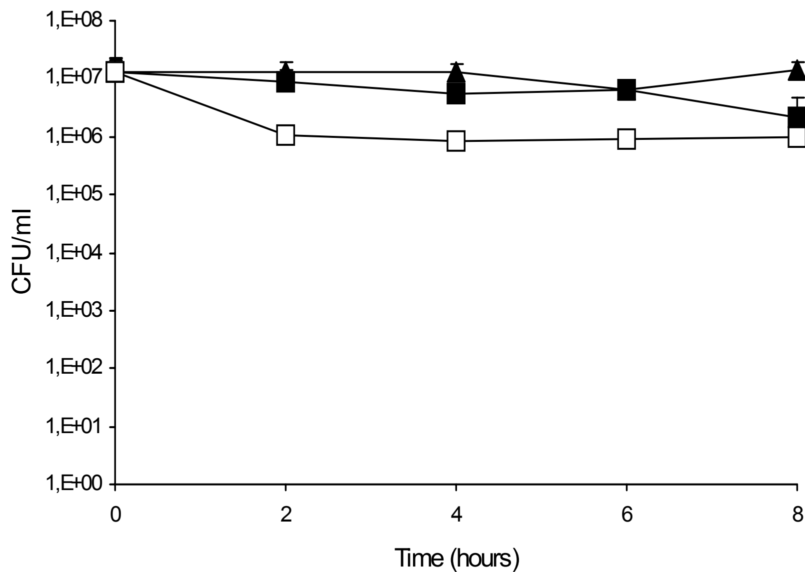

Figure 2. The influence of metronidazole and anaerobic incubation on cell viability. *H. pylori* was grown microaerophilically in the presence (filled triangle) and absence (filled square) of 16 mg/L metronidazole, and anaerobically (open square). At different time point the CFU/mL was determined. Results shown are a representative example of one of the Mtz^R isolates, BH9714-19 and are the means (± SD) of two independent experiments performed in duplicate.

However, under anaerobic conditions in the presence of metronidazole, the amount of viable cells dropped more then 1000-fold when the Mtz^R isolates were incubated for 4 hours anaerobically. After 8 hours of anaerobic incubation in the presence of metronidazole there were no viable cells present anymore (Figure 3). Since there were no differences in cell viability or time-course observed between the Mtz^R isolates containing mutations that resulted in either a truncated RdxA protein or FrxA protein, or a truncation in both, this suggests that neither the *rdxA* gene nor the *frxA* gene are involved in the reversibility of metronidazole resistance in *H. pylori.*

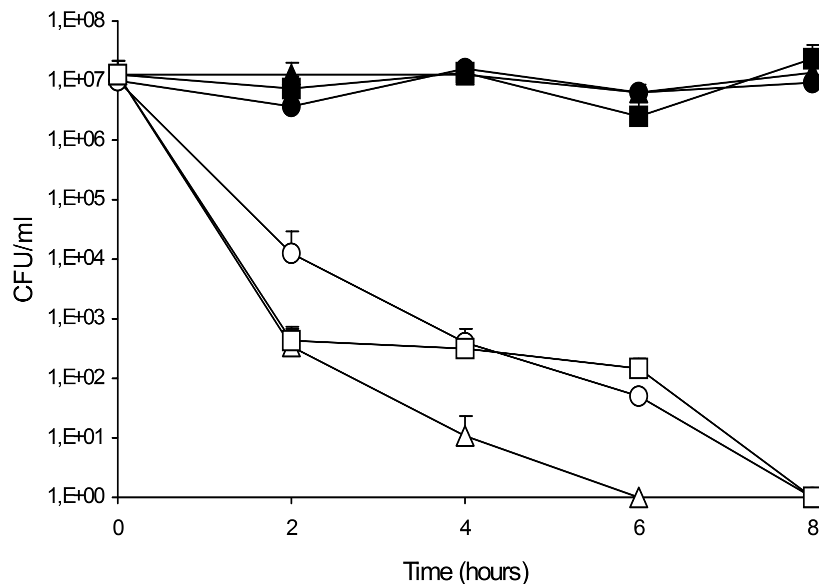

Figure 3. Involvement of the *rdxA* and/or *frxA* gene in the reversibility of metronidazole resistance under anaerobic conditions. *H. pylori* was grown in microaerophilic (filled symbols) and anaerobic (open symbols) conditions in the presence of 16 mg/L metronidazole, and the CFU/mL were determined. Results shown are for representative examples of the Mtz^R isolates tested. Symbols: square, BH9714-19 (containing mutations resulting in a truncated RdxA and FrxA protein); triangle, DM9735-58 (containing mutations resulting in a truncated RdxA); circle, BH9713-141 (containing mutations resulting in a truncated FrxA). Results shown are the means (± SD) of two independent experiments performed in duplicate.

Effect of *de novo* protein synthesis on reversibility of metronidazole resistance during anaerobic incubation.

To determine whether *H. pylori* requires *de novo* protein synthesis for the reversibility of metronidazole resistance, all experiments were repeated in broth in the presence of the bacterial protein synthesis inhibitor chloramphenicol (10 mg/L). This concentration of chloramphenicol was optimized previously, and its effects on the inhibition of the protein synthesis and cell viability are known [20]. When the Mtz^R isolates were incubated anaerobically in the presence of metronidazole and chloramphenicol, cell viability was reduced (Figure 4). As there was no significant difference in cell viability between the cultures with and without chloramphenicol these results indicate that factors that are involved in the reversibility of metronidazole resistance are already present under microaerophilic conditions. *De novo* protein synthesis is not required for this phenomenon.

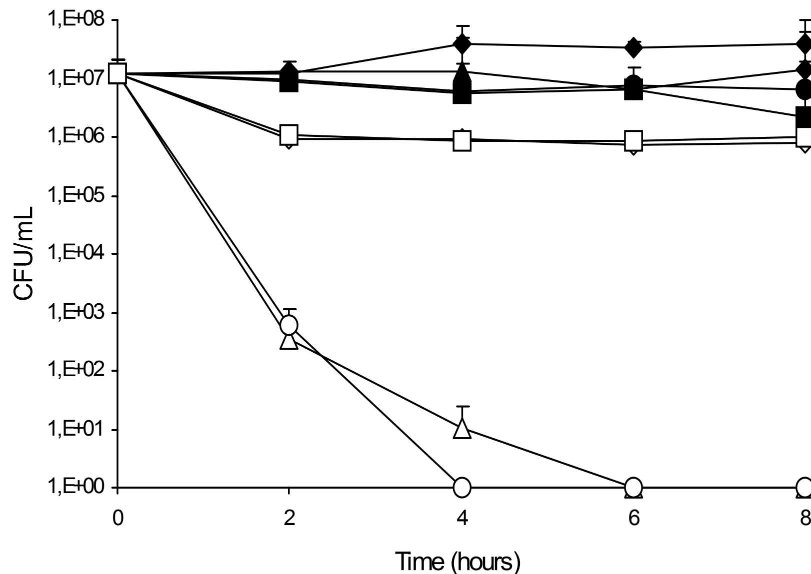

Figure 4. The effect of blocking *de novo* protein synthesis on the reversibility of metronidazole resistance. *H. pylori* was grown in broth either with or without metronidazole (16 mg/L) and/or chloramphenicol (10 mg/L) under microaerophilic (filled symbols) or anaerobic (open symbols) conditions. Results shown are for a representative example (strain BH9714-19) of the Mtz^R isolates tested. Symbols: square, standard; diamond, with metronidazole; triangle, with chloramphenicol; circle, with metronidazole and chloramphenicol. Results shown are the means (± SD) of two independent experiments performed in duplicate.

Implications of experimental data

Metronidazole, a nitroimidazole, is administrated as a prodrug that is activated by the reduction of the nitro group that is attached to an imidazole ring [9]. Since oxygen has a higher reduction potential than metronidazole, this reduction step works out most effectively in an environment with low oxygen tension, such as anaerobic cells and protozoa [15]. Surprisingly, they were also found to be active against the microaerophilic pathogen *H. pylori* [24]. In many strictly anaerobic bacteria, the activation of metronidazole is mediated by the pyruvate:ferredoxin oxidoreductase complex [32]. In *H. pylori*, this function might be fulfilled by the electron carriers, RdxA (HP0954), FrxA (HP0642), ferredoxin (FdxA, HP0277), flavodoxin (FldA, HP1161), pyruvate:ferredoxin oxidoreductase (PorD, HP1109) and 2-oxoglutarate ferredoxin oxidoreductase (OorD, HP0588). As mutations of the latter four nitroreductases were found to be lethal [18, 21], we only tested the involvement of the *rdxA* and *frxA* genes. In contrast with the findings under normal microaerophilic conditions [23, 29], we showed that neither the *rdxA* nor the *frxA* gene is required for the activation of metronidazole under low oxygen conditions, since strains with one or both genes inactivated still become susceptible to metronidazole under anaerobic conditions.

As *in vitro* Mtz^R *H. pylori* isolates lose their resistance to metronidazole after exposure to short periods of anaerobiosis [4, 31], it is suggested that this reversibility is mediated by

compensatory metabolic pathways that are induced under anaerobic conditions [15, 30]. This hypothesis is not supported by our data obtained using the protein synthesis inhibitor chloramphenicol. The loss of metronidazole resistance is mediated by a pre-existing mechanism that functions under anaerobic conditions and is not dependent on *de novo* protein synthesis, when *H. pylori* is exposed to these conditions. Since our data excluded the role of the RdxA and FrxA proteins in this process, we assume that in *H. pylori* metronidazole is reduced by one of the other known nitroreductases.

In summary, Mtz^R *H. pylori* isolates become fully metronidazole susceptible at low oxygen conditions, and this does not require *de novo* protein synthesis. This reversibility in metronidazole resistance also occurred in *H. pylori* isolates that contained mutations in the *rdxA* and/or *frxA* genes. Exposure of *H. pylori* to such low oxygen conditions in the gastric mucosa or gastric pit are likely to induce reduction of metronidazole, and thus assist in the eradication of Mtz^R *H. pylori.*

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CHAPTER 8

Stable amoxicillin resistance in *Helicobacter pylori*

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Helicobacter pylori infection is often diagnosed by a rapid urease test or histology of gastric biopsy samples; neither test allows testing for susceptibility to antibiotics. For amoxicillin, an antibiotic often used in these infections, this is not yet considered a problem because stable resistance to amoxicillin has not yet been reported, although several amoxicillin-tolerant strains have been isolated from gastric biopsy specimens from patients in Italy and the USA [1].

We report the isolation of an amoxicillin-resistant strain of *H. pylori* from an antral biopsy sample of a dyspeptic patient in the Netherlands. An 82-year-old man had chronic obstructive pulmonary disease for many years, and had received twelve courses of amoxicillin during the past 6 years. Biochemical testing and sequencing of 16S rDNA confirmed that this strain was indeed *H. pylori.* The minimal inhibitory concentration (MIC) of amoxicillin was determined by E-test, and was 8 mg/L. β-lactamase activity was not detected by nitrocephin or cephalosporinase assay. Resistance to amoxicillin remained stable after repeated cycles of storage at -80°C and culture, and after repetitive subculture on amoxicillin-free blood agar medium.

Most strains of *H. pylori*, unlike other human pathogens, are able to take up chromosomal DNA from the environment by natural transformation [3]. We isolated chromosomal DNA from the amoxicillin-resistant *H. pylori* strain and introduced it into an amoxicillin-susceptible *H. pylori* strain by natural transformation [3]. In a typical experiment, transformation with 1 μg DNA from the resistant strain resulted in the occurrence of amoxicillin-resistant colonies at a frequency of one transformant per 100000 viable bacteria; no amoxicillin-resistant colonies were observed when TE (1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA) or DNA from an amoxicillin-susceptible strain was used. The MIC of the resistant mutant colonies was increased more than 100-fold compared with the susceptible strain, and only slightly lower than that of the resistant donor strain. Repetitive subculturing of the resistant mutants proved also that this acquired resistance was stable.

