The *Helicobacter hepaticus* hefA Gene is Involved in Resistance to Amoxicillin

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amoxicillin, enteric Helicobacter species, treatment, efflux, antibiotic resistance, bile acids.

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Abstract

**Background:** Gastrointestinal infections with pathogenic *Helicobacter* species are commonly treated with combination therapies, which often include amoxicillin. Although this treatment is effective for eradication of *Helicobacter pylori*, the few existing reports are less clear about antibiotic susceptibility of other *Helicobacter* species. In this study we have determined the susceptibility of gastric and enterohepatic *Helicobacter* species to amoxicillin, and have investigated the mechanism of amoxicillin resistance in *Helicobacter hepaticus*.

**Materials and methods:** The minimal inhibitory concentration (MIC) of antimicrobial compounds was determined by E-test and agar/broth dilution assays. The *hefA* gene of *H. hepaticus* was inactivated by insertion of a chloramphenicol resistance gene. Transcription was measured by quantitative real-time polymerase chain reaction.

**Results:** Three gastric *Helicobacter* species (*H. pylori, H. mustelae, and* *H. acinonychis*) were susceptible to amoxicillin (MIC < 0.25 mg/L). In contrast, three enterohepatic *Helicobacter* species (*H. rappini, H. bilis, and* *H. hepaticus*) were resistant to amoxicillin (MIC of 8, 16, and 6–64 mg/L, respectively). There was no detectable β-lactamase activity in *H. hepaticus*, and inhibition of β-lactamases did not change the MIC of amoxicillin of *H. hepaticus*. A *H. hepaticus hefA* (*hh0224*) mutant, encoding a TolC-component of a putative efflux system, resulted in loss of amoxicillin resistance (MIC 0.25 mg/L), and also resulted in increased sensitivity to bile acids. Finally, transcription of the *hefA* gene was not responsive to amoxicillin, but induced by bile acids.

Conclusions: Rodents are frequently colonized by a variety of enterohepatic *Helicobacter* species, and this may affect their global health status and intestinal inflammatory responses. Animal facilities should have treatment strategies for *Helicobacter* infections, and hence resistance of enterohepatic *Helicobacter* species to amoxicillin should be considered when designing eradication programs.

Members of the genus *Helicobacter* chronically colonize the different organs of the digestive tract in mammals. *Helicobacter* species are subdivided in the gastric *Helicobacter* species, which colonize the gastric mucosa (e.g. *H. pylori* in humans and primates), and the enterohepatic *Helicobacter* species, which colonize the intestines and/or hepatobiliary tract [1,2]. Colonization with enterohepatic *Helicobacter* species usually results in chronic intestinal and hepatic inflammation, and is associated with diseases like typhlocolitis, inflammatory bowel disease, hepatitis, and development of hepatic carcinoma [2–5] or can contribute to the formation of cholesterol gallstones [6–8]. *Helicobacter hepaticus* is the best studied enterohepatic *Helicobacter* species, and colonizes the intestine and bile ducts of rodents [3–5]. Colonization of laboratory mice with *H. hepaticus* or other enterohepatic *Helicobacter* species is a serious problem for many animal facilities worldwide [9]. Not much is known about the antimicrobial resistance of *H. hepaticus* or other enterohepatic *Helicobacter* species, although therapies for treatment of *H. hepaticus* in laboratory mice include a combination of antibiotics [10–13].
Cells have to handle the accumulation of toxic substances in the cell. One of the solutions to this problem is the extrusion of such toxic substances out of the cytoplasm, a process that is usually mediated by efflux pumps [14]. Efflux pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds. Toxic substances targeted by efflux pumps include bile acids and all classes of clinically relevant antibiotics, and efflux pumps are often associated with multiple drug resistance in both eukaryotic and prokaryotic cells [15]. Bacterial efflux systems are classified into several families based on sequence similarities [16]. One such family, the resistance-nodulation-division (RND) family of efflux systems is of particular interest with regard to their occurrence in pathogenic bacteria, because of their unusually broad substrate specificity [17]. This group of efflux proteins is ubiquitous among Gram-negative bacteria [18] where they are often found to be involved in the resistance to multiple antibiotics and antimicrobial compounds [14]. The activity of RND pumps is energized via the proton motive force, and RND pumps consist of three components that include an integral membrane pump protein (IMP), a periplasmatic membrane fusion protein (PMP) and an outer membrane pore protein (OMP). These three proteins together function as a continuous channel for extrusion of substrates from within the cell envelope back to the external environment [18].