We conclude that the amoxicillin resistance we report is a stable genetic feature, which can be easily transferred. Because *H. pylori* can exchange DNA through natural transformation and also through conjugation [2, 3], spread of amoxicillin resistance among *H. pylori* is a potential threat. Our findings emphasize the importance of amoxicillin susceptibility testing for *H. pylori* in clinical practice.

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CHAPTER 9

Alterations in penicillin-binding protein 1A confer resistance to β-lactam antibiotics in *Helicobacter pylori*

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ABSTRACT

Most *Helicobacter pylori* strains are susceptible to amoxicillin, an important component of combination therapies for *H. pylori* eradication. The isolation and initial characterization of the first reported stable amoxicillin-resistant (Amx^R) *H. pylori* clinical isolate (the Hardenberg strain) have been published previously, but the underlying resistance mechanism was not described. Here evidence is presented that the β-lactam resistance of the Hardenberg strain results from a single amino acid substitution in HP0597, a penicillin-binding protein 1A (PBP1A) homolog of *Escherichia coli*. Replacement of the wild-type *pbp1A* (HP0597) gene of the amoxicillin-susceptible (Amx^S) *H. pylori* strain 1061 by the Hardenberg *pbp1A* gene resulted in a 100-fold increase in the minimal inhibitory concentration (MIC) of amoxicillin. Sequence analysis of PBP1A of the Hardenberg strain, the Amx^S *H. pylori* strain 1061, and four Amx^R 1061 transformants revealed a few amino acid substitutions, of which only a single $Ser_{414}Arg$ substitution was involved in amoxicillin resistance. Although we cannot exclude that mutations in other genes are required for high-level amoxicillin resistance of the Hardenberg strain, this amino acid substitution in PBP1A resulted in an increased MIC of amoxicillin that was almost identical to that of the original Hardenberg strain.

INTRODUCTION

Helicobacter pylori, a spiral shaped Gram-negative bacterium which colonizes the human stomach, is the causative agent of chronic active gastritis, peptic ulcer disease, and is associated with increased risk for gastric cancer and gastric lymphoma [3, 9, 21]. Anti-*H. pylori* therapy often consists of the β-lactam antibiotic amoxicillin in combination with one or more antimicrobial drugs, a bismuth component and/or a proton pump inhibitor [9, 12]. *H. pylori* is usually susceptible to amoxicillin, but occasionally strains with an increased MIC (minimal inhibitory concentration) have been reported. The first reported amoxicillin-resistant (Amx^R) strains (MIC > 256 mg/L) were isolated from dyspeptic patients in Italy and the USA [10], but they all rapidly lost their amoxicillin resistance *in vitro* [8]. Subsequently the isolation of a stable Amx^R *H. pylori* clinical isolate, the Hardenberg strain, from an 82-year-old Dutch dyspeptic patient was reported [27].

Bacterial resistance against β-lactam antibiotics mostly results from either the production of β-lactamase [16], structural alterations in one of the penicillin-binding proteins (PBPs) [24], or changes in other proteins involved in the cell wall synthesis [4, 17, 29]. Initial studies with *H. pylori* indicated the presence of three high-molecular weight PBPs, designated PBP1A, PBP2 and PBP3 [6]. Later a fourth, low-molecular weight PBP, PBP4, was identified [14], and subsequently five other potential PBPs [13] and a *H. pylori* specific β-lactamase [19] have been described. Amoxicillin resistance in *H. pylori* was found not to rely on the acquisition or expression of β-lactamase [8, 27]. The nonstable amoxicillin resistance of *H. pylori* [8] is probably due to a decreased expression of the penicillin-binding protein, PBP-D [7]. In the recently described *in vitro*-selected Amx^R *H. pylori* strains amoxicillin resistance was suggested to result from alterations in PBP1A [5, 22].

In this study, we describe the molecular mechanism of amoxicillin resistance of the Hardenberg strain. Ten genes from the published *H. pylori* genomes were selected as potential candidates based on their putative role in cell wall synthesis (Table 1). After genetic transformation of the amoxicillin-susceptible (Amx^S) strain 1061, with PCR products of the selected candidate genes of the Amx^R Hardenberg strain, the minimal PCR fragment able to transfer amoxicilline resistance was sequenced and the amino acid alterations responsible for amoxicillin resistance were identified.

MATERIALS AND METHODS

Bacterial strains and growth conditions

H. pylori strains used in this study were the Amx^R Hardenberg strain [27], and the Amx^S *H. pylori* strains 1061 [11], J99 [2], 26695 [25], and SS1 [15]. Bacteria were routinely grown on Columbia agar plates (Becton Dickonson, Cockeysville, MD, USA) supplemented with 7% lysed horse blood (BioTrading, Mijdrecht, the Netherlands) and *H. pylori* selective supplement (Oxoid, Basingstoke, UK) for 48 to 72 hours at 37ºC in an atmosphere of 5% O_2, 10% CO_2, 85% N_2. *Escherichia coli* strain DH5α MCR (Life technologies BV, Paisley, UK) was grown on Luria-Bertani agar plates [23] for 24 hours at 37ºC in an aerobic environment. Selection of *E. coli* DH5α MCR transformed with pGEM-T Easy clones was performed on ampicillin-containing (100 mg/L) Luria-Bertani agar plates.

Determination of minimal inhibitory concentration
The minimal inhibitory concentration (MIC) was routinely determined by E-test (AB Biodisk, Solna, Sweden) [10] or by agar dilution [8].

DNA manipulation
Recombinant DNA techniques were performed according to standard protocols [23]. PCR fragments and pGEM-T Easy clones were sequenced on an Amersham Vistra 725 DNA sequencer using Thermo-Sequenase pre-mixed cycle sequencing kit (Amersham, Buckinghamshire, UK).

Natural transformation of *H. pylori*
Bacteria were transformed with approximately 1 µg total DNA, or 200 to 500 ng PCR product essentially as described by Wang *et al.* [28]. Transformants were selected on Dent plates containing 0.2 to 2 mg/L amoxicillin (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands). As a control, bacteria were transformed with TE (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA) or DNA from the Amx^S strain 1061.

PCR
The oligonucleotide primers (Isogen, Maarsen, The Netherlands) used in this study are indicated in Table 1 and Figure 1. PCR was performed in an automated thermal cycler (GeneAmp PCR system 9700, Perkin-Elmer), using the PCR-core system I (Promega, Madison, WI, USA).

Table 1. Transformation with PCR products of putative Amx^R genes.

Putitative function of selected gene (gene name)[a]	Gene number[b]	Position fragment[c]	Transformation frequency[d]
Penicillin-binding protein (*pbp4)*	HP0160	168848-169882	None
H. pylori cysteine-rich protein A (*hcpA*)	HP0211	218980-218291	None
Lysis tolerance protein *(lytB*)	HP0400	411955-411354	None
Penicillin-binding protein (*pbp1A*)	HP0597	632742-631000	$5×10^{-6}$
Rod-shape determining protein (*rodA1*)	HP0743	798879-797794	None
Rod-shape determining protein *(mreC*)	HP1372	1436921-1436354	None
Rod-shape determining protein *(mreB*)	HP1373	1438015-1437011	None
Cell division protein (*ftsI*)	HP1556	1639818-1638044	None
Penicillin-binding protein (*pbp2*)	HP1565	1648924-1647271	None
Methicillin resistance protein (*llm*)	HP1581	1660483-1659552	None

[a] Genes were selected from the published *H. pylori* genomes [2, 25] as potential candidates based on their putative role in cell wall synthesis.
[b] HP gene numbers corresponding to the *H. pylori* 26695 genome sequence [25].
[c] Position of duplicated fragment, corresponding to the *H. pylori* 26695 genome sequence [25]. Primers used for the amplification of the PCR fragments started at the outside of the fragment and had each a length of twenty basepairs.
[d] Determined as the number of Amx^R colonies per microgram of DNA per recipient CFU. Data represent the mean of three experiments.

Site-directed mutagenesis
Four different 135 bp oligonucleotides (Isogen) were designed based on the sequence of the *pbp1A* gene of *H. pylori* strain 1061, except that they contained mismatches resulting in none, one, or two amino acid substitutions (Glu_{406}Ala and/or Ser_{414}Arg). For amplification, 5 pmol of the 135 bp oligonucleotides was used as template, with 25 pmol of each terminal primer, 5'-GCTATTCCACGACTTCTAA-3' and 5'-GCAAGGTTACAAGCCCT AAA-3'.

Nucleotide sequence accession numbers
The *pbp1A* sequences of the *H. pylori* Hardenberg and 1061 strains have been deposited into to the Genbank-database, under accession no. AF479617 and AF479618, respectively.

RESULTS AND DISCUSSION

Determination of the MIC of various antibiotics
The MIC of the four commonly used antibiotics in the anti-*H. pylori* therapy, as well as several β-lactam antibiotics are presented in Table 2. The Hardenberg strain had an MIC of amoxicillin of 8 mg/L, while the MIC for strain 1061 was well below 0.016 mg/L. The MIC of the other β-lactam antibiotics was significantly higher for the Hardenberg strain than for *H. pylori* strain 1061. MIC values of clarithromycin and tetracycline were identical for both strains, but the Hardenberg strain was found to be susceptible to metronidazole, while strain 1061 is known to be resistant to that drug [11].

As the E-test was reported to generate discrepant results when compared to the agar dilution method [1, 20, 26], and as the E-test does not allow determination of amoxicillin MICs below 0.016 mg/L, the susceptibility to amoxicillin was also tested by agar dilution. The MIC of amoxicillin for the Hardenberg strain by agar dilution was slightly higher (10 mg/L) than by E-test. For *H. pylori* strain 1061, the MIC of amoxicillin was 0.01 mg/L.

Table 2. MIC values of the Hardenberg strain, *H. pylori* strain 1061 and Amx^R 1061 transformants determined by E-test.

Antibiotic	MIC (mg/L)[a]		
	Hardenberg	1061	Amx^R 1061 transformants[b]
Amoxicillin	8	< 0.016	0.75
Amoxicillin-clavulanate	2	< 0.016	0.25
Cefotaxime	0.75	0.023	0.75
Ceftazidime	4	0.25	3
Penicillin	2	0.032	0.25
Piperacilin	4	< 0.016	0.38
Clarithromycin	< 0.016	< 0.016	< 0.016
Metronidazole	0.023	192	192
Tetracycline	< 0.016	0.023	0.032

[a] Data shown are the averages of three separate experiments.
[b] MIC values are the average of ten randomly selected transformants (obtained after transformation with total DNA of the Hardenberg strain) derived from three independent transformation experiments.

Transfer of amoxicillin resistance by natural transformation

Transformation of *H. pylori* strain 1061 with total DNA of the Hardenberg strain resulted in Amx^R colonies at a frequency of 1×10^{-5} CFU/μg DNA. The MICs of amoxicillin of ten at random selected transformants (obtained from three independent transformation experiments) as determined by E-test (Table 2) and agar dilution was 0.75 mg/L and 1.0 mg/L, respectively. In the agar dilution method, for these transformants the MIC of amoxicillin was 100-fold higher than that for the 1061 acceptor strain, and it was only slightly lower than that of the Hardenberg donor strain. For the Amx^R transformants, the MICs of the other β-lactam antibiotics also displayed increases (Table 2).

Transformation with PCR products of cell wall synthesis-encoding genes

Based on their putative role in cell wall synthesis, ten genes from the published *H. pylori* genome sequences of strain 26695 [25] and strain J99 [2] were selected as potential Amx^R genes (Table 1). Amx^S strain 1061 was transformed with PCR amplified products of the selected genes of the Amx^R Hardenberg strain. Only transformation with the *pbp1A* gene resulted in Amx^R colonies with a frequency of 5×10^{-6} CFU/μg DNA (Table 1). PCR products of the other putative amoxicillin resistance genes, TE, and DNA from strain 1061 were unable to transfer amoxicillin resistance. The MIC of amoxicillin of ten at random selected Amx^R colonies (obtained from three independent transformation experiments) was identical to that of strain 1061 transformed with total DNA of the Hardenberg strain. Repeated transformation of these transformants with either the total DNA or the other nine PCR products of the Hardenberg strain did not result in an increase of the MIC (data not shown). The MIC of these transformants could be increased to the same level as the Hardenberg strain only by continued exposure to amoxicillin, but this additional increase of the resistance was not stable. This indicated that the additional increase in MIC of amoxicillin for the Amx^R 1061 transformants was due to a transient physiological change. Apparently a stable increase of amoxicillin resistance to the level of the Hardenberg strain requires mutations in more than one locus, and the likelihood that these are all transferred by transformation is relatively small.

Identification of *pbp1A* gene mutations involved in amoxicillin resistance

To determine the mutations of *pbp1A* gene responsible for amoxicillin resistance, natural transformation experiments were performed using small overlapping PCR fragments of the *pbp1A* gene (Figure 1). Amx^R 1061 transformants (7×10^{-7} CFU/μg DNA) were only observed with the 361-bp DNA fragment that spanned nucleotides 953 to 1313 (numbering according to the *pbp1A* gene of *H. pylori* strain 26695). No Amx^R colonies were observed after transformation with the seven other *pbp1A* PCR fragments, TE, or DNA from strain 1061. The MIC of amoxicillin of ten randomly selected transformants (obtained from three independent transformation experiments) was 1.0 mg/L.

Analysis of PBP1A sequences obtained from the Hardenberg strain, the Amx^S 1061 strain and four Amx^R 1061 transformants (obtained with total DNA from the Hardenberg strain) showed several amino acid substitutions in the Hardenberg strain as well as in the transformants, that did not occur in the Amx^S strain. Three transformants had incorporated the complete Hardenberg *pbp1A* gene sequence, while the fourth transformant only contained the second half of the gene. For this fourth transformant the DNA exchange

apparently occurred between nucleotide 793 and 854. This was independent proof that the first 792 bp of the *pbp1A* gene of the Hardenberg strain are not involved in amoxicillin resistance, as was already indicated by transformation with the *pbp1A* PCR fragments.

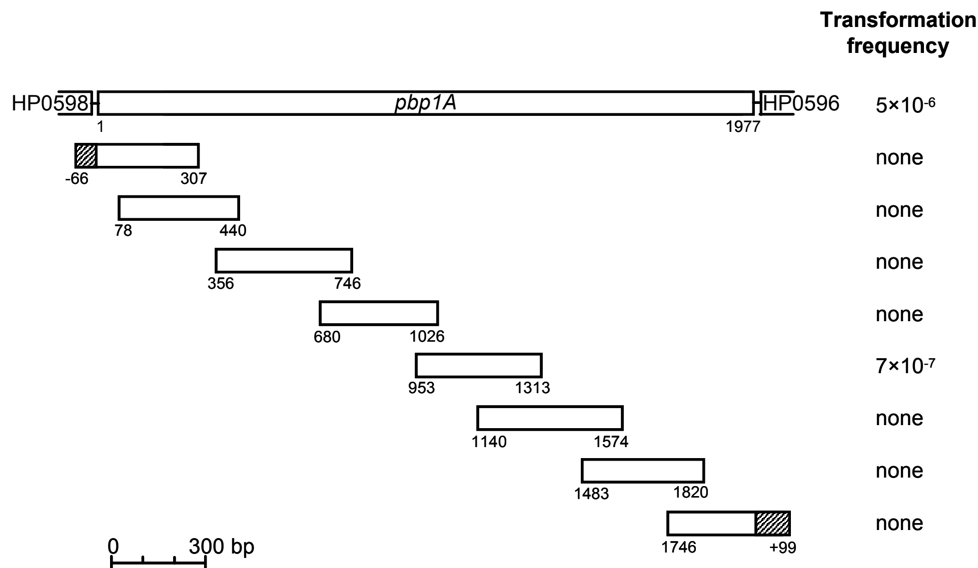

Figure 1. Identification of the region of *pbp1A* required for amoxicillin resistance. Transformants were obtained by transformation of overlapping PCR fragments of the Amx^R Hardenberg strain to the Amx^S strain 1061. The transformation frequency was determined as the number of Amx^R colonies per microgram of DNA. Data represent the mean of three experiments. Primers used for the amplification of the smaller overlapping PCR fragments started at the outside of the fragment and had each a length of twenty base pairs. The first and last fragment (hatched area's) partially consists of the flanking genes of the *pbp1A* gene, namely HP0596 and HP0598.

Amoxicillin resistance of *pbp1A* is due to the $Ser_{414}Arg$ substitution

Sequence analysis of PBP1A showed two amino acid differences in the region between nucleotide 953 and 1313, a glutamic acid by an alanine ($Glu_{406}Ala$) and a serine by an arginine ($Ser_{414}Arg$). In order to identify which of these amino acid changes was responsible for the increase in amoxicillin resistance, site-directed mutagenesis was used. Strain 1061 was transformed with four different 135 bp fragments coding for amino acids 381 to 426 (numbering according to the *pbp1A* gene of *H. pylori* strain 26695 [25]) containing either no, one ($Glu_{406}Ala$ or $Ser_{414}Arg$), or both amino acid substitutions. Amx^R colonies were observed only after natural transformation with the two fragments that contained the $Ser_{414}Arg$ substitution with transformation frequencies of 7×10^{-8} and 4×10^{-8} CFU/µg DNA, respectively. Sequence analysis of PBP1A of four randomly selected transformants, obtained from two independent transformation experiments with the 135 bp $Ser_{414}Arg$ fragment, confirmed that only this substitution was present in these Amx^R transformants. The MIC of amoxicillin for these transformants was 1.0 mg/L. Beside MIC values of amoxicillin, the growth rates (optical density at 600 nm measured at regular intervals

during a time period of 48 hours), sizes, and shapes of the bacteria were determined. In the absence of amoxicillin no differences between the Hardenberg strain, the four Amx^R transformants, and strain 1061 were found in growth rates, sizes, or shapes of the bacteria. Under amoxicillin pressure (1 mg/L), the bacteria became a little bit shorter and thicker, but in spite of this morphological change there was no significant change in growth rates (data not shown).

Site-directed mutagenesis, indicated that the $Ser_{414}Arg$ substitution in PBP1A represents the main factor in amoxicillin resistance of the Hardenberg strain. Recently, other investigators [22] reported that amino acid substitutions, $Tyr_{484}Cys$, $Thr_{541}Ile$ and $Pro_{600}Thr$ in PBP1A are responsible for amoxicillin resistance of an *in vitro*-selected Amx^R *H. pylori* strain. None of these amino acid substitutions play a role in amoxicillin resistance of the Hardenberg strain, since these substitutions were not present in the Amx^R Hardenberg strain. Further, the $Ser_{414}Arg$ substitution in PBP1A in the Hardenberg strain and Amx^R 1061 transformants had no influence on the expressed amount of PBP1A molecules compared to the wild-type PBP1A of *H. pylori* strain 1061 (data not shown). Probably the amino acid substitutions in PBP1A of the Hardenberg strain and the *in vitro*-selected Amx^R strain [22] affect the binding affinity of PBP1A for penicillins, but this was not tested.

Role of the *pbp1A* gene in amoxicillin resistance of other *H. pylori* strains

To test the generality of our findings we also transformed the *H. pylori* strains J99, 26695 and SS1 (MICs of amoxicillin of 0.01 mg/L, 0.01 mg/L, and 0.2 mg/L, respectively) either with PCR product of the *pbp1A* gene or with total DNA from the Hardenberg strain. With all strains, Amx^R transformants were isolated at high frequency. Ten Amx^R transformants of each strain (obtained from two independent transformation experiments) were selected at random, and the MIC of amoxicillin was determined. All transformants had a MIC of 1.0 mg/L, which was identical to the MIC of the Amx^R *H. pylori* 1061 transformants, but lower than the MIC of the Amx^R Hardenberg strain. These results indicated that also in other strains than 1061, the introduction of the *pbp1A* gene from the Hardenberg increased their MIC of amoxicillin.

The results of our study showed that a single serine to arginine substitution in PBP1A induces high-level amoxicillin resistance in *H. pylori*. Since the existence of *H. pylori* strains with a moderate resistance level against amoxicillin already has been reported [18, 30], and apparently a single mutation is sufficient to increase the MIC of amoxicillin, an increase of naturally occurring high-level Amx^R *H. pylori* strains can be expected in the near future.

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CHAPTER 10

Mutational changes in the penicillin-binding protein 1A gene mediate amoxicillin resistance in *Helicobacter pylori*

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ABSTRACT

Amoxicillin-based therapies are still highly effective in the treatment of *Helicobacter pylori* infections. However, the incidence of amoxicillin resistance in *H. pylori* is increasing, resulting in reduced therapy efficacies. In the present study, the molecular mechanism underlying amoxicillin resistance of seven naturally occurring amoxicillin-resistant (Amx^R) *H. pylori* isolates was investigated. All seven *H. pylori* isolates were negative for β-lactamase activity. Gene transfer experiments indicate that in these Amx^R *H. pylori* isolates, resistance is mediated via mutations in the gene encoding the penicillin-binding protein 1A (PBP1A). Replacement of the wild-type *pbp1A* gene of the amoxicillin-susceptible (Amx^S) *H. pylori* reference strain 26695 by the *pbp1A* gene of the Amx^R *H. pylori* isolates resulted in an increased MIC of amoxicillin (MIC 0.5-1.0 mg/L). Analysis of the proteins encoded by the *pbp1A* genes of the Amx^S *H. pylori* strain 26695, the seven Amx^R isolates, and their Amx^R 26695 transformants revealed that three of the Amx^R *H. pylori* isolates contained the previously reported serine to arginine substitution at amino acid position 414, whereas the four other isolates contained amino acid differences within or adjacent to one of the conserved penicillin-binding motifs. These differences may affect the structural conformation and stability of the PBP1A protein, and thereby cause amoxicillin resistance in *H. pylori*. Although we cannot exclude the role of other genes in amoxicillin resistance of *H. pylori*, our results indicate that mutational changes in the *pbp1A* gene are the major cause of amoxicillin resistance in *H. pylori*.

INTRODUCTION

Helicobacter pylori is a common human pathogen that is strongly associated with the development of active gastritis, peptic ulcer diseases and gastric cancer [29]. Successful treatment of *H. pylori* infections not only leads to eradication of the bacterium, but also to regression of the associated diseases [14, 30]. In the United States and Europe most anti-*H. pylori* therapies consist of two antibiotics in combination with a proton pump inhibitor and/or bismuth component [19]. The antibiotic amoxicillin is one of the key components of such *H. pylori* eradication therapies [13].

Until the end of the 20th century it was generally accepted that amoxicillin resistance in *H. pylori* was very rare [20], but this is no longer the case. At first, amoxicillin-resistant (Amx^R) *H. pylori* isolates were obtained from dyspeptic patients in Italy and the United States [6]. As these isolates lost their amoxicillin resistance phenotype upon freezing at -80°C, they are often referred to as amoxicillin-tolerant [6]. Later, stable Amx^R *H. pylori* isolates were obtained from dyspeptic patients living in different geographic regions [21, 33, 34]. Currently available amoxicillin resistance rates vary between 0.8% to 1.4% [11, 22]. If amoxicillin resistance in *H. pylori* spreads further, serious problems would arise, resul-ting in reduced efficacy of amoxicillin-containing regimens [7].

In Gram-negative bacteria, resistance to β-lactam antibiotics like amoxicillin is often due to the enzymatic degradation of the antibiotic by β-lactamase [18]. Other mechanisms such as structural alterations in penicillin-binding proteins (PBPs), decreased membrane permeability of antibiotics into the bacterial cell and/or active efflux of the antimicrobial drug out of the bacterial cell have also been reported [3, 8, 24]. Although the *H. pylori* genome contains β-lactamase-like genes [23], no significant ß-lactamase activity has been detected and thus it seems that amoxicillin resistance in *H. pylori* does not rely on the acquisition or expression of a β-lactamase [5, 33].

To our knowledge the molecular mechanism underlying amoxicillin resistance has thus far only been described for five amoxicillin-tolerant *H. pylori* isolates [5], three natu-rally occurring amoxicillin-resistant (Amx^R) *H. pylori* isolates [9, 17, 26], and two *in vitro*-selected Amx^R mutants [4, 27]. In the amoxicillin-tolerant *H. pylori* isolates the resistance depends on a lack of the low-molecular-weight penicillin-binding protein PBP-D (also called PBP 4, HP0160) [5], whereas in the Amx^R *H. pylori* isolates and *in vitro*-selected mutants the resistance is mediated by mutational changes in the gene encoding the penicillin-binding protein 1A (*pbp1A*; HP0597) [4, 9, 17, 26, 27].

In this study we describe the molecular mechanism of amoxicillin resistance in seven naturally occurring Amx^R *H. pylori* isolates that were obtained between 1999 and 2003 from dyspeptic patients from Brazil (n=4) and the Netherlands (n=3).

Table 1. *H. pylori* isolates used in this study, their MICs of amoxicillin determined by agar dilution, and PBP1A sequence accession numbers.

Isolates/reference strain	Origin/Reference	Gastric disorders	MIC (mg/L)[a]	Accession number[b]
Clinical isolates				
BH13	Brazil	Duodenal ulcer	4	AY743230
13	Brazil	Duodenal ulcer	2	AY743231
60	Brazil	Gastroesophageal Reflux Disease	4	AY743232
219	Brazil	Gastric Ulcer	8	AY743233
SZ79	The Netherlands	Gastric cancer	4	AY743234
Hardenberg strain				
Original isolate	[9]	Gastritis	8	AF479617
H-I[c]	The Netherlands	Gastritis	16	AY743235
H-II[c]	The Netherlands	Gastritis	16	AY743236
Reference strain				
26695	[31]	Gastritis	< 0.125	

[a] MIC shown are means of results from three independent experiments. The isolates were considered to be resistant when the MIC of amoxicillin was > 0.25 mg/L [11].
[b] The *pbp1A* gene sequences have been deposited into the Genbank database.
[c] Follow-up isolate the Hardenberg strain, obtained from the same patient nine months after the isolation of the original Hardenberg strain.

MATERIAL AND METHODS

Bacterial strains and growth conditions

H. pylori isolates used in this study are listed in Table 1. The *H. pylori* isolates were routinely cultured on Columbia agar plates (Becton Dickonson and Company, Cockeysville, MD, USA) supplemented with 7% lysed horse blood (BioTrading, Mijdrecht, the Netherlands) and *H. pylori* selective supplement (Oxoid, Basingstoke, UK). *H. pylori* was grown for 48 to 72 hours at 37ºC in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2.

Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of amoxicillin was routinely determined by agar dilution using Mueller-Hinton (Difco, Sparks, MD, USA) plates supplemented with 5% sheep blood (BioTrading), according to the protocols of the National Committee for Clinical Laboratory Standard (NCCLS) [25]. Two µL of 2.0 MacFarland bacterial suspensions were inoculated on plates containing a 2-fold serial dilution of amoxicillin (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands). As controls, the amoxicillin-susceptible (Amx^S) strain 26695 and Amx^R Hardenberg strain were always included, and MIC determination was only accepted if the MIC of amoxicillin for these two strains were < 0.125 mg/L and > 4 mg/L, respectively. Isolates were considered to be resistant when the MIC of amoxicillin exceeded 0.25 mg/L [11]. All MICs of amoxicillin were determined in three independent experiments.

Detection of β-lactamase activity

Production of β-lactamase by *H. pylori* was tested using the Nitrocefin BBL™ DrySlide™ (Becton Dickinson and Company, sparks, MD, USA) according to the manufacturer's instructions.

Natural transformation of *H. pylori*

The Amx^S *H. pylori* reference strain 26695 was transformed with ~1 µg of genomic DNA from an Amx^R strain (Table 1), or ~250 ng of *pbp1A* gene products amplified from genomic DNA dissolved in TE (1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA). Amx^R transformants were selected on Columbia agar-plates supplemented with 7% lysed horse blood and 0.25 mg/L amoxicillin. As controls, bacteria were transformed with either DNA from the original Hardenberg strain (Amx^R), DNA from strain 26695 (Tet^S), or TE without DNA.

General DNA techniques

Oligonucleotide primers (Isogen, Maarsen, the Netherlands) used in this study are listed in Table 2. PCR was performed in an automated thermal cycler (I-cycler, Biorad Laboratories BV, Veenendaal, The Netherlands) using the PCR-core system I (Promega, Madison, WI, USA). Direct sequencing of the obtained PCR products was performed by Baseclear Inc. (Leiden, The Netherlands).

Nucleotide sequence accession numbers

The *pbp1A* gene sequences of the seven Amx^R *H. pylori* isolates have been deposited into the GenBank database (Table 1).

Table 2. Oligonucleotides used in this study.

Primer name	Primer sequence (5'-3')[a]	Position *pbp1A* gene[b]
Hp0597-F1	AGTTTGGGTAACTACGGATA	78 to 97, forward
Hp0597-F2	TTTGGGCATGGGTATTATGG	505 to 524, forward
Hp0597-F3	CTAACGCGTCTAATGAAGAT	953 to 972, forward
Hp0597-F4	TAACCCTTTCAGYGCTGATG	1583 to 1602, forward
Hp0597-R1	CGCATGAAATACGAATACAC	1820 to 1801, reverse
Hp0597-R2	AATCTTACCGGTGCTCGTGT	1026 to 1007, reverse
Hp0597-R3	CTCGTGTGAGCACCATGTTT	400 to 381, reverse

[a] Oligonucleotides used for amplification of the *pbp1A* gene were based on the published genome sequence of *H. pylori* strain 26695 [31].
[b] Position of oligonucleotides are given to the relative start point of the *pbp1A* gene of *H. pylori* strain 26695 [31].

RESULTS

MICs of amoxicillin and determination of β-lactamase activity

The MICs of amoxicillin for four Brazilian and three Dutch Amx^R *H. pylori* isolates were determined by agar dilution, and compared with previously described Amx^R *H. pylori* strain Hardenberg (MIC 8 mg/L) and the Amx^S *H. pylori* reference strain 26695 (MIC < 0.125 mg/L) (Table 1). The MICs of amoxicillin for the four Brazilian Amx^R *H. pylori* isolates ranged from 2 to 8 mg/L (susceptibility breakpoint > 0.25 mg/L), whereas the MICs of amoxicillin for the three Dutch Amx^R *H. pylori* isolates (SZ79 and the two follow-up isolates of the Harderberg strain, H-I and H-II) ranged from 4 to 16 mg/L. All MICs remained stable after storage -80°C and repetitive subculturing on amoxicillin-free agar plates, indicating that amoxicillin resistance in these seven novel Amx^R *H. pylori* isolates is stable.

There was no β-lactamase activity detectable in any of the isolates when tested by using the chromogenic cephalosporin method, excluding a role for β-lactamase in amoxicillin resistance in these Amx^R isolates.

Transfer of amoxicillin resistance by natural transformation

To examine whether the resistance to amoxicillin of the seven Amx^R strains was transferable to Amx^S *H. pylori*, natural transformation was performed using genomic DNA isolated from the Amx^R strains, with the Amx^S *H. pylori* reference strain 26695 as a recipient. Amx^R colonies were consistently obtained after transformation with genomic DNA isolated from Amx^R *H. pylori* strains, but not when DNA from the Amx^S 26695 strain or TE without DNA was used. The transformation frequencies with DNA from the Amx^R isolates were identical to that of the Hardenberg positive control and ranged from 10^{-4} to 10^{-7} CFU/µg DNA. MICs of amoxicillin for the Amx^R 26695 transformants (two transformants from two independent transformation experiments for each isolate tested) were determined by agar dilution, and ranged from 0.5 to 1.0 mg/L.

The *pbp1A* gene is involved in amoxicillin resistance

As stable amoxicillin resistance in all Amx^R *H. pylori* isolates studied thus far is mediated by mutations in the *pbp1A* gene, *H. pylori* reference strain 26695 (Amx^S) was transformed with *pbp1A* PCR fragments amplified from the genomic DNA of the seven Amx^R isolates. Amx^R colonies were obtained for all seven isolates with a transformation frequency of 10^{-4} to 10^{-7} CFU/µg DNA. No Amx^R transformants were observed after transformation with total DNA or PCR-amplified *pbp1A* of the Amx^S *H. pylori* reference strain 26695 or TE without DNA.

The MICs of amoxicillin for the obtained transformants ranged between 0.5 and 1.0 mg/L. Repeated transformation of these transformants with total DNA of their Amx^R donor strain did not result in an increase of the MIC of amoxicillin. Consistent with previous observations [9, 26, 27], the MICs of amoxicillin for the Amx^R transformants were still five to ten-fold lower than the MICs of the original strains. All Amx^R transformants were then transformed with either total DNA from the Hardenberg strain or total DNA from one of the other six Amx^R isolates, and plating out on agar plates containing 1-2 mg/L amoxicillin to assess an increase in resistance. No Amx^R colonies were obtained after transformation on agar plates containing 1-2 mg/L amoxicillin.

Identification of PBP1A mutations by sequence analysis

Sequence analysis of the *pbp1A* gene PCR fragments obtained from the Amx^S *H. pylori* reference strain 26695, the seven Amx^R clinical isolates, and their Amx^R 26695 transformants (obtained with chromosomal DNA) revealed multiple amino acid differences (Table 3). Most of them were located in the C-terminal part (encoded by nucleotide 960 to 1980 [12]) of the PBP1A protein (Table 3). Of the in total 52 amino acid differences, the involvement of 24 amino acid differences in amoxicillin resistance can be excluded, since 19 of them were present both in Amx^S and Amx^R *H. pylori* isolates (Figure 1), and the five others were not exchanged by natural transformation (Table 3). Of all amino acid differences, there are two amino acid substitutions that stand out, (i) a serine to a glycine substitution observed within the PBP1A motif $SKN_{402-404}$ of two Brazilian Amx^R isolates (BH13, and 13) and (ii) the previous described [9] serine to arginine substitution at position 414 found in the three Dutch Amx^R isolates.

DISCUSSION

Most *H. pylori* isolates are still susceptible to amoxicillin, an antibiotic commonly used in eradication therapy of *H. pylori*. Nevertheless, amoxicillin resistance is emerging in clinical isolates, especially in countries where this antibiotic can be obtained without prescription. Thus far, the molecular mechanism underlying amoxicillin resistance has only been identified for a few Amx^R and amoxicillin-tolerant *H. pylori* isolates [4, 5, 9, 17, 26]. In the Amx^R *H. pylori* isolates the resistance was mediated by differences in the gene encoding the PBP1A [9, 17, 26], whereas in the two amoxicillin-tolerant *H. pylori* isolates the resistance was mediated by a lack in PBP-D [5]. In this study, the mechanism of amoxicillin resistance was established for seven additional naturally occurring Amx^R *H. pylori* isolates.

Table 3. Overview on amino acid differences in PBP1A proteins from Amx^R *H. pylori* isolates and their Amx^R transformants.

Isolate	Amino acid position[a] 44	70	79	101	125	138	141	148	207	242	250	269	310	315	317	318	322 +	324	325	336	366	374	402	406	414
26695	G	R	I	I	F	T	L	I	S	G	V	K	K	K	K	T	-	D	E	D	F	V	S	E	S
BH13	[S]	[H]	[V]	V	L	I		L		S	I						D						G	A	
13	[S]		[V]		[L]			[L]															G	A	
60	S			V	L			L		S							D			E				V	
219					[L]			[L]		[S]		R		E	E	A		N	K						
SZ79					[L]		[I]														L				R
H-I					L				N				Q									L		A	R
H-II	[S]				[L]				[N]				Q									L		A	R

Isolate	417	450	469	473	479	480	491	504	508	509	511	515	518	530	532	535	543	558	562	563	589	591	593	595
26695	S	I	V	F	D	A	T	N	D	V	T	M	K	L	V	D	S	T	N	I	S	G	T	G
BH13	T							D	N	I	V	I			A						G		P	
13	T								[E]			[I]			[A]	[N]	[R]							
60	T							D	N	I	V				A					V	G			
219		V			E			D		I			R			N	R				G	K	A	S
SZ79			M	L										[I]									[A]	
H-I						V	S	D	N	I		I				N		S	Y		G			
H-II						V	S	D	N	I		I				N		S	Y		[G]			

[a] Position of amino acid differences are given to the relative start point of *pbp1A* gene of *H. pylori* strain 26695 [31]. Boxed capitals represent amino acid differences between the original Amx^R isolates and their Amx^R transformants, and are thus unlikely to contribute to amoxicillin resistance in *H. pylori*.

```
             .       I .         .         .         .         .         .         .         .         .
S:                  IV   V V     S N           S                                  V
1   MLKKIFYGFIVLFLIVMGLLAILIAQVWVTTDKDIAKIKDYRPGVASQILDRKGRLIANIYDKEFRFYARFEEIPPRFIESLLAVEDTLFFEHGGINLDA
R:           M  V   II                       G S                        VH    R   V

S:  V     R     H       I   L                      L                                                A
101 IMRAMIKNAKSGRYTEGGSTLTQQFVKNMVLTREKTLTRKLKEAIISIRIEKVLSKEEILERYLNQTFFGHGYYGVKTASLGYFKKPLDKLTLKEITMLV
R:  V                       L            I  I      L

S:                                           S       I                                I
201 ALPRAPSFYDPTKNLEFSLSRANDILRRLYSLGWISSNELKGALNEVPIVYNQTSTQNIAPYVVDEVLKQLDQLDGLKTQGYTIKLTIDLDYQRLALESL
.R:       N                                  S       I                  R

S:                         DKN           E
301 RFGHQKILEKIAKEKPKTNASN-E-DEDNLNASMIVTDTSTGKILALVGGIDYKKSAFNRATQAKRQFGSAIKPFVYQIAFDNGYSTTSKIPDTARNFEN
R:           Q    E EA    D  NK          E                             L       L       A

           K
S:         V          T
399 GNYSKNSEQNHAWHPSNYSRKFLGLVTLQEALSHSLNLATINLSDQLGFEKIYQSLSDMGFKNLPKD-LSIVLGSFAISPIDAAEKYSLFSNYGTMLKPM
R:     G   V       R  T                    M           V               E   M   L     EV   C       S
           A

S:   E    D   E      I              I A  N          H     V     S                   M          G
498 lIESITNQQNDVKTFTPMETKKITSKEQAFLTLSVLMDAVENGTGSLARIKGLEIAGKTGTSNNNIDAWFIGFTPTLQSVIWFGRDDNTPISKGATGGVV
R:        D   NI V   I  R           I A  N     I R            S S    YV                        G K A S
              E                                                                                    P

S:               S                           S        QK   G  P
598 SAPVYSYFMRNILAIEPSLKRKFDVPKGLRKEIVDKIPYYSTPNSITPTPKRTDDSEERLLF
R:    T          S                                    QK      P
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Figure 1. The sequence of the PBP1A protein of *H. pylori* reference strain 26695, with all amino acid differences detected so far, indicated in capitals above and below the sequence. Amino acid differences present in amoxicillin-susceptible isolates are indicated above the sequence (S:), whereas those present in amoxicillin-resistant isolates are given below the sequence (R:). Boxed residues represent amino acid substitutions potentially involved in amoxicillin resistance (present paper and [9, 17, 26]); underlined residues represent the PBP motifs [15]; italicized residues represent amino acid changes found in *in vitro*-selected Amx^{R} mutants [4, 27].

In Gram-negative bacteria, resistance to β-lactams is mostly due to the production of β-lactamase, either chromosomally encoded, or plasmid-borne [2]. In contrast, alterations in PBPs, have mainly been described in β-lactam-resistant Gram-positive bacteria, but they have also been reported in some Gram-negative bacterial species [10, 28]. Consistent with earlier findings in *H. pylori* [5, 17, 26, 33], amoxicillin resistance in all seven Amx^R *H. pylori* isolates did not rely on an increased β-lactamase activity. In this study, we have demonstrated that amoxicillin resistance in these seven Amx^R *H. pylori* isolates is stable and mediated by various mutational changes in the gene encoding the PBP1A protein.

Sequence analysis of the PBP1A from the obtained Amx^R 26695 transformants showed that the majority of the amino acid differences are located in the C-terminal part of this protein (Table 3). This region, also called transpeptidase domain of the PBP1A [12], contains three conserved PBP motifs: SXXK, SXN and KTG [15]. In *H. pylori* it is thought that the PBP motifs $SXN_{402-404}$ and $KTG_{555-557}$ are involved in the formation and stabilization of an active cleft [15]. Since in several other bacterial species β-lactam resistance is caused by mutations in or adjacent to one of the two motifs [1, 16, 32], it is likely that the amino acid changed $Ser_{402}Gly$, $Ser_{414}Arg$, $Asn_{562}Tyr$, or $Ile_{563}Val$ (marked in Figure 1) are involved in amoxicillin resistance of *H. pylori*, but this remains to be confirmed by targeted mutational studies.

In summary, in naturally occurring Amx^R *H. pylori* isolates, amoxicillin resistance is mediated by various mutational changes in the *pbp1A* gene. Although we cannot exclude the role of other genes in amoxicillin resistance, our results indicate that mutational changes in the *pbp1A* gene are the major contributing factor to this resistance.

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CHAPTER 11

Summary and concluding remarks

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An estimated 4 to 5 million individuals in the Netherlands are actively infected with *Helicobacter pylori*. Eradication of this bacterium becomes more difficult as the prevalence of antibiotic resistance is increasing worldwide. Most *H. pylori* infections are now diagnosed by non-invasive testing (i.e. urea breath test, serology, stool test), and thus data on antibiotic susceptibility are lacking. Furthermore, once the antibiotic susceptibility is assessed using conventional culture-based methods by means of an E-test, agar dilution or disc-diffusion, then data are difficult to compare between different centers due to lack of standardization. Molecular-based methods are reproducible and easily standardized, and thus they can offer an attractive alternative. To develop molecular-based methods knowledge of molecular mechanisms underlying antibiotic resistance is mandatory. The research presented in this thesis aims to obtain information on molecular mechanisms of antibiotic resistance in *H. pylori.* The clinical implications, the molecular mechanisms of antibiotic resistance in *H. pylori* and molecular detection methods developed thus far are presented in **Chapter 2**.

Molecular mechanism of tetracycline resistance in *H. pylori*

Most *H. pylori* strains are susceptible to tetracycline, but currently the incidence of tetracycline resistance in *H. pylori* seems to increase. In **Chapter 3** the isolation and characterization of the first Dutch tetracycline-resistant *H. pylori* isolate (strain 181) is described. In this study, twelve genes were selected from the published *H. pylori* genome sequences as potential candidates (i.e. seven efflux genes, three ribosomal protection genes and two 16S rRNA genes), and tested for the ability to transfer tetracycline resistance to tetracycline-susceptible *H. pylori* strains. Only the two 16S rRNA genes (*rrnA* and *rrnB*) were able to transfer the resistance. Subsequently it was shown that a single triple-base-pair substitution AGA$_{926-928}$TTC (numbering according to 16S *rrnA* of *H. pylori* strain 26695), present within both copies of the 16S rRNA genes mediates tetracycline resistance in *H. pylori* strain 181. As the minimal inhibitory concentration (MIC) of tetracycline for the tetracycline-resistant *H. pylori* strain 181 and the obtained tetracycline-resistant transformants containing the AGA$_{926-928}$TTC substitution were similar, we believe that this substitution is fully responsible for tetracycline resistance in *H. pylori* strain 181.

In **Chapter 4**, site-directed mutants containing single-, double- or triple-base-pair substitutions at position 926 to 928 of the 16S rRNA genes were constructed in the tetracycline-susceptible *H. pylori* reference strain 26695. In a similar isogenic background, the effect of the different 16S rRNA mutations on the growth rate, stability and the level of tetracycline resistance (MIC) were studied. Replacement of one or two base pairs in the 16S rRNA region 926 to 928 of the tetracycline-susceptible *H. pylori* strain reduced growth rates without inducing tetracycline resistance. Mutants that contained the naturally occurring TTC substitution had the highest growth rates and highest MIC values (MIC of tetracycline 8 mg/L). Apart from the TGA and AGC mutations, all other 16S rRNA mutations were stable upon repeated subculturing both in the presence and in the absence of tetracycline. The preference in *H. pylori* for the AGA$_{926-928}$TTC triple-base-pair substitution as main mechanism for tetracycline resistance in *H. pylori* most likely results from differences in growth rates, levels of resistance, and the stability of the mutations.

The study presented in **Chapter 5** confirms and extends the conclusions of **Chapter 3.** In five unrelated tetracycline-resistant *H. pylori* clinical isolates obtained from dyspeptic patients from Brazil, tetracycline resistance was mediated by the exact same triple-base-pair substitution as was present in the Dutch isolate, *H. pylori* strain 181. Both studies imply that, to confer tetracycline resistance, *H. pylori* requires the triple-base-pair $AGA_{926\text{-}928}\underline{TTC}$ substitution within both copies 16S rRNA genes.

Tetracycline resistance in *H. pylori* probably emerges by a stepwise process that is driven by selection depending on the duration and the dose of tetracycline in treatment. Therefore, it was not a surprise that low-level tetracycline-resistant *H. pylori* strains (MICs below the tetracycline susceptibility breakpoint of 4 mg/L) were isolated that contain single- or double-base-pair substitutions at position 926 to 928, i.e. $A_{926}\underline{G}$, $A_{928}\underline{C}$ $AG_{926\text{-}927}\underline{GT}$, and $A_{926}\underline{G}/A_{928}\underline{C}$ (**Chapter 6** and [5]).

The finding that *H. pylori* requires for tetracycline resistance a triple-base-pair substitution within both copies of the 16S rRNA genes, may explain the low prevalence of this resistance. However, we expect a higher incidence of low-level tetracycline-resistant *H. pylori* isolates with single- and double-base-pair substitutions as a result of insufficient antibiotic therapy. In order to evaluate the prevalence of single-, double-, and triple-base-pair substitutions in this region, we need to develop quick molecular screening techniques.

Molecular detection methods for tetracycline resistance in *H. pylori*

Conventional methods to assess levels of tetracycline resistance of *H. pylori* are culture-based. These methods are slow (data are usually obtained only after 6 to 10 days), cumbersome, and fail in approximately 10% of the cases due to contamination of the biopsy samples or growth failure. Since tetracycline resistance seems to be restricted to the occurrence of specific 16S rRNA mutations, molecular methods offer an attractive alternative. In **Chapter 5** a PCR-based restriction fragment length polymorphism (RFLP) was used to detect the triple-base-pair substitution $AGA_{926\text{-}928}\underline{TTC}$. This simple assay distinguishes tetracycline-resistant *H. pylori* isolates from the low-level tetracycline-resistant and tetracycline-susceptible *H. pylori* isolates, and thus allows rapid detection of tetracycline resistance in *H. pylori*.

In **Chapter 6**, a real-time PCR followed by melting curve analysis using LightCycler technology was developed. In contrast to the PCR-RFLP develop in **Chapter 5**, this assay was able to detect all previous reported mutations. The wild-type AGA sequence showed the highest melting temperature (57.4°C), whereas the single- (49.6-53.8°C), double- (46.3-49.1°C) and triple-base-pair (46.0°C) substitution displayed lower melting points. This method was originally developed on DNA extracts from cultured *H. pylori* isolates, and is now optimized for direct detection of *H. pylori* tetracycline resistance in gastric biopsy samples.

Oxygen-dependent metronidazole resistance in *H. pylori*

Metronidazole resistance in *H. pylori* reduces the efficacy of metronidazole-containing regimens. Nevertheless, it does not make them completely ineffective as therapeutically agent. The discrepancy observed between *in vitro* metronidazole resistance and treatment outcome may partially be explained by the antimicrobial activity of other components in the regimens and/or duration and doses of the therapy. Apart from these factors, it is thought that a low oxygen tension in the gastric environment also participates in this process, as *in vitro* metronidazole-resistant *H. pylori* strains become metronidazole-susceptible after short periods of anaerobiosis [4].

Metronidazole is administrated as a prodrug that needs to be activated by a reduction reaction. In *H. pylori* the *rdxA* and *frxA* genes encode reductases have been associated with the activation of metronidazole. Inactivation of these genes potentially results in metronidazole resistance. In **Chapter 7** the role of inactivating mutations in these genes on oxygen-dependent metronidazole resistance was investigated. Clinical isolates of *H. pylori* containing null mutations in the *rdxA* and *frxA* gene were incubated under anaerobic conditions either in the presence or absence of metronidazole, and the effect on reversibility of metronidazole resistance was determined. All metronidazole-resistant isolates became susceptible under anaerobic conditions, which suggests that metronidazole susceptibility may be restored at low oxygen conditions through the activation of potential anaerobic reduction pathways. However the loss of metronidazole resistance also occurred in the presence of the protein synthesis inhibitor chloramphenicol. This indicates that metronidazole is reduced by a pre-existing mechanism that functions under anaerobic conditions, and does not require *de novo* protein synthesis. Moreover, the results suggest that the RdxA and the FrxA proteins do not participate in oxygen-dependent metronidazole resistance, since metronidazole-resistant *H. pylori* containing inactivated *rdxA* and/or *frxA* genes become metronidazole-susceptible under anaerobic conditions. It is likely that metronidazole is activated by one of the other known nitroreductases such as ferredoxin (FdxA, HP0277), ferredoxin-like protein (FdxB, HP1508), flavodoxin (FldA, HP1161), pyruvate:ferredoxin oxidoreductase (PorD, HP1109) and/or 2-oxoglutarate ferredoxin oxidoreductase (OorD, HP0588).

Molecular mechanism of amoxicillin resistance in *H. pylori*

Amoxicillin-based therapies are still highly effective for the treatment of *H. pylori* infections, but the incidence of amoxicillin resistance is increasing, resulting in reduced therapy efficacies. **Chapter 8** describes the isolation of the first reported stable amoxicillin-resistant *H. pylori* clinical isolate (the Hardenberg strain). This strain was isolated from an 82-year-old Dutch dyspeptic patient who had received twelve courses of amoxicillin over the past 6 years for the treatment of chronic obstructive pulmonary disease. The Hardenberg strain was negative for β-lactamase activity. In a transformation experiment, it was shown that the resistance in the Hardenberg strain could be transferred to an amoxicillin-susceptible *H. pylori* strain, indicating that a genetic feature achieves the resistance. **Chapter 9** describes the characterization of the molecular mechanism underlying amoxicillin resistance in the Hardenberg strain. Based on their putative role in cell wall synthesis, ten genes were selected from the published *H. pylori* genome

sequences as potential candidates in amoxicillin resistance. These genes were amplified from genomic DNA of the Hardenberg strain and tested for their ability to transfer amoxicillin resistance to amoxicillin-susceptible *H. pylori* strains. Only the replacement of the wild-type *pbp1A* gene (encoding the penicillin-binding protein 1A, HP0597) by the *pbp1A* gene of the Hardenberg resulted in an increased MIC of amoxicillin. Sequence analysis of the minimal fragment able to transfer amoxicillin resistance revealed two amino acid substitutions (Glu_{406}Ala and Ser_{414}Arg) that either participates alone or together in amoxicillin resistance of the Hardenberg strain. Using site-directed mutagenesis we showed that amoxicillin resistance of the Hardenberg strain was mediated by the Ser_{414}Arg substitution in PBP1A.

Chapter 10 describes the molecular mechanism underlying amoxicillin resistance of seven additional amoxicillin-resistant *H. pylori* clinical isolates, obtained between 1999 and 2003 from dyspeptic patients from Brazil and the Netherlands. Consistent with the findings of **Chapter 9**, amoxicillin resistance in these seven amoxicillin-resistant *H. pylori* isolates was mediated by mutational changes in the gene encoding the PBP1A protein. Three of the amoxicillin-resistant *H. pylori* isolates contained the previously mentioned Ser_{414}Arg substitution, whereas the four other isolates contained mutations within or adjacent to one of the conserved penicillin-binding motifs. These mutations may affect the structural conformation of the PBP1A protein and consequently reduce the affinity of the PBP1A protein for amoxicillin, thereby causing amoxicillin resistance in *H. pylori*. However, this hypothesis remains to be investigated by targeted mutational studies and/or affinity binding studies with amoxicillin and PBP1A.

As the MICs of amoxicillin for the obtained amoxicillin-resistant transformants were slightly lower than that of the original amoxicillin-resistant *H. pylori* strains (**Chapter 8, 9 and 10)**, we cannot exclude the role of additional genes in *H. pylori* amoxicillin resistance. In this context, it has been demonstrated that decreased membrane permeability enhances amoxicillin resistance in *H. pylori* [6, 14]. Changes in lipopolysaccharides, phospholipid bilayers and outer membrane proteins, like porins HopA to HopE, may all affect the permeability of the membrane and thereby mediate the decreased uptake of amoxicillin in *H. pylori.* However, this issue remains to be investigated.

Concluding remarks

H. pylori infections is now generally accepted as an important cause of chronic active gastritis, peptic ulcer disease and gastric cancer. Infection with *H. pylori* is a worldwide problem, and currently, the only feasible cure for *H. pylori* is antibiotics treatment. The extensive use and limited choice of antibiotics for this therapy contributes to the development of antibiotic resistance, and may threaten the treatment of *H. pylori*-related disorders. To gain a better understanding of the effect of resistance on therapy outcome and to guide anti-*H. pylori* therapy, antibiotic resistance in *H. pylori* should be monitored. Data obtained by national and international surveillance programs can be used to improve therapy strategies, and slow down the development of antibiotic resistance in *H. pylori*. As in most countries, including the Netherlands, *H. pylori* infections are diagnosed by non-invasive testing (no data available on antibiotic susceptibility), and surveillance programs to monitor antibiotic resistance in *H. pylori* are lacking, most therapies are prescribed on insufficient data.

The research described in this thesis has primarily been focused on the elucidation of the molecular mechanisms underlying tetracycline, metronidazole and amoxicillin resistance in *H. pylori*. In summary, tetracycline resistance in *H. pylori* is mediated by the triple-base-pair substitution $AGA_{926\text{-}928}\underline{TTC}$ in both 16S rRNA genes (**Chapter 3, 4 and 5**), whereas metronidazole resistance in *H. pylori* is caused on mutational changes in the *rdxA* and *frxA* genes **(Chapter 7)**, and amoxicillin resistance in *H. pylori* seems to be based by various mutations in the *pbp1A* gene (**Chapter 9 and 10**). These findings are consistent with previous reported antibiotic resistance mechanisms of *H. pylori*; they all rely on point mutations of genes located in the chromosome [8, 11, 16, 20]. Although in *H. pylori* horizontal gene transfer among susceptible and resistant strains has been described [18], it appears that most mutations conferring antibiotic resistance arise *de novo* [21]. Therefore, mono- and dual-therapies based on one antibiotic should be avoided, as it will promote the induction and spread of antibiotic resistance in *H. pylori*.

Conventional culture-based susceptibility testing of *H. pylori* is not only time-consuming, but also standardization of the tests is lacking. As the majority of antibiotic resistance mechanisms in *H. pylori* are restricted to specific point mutations, molecular-based methods offer an attractive alternative. In contrast to culture-based susceptibility assays, these methods are reproducible and easily standardized, as they are independent of cell viability and growth rate of the bacteria. Moreover, they are faster than the conventional culture-based assays. When directly applied on gastric biopsy samples (transport time excluded), data can be obtained at the day of endoscopy. Using the knowledge on molecular mechanisms of antibiotic resistance in *H. pylori*, several molecular-based methods are now for hand to assess clarithromycin, tetracycline, and ciprofloxacin resistance in *H. pylori*. For metronidazole and amoxicillin resistance such methods are lacking, and do not seem to be forthcoming.

As nowadays most *H. pylori* infections are diagnosed by non-invasive testing, time and effort should be invested in the development of simple non-invasive molecular-based techniques that are able to detect antibiotic resistance of *H. pylori* in alternative clinical samples such as feces.

Future perspectives

In *H. pylori*, several virulence factors, such as urease, the vacuolating toxin A (VacA), the cytotoxin-associated antigen (CagA), and the blood-group-antigen binding adhesin (BabA) have all been used for the development of vaccines. Although phase I and II clinical trials are performed in humans [2, 7, 15], a commercial vaccine is still not available. Other potential developments in *H. pylori* treatment are: (i) antimicrobial peptides such as magainins, LL-37/hCAP18, and defensins [1, 10, 12]. They participate in the innate and adaptive immunity of nearly all living creatures [13]; (ii) porphyrins, they are a class of naturally occurring compounds that exhibit antimicrobial activity through catalyzing peroxidase and oxidase reactions [19]; and (iii) new diets, based on essential oils [3, 17] or probiotics [9]. These agents may be used as monotherapy, or synergistically to enhance the efficacy of existing anti-*H. pylori* therapy. Initial studies using these components are promising, and open a new field of research to improve the treatment of *H. pylori* infections.

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Samenvatting en conclusies, dankwoord, curriculum vitae, list of publications and conferences

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SAMENVATTING EN CONCLUSIES

Ruim 4 miljoen Nederlanders zijn besmet met *Helicobacter pylori*. Door de wereldwijde toename van antibioticum resistentie wordt de behandeling van een maaginfectie met deze bacterie steeds gecompliceerder. Daar op dit moment de meeste *H. pylori* infecties gediagnostiseerd worden met behulp van niet-invasieve testen (b.v. ureum ademtest, serologie, stool antigen test), is informatie over antibioticum gevoeligheid van deze bacterie vaak niet voor handen. Echter, indien de gevoeligheid wordt bepaald met op kweek gebaseerde technieken (E-test, agar dilutie en disk diffusie), kunnen de verkregen waarden moeilijk met elkaar worden vergeleken door het ontbreken van standaardisatie. Moleculaire testen daarentegen zijn reproduceerbaar en gemakkelijk te standaardiseren; zij bieden dus een aantrekkelijk alternatief. De ontwikkeling van deze technieken vereist echter inzicht in moleculaire mechanismen van antibioticum resistentie. Het onderzoek gepresenteerd in dit proefschrift was voornamelijk gericht op het ontrafelen van moleculaire mechanismen van antibioticum resistentie in *H. pylori*. De klinische implicaties, de moleculaire mechanismen van antibioticum resistentie in *H. pylori* en de tot op heden ontwikkelde moleculaire detectie technieken zijn beschreven in **hoofdstuk 2**.

Moleculair mechanisme van tetracycline resistentie in *H. pylori*

De meeste *H. pylori* stammen zijn gevoelig voor het antibioticum tetracycline, maar in de laatste paar jaar neemt de incidentie van tetracycline resistentie in *H. pylori* gestaag toe. **Hoofdstuk 3** rapporteert de isolatie en karakterisatie van de eerste Nederlandse tetracycline-resistente *H. pylori* stam (181). Voor dit onderzoek werd uit de gepubliceerde *H. pylori* genoom sequentie twaalf genen geselecteerd als potentiële kandidaten voor tetracycline resistentie. Dit betrof zeven efflux genen, drie ribosomale protectie genen en twee 16S rRNA genen. Alle twaalf genen werden in een transformatie experiment getest op hun vermogen om tetracycline resistentie over te dragen naar een tetracycline-gevoelige *H. pylori* stam. Slechts twee genen, de 16S *rrnA* and *rrnB* genen waren hiertoe in staat. Vervolgens werd in deze genen aangetoond dat één enkele drievoudige basenpaar verandering AGA$_{926-928}$TTC verantwoordelijk is voor de resistentie. Aangezien de resistentie waarden (ook wel MIC genoemd) van de tetracycline-resistente *H. pylori* stam 181 en de verkregen tetracycline-resistente AGA$_{926-928}$TTC transformanten gelijk waren, is het aannemelijk dat de AGA$_{926-928}$TTC verandering volledig verantwoordelijk is voor de resistentie in stam 181.

In **hoofdstuk 4** werden zeven gerichte mutanten gecreëerd in de tetracycline-gevoelige *H. pylori* referentie stam 26695. Deze mutanten bevatten één, twee of drie basenpaar veranderingen op de 16S rRNA posities 926 tot 928. In een gelijkwaardige, isogene achtergrond werd het effect van het verschillende 16S rRNA mutaties op de groeisnelheid, stabiliteit en het resistentie niveau gemeten. Vervanging van één of twee basenparen in de 16S rRNA regio 926 tot 928 van de tetracycline-gevoelige *H. pylori* stam leidde tot een verlaging van de groeisnelheid, echter zonder inductie van tetracycline resistentie. De mutanten die de natuurlijk voorkomende TTC mutatie bevatten, hadden de hoogste groeisnelheden en de hoogste MIC waarden (MIC van 8 mg/L). Met uitzondering van de TGA en AGC mutaties, waren alle 16S rRNA mutaties stabiel tijdens herhaalde kweekexperimenten in de aan- en afwezigheid van tetracycline. De voorkeur van *H. pylori*

voor de drievoudige $\mathrm{AGA}_{926-928}\underline{\mathrm{TTC}}$ mutatie als voornaamste mechanisme van tetracycline resistentie in *H. pylori* wordt kennelijk veroorzaakt door verschillen in groeisnelheid, niveau van resistentie en stabiliteit van de mutatie.

De studie gepresenteerd in **hoofdstuk 5** ondersteunt de conclusies van **hoofdstuk 3**. In vijf niet-verwante klinische tetracycline-resistente *H. pylori* isolaten, verkregen van Braziliaanse dyspeptische patiënten, werd tetracycline resistentie veroorzaakt door dezelfde drievoudige basenpaar verandering als gevonden in stam 181. Beide studies impliceren dat *H. pylori* de drievoudige $\mathrm{AGA}_{926-928}\underline{\mathrm{TTC}}$ mutatie in beide 16S rRNA genen vereist voor tetracycline resistentie.

In *H. pylori* ontstaat tetracycline resistentie waarschijnlijk door een stapsgewijs proces, dat berust op selectie afhankelijk van de duur en de dosis van tetracycline in de therapie. Om deze reden was het ook geen verrassing dat laag-resistente *H. pylori* stammen (MIC waarden beneden het tetracycline gevoeligheidsbreekpunt van 4 mg/L) werden geïsoleerd met één of twee basenpaar veranderingen op positie 926 tot 928 namelijk $\mathrm{A}_{926}\underline{\mathrm{G}}$, $\mathrm{A}_{928}\underline{\mathrm{C}}$, $\mathrm{AG}_{926-927}\underline{\mathrm{GT}}$ en $\mathrm{A}_{926}\underline{\mathrm{G}}/\mathrm{A}_{928}\underline{\mathrm{C}}$ (**hoofdstuk 6** en [5]).

Het gegeven dat in *H. pylori* voor tetracycline resistentie meerdere mutaties in twee genen vereist zijn, verklaart mogelijk de lage prevalentie van deze resistentie. Tevens is het aannemelijk dat het aantal *H. pylori* isolaten met één en twee basenpaar veranderingen hoger is dan de tot nu toe gerapporteerde aantal, als gevolg van falende antibioticum therapieën. Voor de evaluatie van de prevalentie van één, twee en drievoudige basenpaar veranderingen in deze regio, dienen snelle moleculaire detectie technieken te worden ontwikkeld.

Moleculaire detectie technieken voor tetracycline resistentie in *H. pylori*

De traditionele technieken voor de bepaling van tetracycline resistentie in *H. pylori* zijn op kweek gebaseerd. Deze technieken zijn langzaam (resultaten worden meestal pas verkregen na 6 tot 10 dagen) en falen in ongeveer 10% van de gevallen door contaminatie van het biopt of door groeiproblemen. Aangezien tetracycline resistentie waarschijnlijk beperkt is tot het voorkomen van specifieke 16S rRNA veranderingen, bieden moleculaire technieken een aantrekkelijk alternatief. In **hoofdstuk 5** werd een PCR gebaseerde restrictie fragment lengte polymorfisme (RFLP) gebruikt voor de detectie van de drievoudige basenpaar verandering $\mathrm{AGA}_{926-928}\underline{\mathrm{TTC}}$. Deze eenvoudige methode onderscheidt tetracycline-resistente *H. pylori* isolaten met de $\mathrm{AGA}_{926-928}\underline{\mathrm{TTC}}$ mutatie van laag-resistente en gevoelige *H. pylori* isolaten.

In **hoofdstuk 6** werd een real-time PCR gevolgd door een smeltpunt analyse beschreven op basis van LightCyler technologie. In tegenstelling tot de PCR-RFLP beschreven in **hoofdstuk 5** is deze methode in staat om alle tot nu toe gerapporteerde veranderingen te detecteren. De wild-type AGA sequentie vertoonde de hoogste smelttemperatuur (57,4ºC), terwijl de smelttemperaturen van de enkele (49,6-53,8ºC), dubbele (46,3-49,1ºC) en drievoudige (46,0ºC) basenpaar veranderingen lager waren. Deze techniek was oorspronkelijk ontwikkeld op geëxtraheerd DNA van gekweekte *H. pylori* isolaten, maar wordt momenteel geoptimaliseerd voor de directe detectie van tetracycline resistentie in *H. pylori* positieve maagbiopten.

Zuurstof-afhankelijke metronidazol resistentie in *H. pylori*

Metronidazol resistentie in *H. pylori* reduceert de effectiviteit van een metronidazol gebaseerde anti-*H. pylori* therapie. Het maakt echter deze therapie niet volledig ongeschikt. De waargenomen discrepantie tussen de metronidazol resistentie waarden en het slagings-percentages van de therapieën kan gedeeltelijk verklaard worden door de antimicrobiële activiteit van de andere componenten, de duur en/of de dosis van de therapie. Aangezien *in vitro* metronidazol-resistente *H. pylori* stammen metronidazol gevoelig worden na een korte periode van anaerobiosis, denkt men dat een lagere zuurstofspanning in de maag tevens een bijdrage levert in dit proces [4].

Het antibioticum metronidazol is een prodrug die geactiveerd dient te worden. In *H. pylori* zijn de *rdxA* en *frxA* gen coderende reductases hiervoor verantwoordelijk. Inactivatie van deze genen leidt dan ook meestal tot metronidazol resistentie. In **hoofdstuk 7** werd de rol van inactiverende *rdxA* en *frxA* genmutaties op zuurstof-afhankelijke metro-nidazol resistentie bestudeerd. Zeven klinische *H. pylori* isolaten met een nul mutatie (geïnactiveerd gen) in het *rdxA* en/of *frxA* gen werden gekweekt onder anaërobe condities, in aan- en afwezigheid van metronidazol. Vervolgens werd het effect van deze condities op de omkeerbaarheid van metronidazol resistentie onderzocht. Alle metronidazol-resistente isolaten werden metronidazol gevoelig onder anaërobe condities. Dit suggereert dat metronidazol gevoeligheid hersteld kan worden onder lage zuurstof condities, mogelijk door de activatie van één of meerdere anaërobe reductie routes. Het verlies van metronidazol resistentie trad echter ook op in aanwezigheid van de eiwitsyntheseremmer chlooramfenicol. Dit impliceert dat metronidazol wordt gereduceerd door een reeds bestaand mechanisme dat functioneert onder anaërobe condities en geen *de novo* eiwit-synthese vereist. Verder suggereren de resultaten dat de RdxA en FrxA eiwitten geen rol spelen in zuurstof-afhankelijke metronidazol resistentie, aangezien metronidazol-resistente *H. pylori* stammen met een geïnactiveerd *rdxA* en/of *frxA* gen metronidazol gevoelig worden onder anaërobe condities. Mogelijk wordt metronidazol onder deze condities geactiveerd door een reeds bekend nitroreductase, bijvoorbeeld ferredoxine (FdxA, HP0277), ferredoxine-achtig eiwit (FdxB, HP1508) flavodoxine (FldA, HP1161), pyruvaat:ferredoxine oxidoreductase (PorD, HP1109) en/of 2-oxoglutarate ferredoxine oxidoreductase (OorD, HP0588).

Moleculaire mechanisme van amoxicilline resistentie in *H. pylori*

Tot voor kort waren de meeste anti-*H. pylori* therapieën gebaseerd op amoxicilline zeer effectief. Echter door de stijgende incidentie van amoxicilline resistentie neemt de effectiviteit van deze therapieën gestaag af. **Hoofdstuk 8** beschrijft de isolatie van het eerste gerapporteerde stabiele amoxicilline-resistente *H. pylori* isolaat, ook wel de Hardenberg stam genoemd. Deze stam werd geïsoleerd bij een 82-jarige Nederlandse dyspeptische patiënt die in de afgelopen 6 jaar twaalf maal een amoxicilline kuur had ontvangen voor de behandeling van een chronische obstructieve longziekte. De Hardenberg stam vertoonde geen β-lactamase activiteit. Tijdens een transformatie experiment werd aangetoond dat de resistentie in de Hardenberg stam overgedragen kon worden naar een amoxicilline-gevoelige *H. pylori* stam. Dit wijst erop dat de resistentie waarschijnlijk berust op een genetische verandering.

Hoofdstuk 9 beschrijft de karakterisatie van het moleculaire mechanisme van amoxicilline resistentie in de Hardenberg stam. Op basis van hun vermeende rol in de celwand synthese werden tien genen als potentiële kandidaten geselecteerd uit de gepubliceerde *H. pylori* genoom sequentie. Deze genen werden geamplificeerd van genomisch DNA van de Hardenberg stam en getest op hun vermogen om amoxicilline resistentie over te dragen op een amoxicilline-gevoelige *H. pylori* stam. Alleen vervanging van het wild-type *pbp1A* gen (codeert voor het penicilline bindende eiwit 1A, HP0597) door het *pbp1A* gen van de Hardenberg stam resulteerde in een verhoging van de MIC. Sequentie analyse van het kleinst mogelijke fragment dat in staat was de resistentie over te dragen, liet twee aminozuur veranderingen zien, een $Glu_{406}Ala$ en $Ser_{414}Arg$. Vervolgens werd, door gebruik te maken van gerichte mutanten, aangetoond dat amoxicilline resistentie in de Hardenberg stam wordt veroorzaakt door de $Ser_{414}Arg$ verandering in het PBP1A eiwit.

Hoofdstuk 10 beschrijft het moleculaire resistentie mechanisme van zeven additionele klinische amoxicilline-resistente *H. pylori* isolaten. Deze isolaten werden tussen 1999 en 2003 geïsoleerd uit Braziliaanse en Nederlandse patiënten met dyspeptische klachten. Overeenkomstig met de bevindingen van **hoofdstuk 9**, werd de resistentie in deze zeven amoxicilline-resistente *H. pylori* isolaten veroorzaakt door mutaties in het *pbp1A* gen. Drie van de isolaten bevatten de hierboven genoemde $Ser_{414}Arg$ verandering, terwijl de vier andere isolaten mutaties bevatten die gelegen zijn in of aangrenzend aan één van de geconserveerde penicilline bindingsmotieven. Deze veranderingen kunnen een effect hebben op structurele samenstelling van het PBP1A eiwit, met als gevolg een reductie van de affiniteit van het PBP1A eiwit voor amoxicilline, wat op hun beurt kan leiden tot amoxicilline resistentie. Echter dient deze hypothese nog onderzocht te worden met gerichte mutant studies en/of affiniteit bindingsstudies met amoxicilline en het PBP1A eiwit.

Aangezien de resistentie waarden van de amoxicilline-resistente *H. pylori* transformanten iets lager waren dan de resistentie waarden van de originele amoxicilline-resistente stammen, kan de rol van additionele genen in amoxicilline resistentie bij *H. pylori* niet worden uitgesloten. In lijn met deze veronderstelling is recent gebleken dat amoxicilline resistentie in *H. pylori* toeneemt door een verlaagde membraanpermeabiliteit [6, 14]. Veranderingen in lipopolysacchariden, fosfolipiden en buitenmembraaneiwitten, zoals de porines HopA tot HopE, kunnen allen effect hebben op de membraanpermeabiliteit en dus een rol spelen in de verlaagde opname van amoxicilline door *H. pylori*. Dit laatste vraagstuk is echter nog niet opgehelderd.

Conclusies

Het is nu algemeen geaccepteerd dat *H. pylori* infecties een belangrijke rol spelen in de ontwikkeling van chronische actieve gastritis, peptische zweren en maagkanker. Infectie met *H. pylori* komt wereldwijd voor. Momenteel berust de enige geschikte behandeling van *H. pylori* op antibiotica. Het intensieve gebruik en de beperkte keuze van antibiotica voor de behandeling van *H. pylori* hebben bijgedragen aan de ontwikkeling van antibioticum resistentie, en dit kan een bedreiging vormen voor de behandeling van *H. pylori* gerelateerde afwijkingen. Om een beter inzicht te krijgen in het effect van resistentie op het slagen van de therapie, dient antibioticum resistentie in *H. pylori* gecontroleerd te worden. De gegevens die verkregen worden via nationale en internationale surveillance

programma's kunnen gebruikt worden ter verbetering van de huidige behandelingsstrategieën. Aangezien in de meeste landen (waaronder Nederland) infecties met *H. pylori* worden vastgesteld met niet-invasieve testen en landelijke surveillance programma's voor de detectie van antibioticum resistentie in *H. pylori* vaak ontbreken, worden de meeste anti-*H. pylori* therapieën voorgeschreven op basis van onvolledige data.

Het onderzoek beschreven in dit proefschrift was voornamelijk gericht op de opheldering van de moleculaire resistentie mechanismen van tetracycline, metronidazole en amoxicilline resistentie in *H. pylori*. In samenvatting, tetracycline resistentie in *H. pylori* wordt veroorzaakt door een drievoudige basenpaar verandering $AGA_{926\text{-}928}\underline{TTC}$ in beide 16S rRNA genen (**hoofdstuk 3, 4** en **5**), terwijl metronidazol resistentie in *H. pylori* wordt veroorzaakt door veranderingen in het *rdxA* en *frxA* gen (**hoofdstuk 7**). Amoxicilline resistentie in *H. pylori* daarentegen lijkt te berusten op verschillende mutaties in het *pbp1A* gen (**hoofdstuk 9** en **10**). Deze data zijn in overeenstemming met de reeds eerder beschreven antibioticum resistentie mechanismen in *H. pylori*, die allen berusten op puntmutaties in genen die gelokaliseerd zijn op het chromosoom [8, 11, 16, 20]. Alhoewel in *H. pylori* horizontale genoverdracht tussen gevoelige en resistente stammen incidenteel is beschreven [18], lijkt het erop dat antibioticum resistentie in deze bacterie ontstaat door spontane mutaties [21]. Om deze reden dient het gebruik van mono- of duo-therapieën gebaseerd op één antibioticum vermeden te worden, aangezien zij het ontstaan en de verspreiding van antibioticum resistentie in *H. pylori* bevorderen.

Antibioticum gevoeligheidsbepalingen gebaseerd op kweek zijn niet alleen tijdrovend, maar moeilijk te interpreteren door het ontbreken van standaardisatie. Aangezien de meeste van de resistentie mechanismen in *H. pylori* veroorzaakt worden de specifieke puntmutaties, bieden moleculair gebaseerde detectie technieken een aantrekkelijk alternatief. In tegenstelling tot op kweek gebaseerde technieken, zijn deze methoden reproduceerbaar en gemakkelijk te standaardiseren, aangezien zij onafhankelijk zijn van de levensvatbaarheid en groeisnelheid van de bacterie. Daarnaast zijn deze technieken sneller dan de op kweek gebaseerde technieken. Indien de detectie direct plaatsvindt op maagbiopten kan de uitslag nog op de dag van endoscopie worden verkregen. Door gebruik te maken van de moleculaire kennis op het gebied van antibioticum resistentie zijn reeds enkele testen voor de detectie van clarithromycine, tetracycline en ciprofloxacine resistentie in *H. pylori* ontwikkeld. Voor metronidazole en amoxicilline resistentie ontbreken deze testen echter, en het lijkt erop dat zij ook niet op korte termijn ontwikkeld gaan worden. Aangezien de meeste *H. pylori* infecties momenteel gediagnostiseerd worden met niet-invasieve testen, dient meer tijd en inspanning geïnvesteerd te worden in de ontwikkeling van simpele niet-invasieve moleculaire technieken die in staat zijn om antibioticum resistentie van *H. pylori* aan te tonen in alternatieve klinische monsters, bijvoorbeeld in faeces.

Toekomstperspectieven

In *H. pylori* zijn verscheidene virulentie factoren, zoals urease, het vacuolating cytotoxine A (VacA), het cytotoxine geassocieerde antigeen (CagA) en het bloedgroep antigeen bindende adhesine (BabA) gebruikt voor de ontwikkeling van vaccins. Hoewel fase I en II klinische trials op mensen zijn uitgevoerd [2, 7, 15], is een commercieel vaccin nog steeds niet beschikbaar. Andere potentiële ontwikkelingen ter verbetering van de huidige *H. pylori* therapieën berusten op (i) antimicrobiële peptiden zoals magainines, LL-37/hCAP18

en defensines [1, 10, 12]. Deze peptiden participeren in de aangeboren en verkregen afweer van bijna alle levende individuen [13]; (ii) porfyrines, zij behoren tot een klasse van natuurlijk voorkomende componenten die antimicrobiële activiteit vertonen doordat zij peroxidase en oxidase reacties versnellen [19]; en (iii) nieuwe diëten, gebaseerd op essentiële oliën [3, 17] of probiotica [9]. Deze componenten kunnen gebruikt worden in de vorm van monotherapieën of in combinatie met de huidige anti-*H. pylori* therapieën. Aangezien de eerste studieresultaten met deze componenten hoopgevend lijken, openen zij nieuwe perspectieven voor de behandeling van *H. pylori*.

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Monique Gerrits

CURRICULUM VITAE

Monique Gerrits is geboren op 4 september 1972 te Zevenaar. Na 4 jaar Sint Willibrord Mavo te Doetinchem, startte zij in 1988 met de opleiding Middelbaar Laboratorium Onderwijs aan het MBO College te Arnhem. Vervolgens werd met succes het Hoger Laboratorium Onderwijs aan Hogeschool van Utrecht afgesloten. Tijdens deze laatste studie specialiseerde zij zich in de Medische Microbiologie en Medische Biotechnologie. In het kader van haar specialisatie, liep zij twee maal stages, één bij het Medisch Microbiologisch Laboratorium van het Diakonessenhuis te Utrecht en één bij de afdeling Medische Microbiologie en Infectiepreventie van de Vrije Universiteit te Amsterdam. Bij haar laatste stageplaats accepteerde zij in april 1996 een functie als medisch research analiste. Hier verrichtte zij onder leiding van dr. J.G. Kusters vierenhalf jaar lang onderzoek aan *Helicobacter pylori*. Per 1 januari 2001 werd deze samenwerking voortgezet bij de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC te Rotterdam. In mei 2002 aanvaarde zij onder leiding van Prof.dr. E.J. Kuipers, dr. J.G. Kusters en dr. A.H.M. van Vliet een promotiepositie. Tijdens haar promotieonderzoek ging zij op internationaal werkbezoek bij de afdeling Clinical Pharmacology and Gastroenterology Unit van de São Francisco University te Bragança Paulista, Brazilië.

LIST OF PUBLICATIONS

1. Gerrits, M. M., E. J. van Der Wouden, D. Bax, A. de Jonge, A. H. van Vliet, J. C. Thijs, J. G. Kusters, A. A. van Zwet, and E. J. Kuipers. 2004. The role of the *rdxA* and *frxA* genes in oxygen-dependent metronidazole resistance in *Helicobacter pylori*. J. Med. Microbiol. 53:1123-1128.

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INTERNATIONAL CONFERENCES

1. 11th International workshop on Campylobacter, Helicobacter and related organisms (CHRO), Freiburg, Germany, 2001.
2. 5th International workshop on pathogenesis and host response in Helicobacter infections, Helsingør, Denmark, 2002. 'Winner Young Scientists Awards, best poster presentation in the session genetics'.
3. 12th International workshop on Campylobacter, Helicobacter and related organisms (CHRO), Aarhus, Denmark, 2003.
4. 17th International workshop of the European Helicobacter study group (EHSG), Vienna, Austria, 2004. 'Best oral presentation in the session drug resistance and novel treatments'.