In this study we report that several enterohepatic Helicobacter species are resistant to the β-lactam antibiotic amoxicillin. Subsequently we have used H. hepaticus as a type species for the group of enterohepatic Helicobacter species, and demonstrate that the gene encoding the HefA component of the putative H. hepaticus HefABC RND efflux system is involved in resistance to amoxicillin and other antimicrobials including bile acids. In addition, transcription of the hefA gene is induced by bile acids but not by amoxicillin. Finally, we discuss the implications of amoxicillin resistance in enterohepatic Helicobacter species on treatment of this infection, especially with respect to mouse strains used in animal experiments.

### Methods

#### Bacterial strains, plasmids, and growth conditions

All Helicobacter species and strains used are listed in Table 1. These were routinely cultured at 37 °C in microaerobic conditions (5% O₂, 7.5% CO₂, 7.5% H₂, and 80% N₂) on Columbia agar plates supplemented with 7% saponin-lysed horse blood and Dent selective supplement (Oxoid, Basingstoke, UK) [19,20]. Liquid growth was performed in Brucella broth (Difco, Sparks, MD, USA) supplemented with 0.2% (w/v) β-cyclodextrins (Fluka, Buchs, Switzerland). Escherichia coli strains DH5α and ER1793 were grown aerobically in Luria-Bertani medium [21] at 37 °C. When required, growth media were supplemented with chloramphenicol to a final concentration of 20 mg/L or ampicillin to a final concentration of 100 mg/L.

#### Assessment of antimicrobial susceptibility

Helicobacter species were grown on horse blood-supplemented Columbia agar plates for 24 hours at 37 °C. Thereafter cells were resuspended in PBS and adjusted to a concentration of approximately 2 × 10⁷ cells/mL. This suspension was spread with a swab stick on horse blood-supplemented

<table>
<thead>
<tr>
<th>Species</th>
<th>Natural host</th>
<th>Site of colonization</th>
<th>MIC (mg/L)</th>
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</thead>
<tbody>
<tr>
<td>H. hepaticus ATCC S1449</td>
<td>Mouse</td>
<td>Enterohepatic</td>
<td>64 (R)</td>
</tr>
<tr>
<td>H. hepaticus 1701</td>
<td>Mouse</td>
<td>Enterohepatic</td>
<td>8 (R)</td>
</tr>
<tr>
<td>H. hepaticus MU94</td>
<td>Mouse</td>
<td>Enterohepatic</td>
<td>6 (R)</td>
</tr>
<tr>
<td>H. rappini NCTC 12461</td>
<td>Mouse</td>
<td>Enterohepatic</td>
<td>8 (R)</td>
</tr>
<tr>
<td>H. bilis NCTC 18449</td>
<td>Mouse, rat, human</td>
<td>Enterohepatic</td>
<td>16 (R)</td>
</tr>
<tr>
<td>H. mustelae NCTC 12198</td>
<td>Ferret</td>
<td>Gastric</td>
<td>0.25 (S)</td>
</tr>
<tr>
<td>H. acinonychis NCTC 12686</td>
<td>Cheetah</td>
<td>Gastric</td>
<td>&lt; 0.016 (S)</td>
</tr>
<tr>
<td>H. pylori 26695</td>
<td>Human</td>
<td>Gastric</td>
<td>0.047 (S)</td>
</tr>
<tr>
<td>H. pylori BH9802-108</td>
<td>Human</td>
<td>Gastric</td>
<td>4 (R)</td>
</tr>
</tbody>
</table>

*Minimal inhibitory concentration (MIC) of amoxicillin of different gastric and enterohepatic Helicobacter species was performed using E-test. All values are means derived from three independent experiments.

*MIC values of the H. pylori amoxicillin-resistant and -sensitive reference strains are similar to previously described values [22,26].

R, resistant to amoxicillin; S, susceptible to amoxicillin. The susceptibility breakpoint of amoxicillin for H. pylori is 0.5 mg/L [22], and was used for all other Helicobacter species tested.
Columbia agar plates (without Dent supplement). Minimal inhibitory concentration (MIC) values were determined by E-test (AB Biodisc, Solna, Sweden) or by agar dilution assays [22]. Susceptibility to ethidium bromide (Promega, Leiden, the Netherlands), sodium dodecyl sulfate (Sigma-Aldrich, Zwijndrecht, the Netherlands), nickel chloride (Sigma-Aldrich), cholic acid (Sigma-Aldrich), and deoxycholic acid (Sigma-Aldrich) was determined by broth dilution assay [23]. In order to test for β-lactamase activity of *H. hepaticus*, cells were grown overnight on horse blood-supplemented Columbia agar plates with or without 32 mg/L amoxicillin. Cells were collected and suspended in PBS, and put on a Dryslide Nitrocefin (BD BBL, Breda, the Netherlands) test. The effect of disturbance of the proton motive force was assessed with the aid of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich). Absence of β-lactamase activity in *H. hepaticus* was confirmed using augmentin (amoxicillin and clavulanate) E-test strips.

The genome sequence [27].

**Construction of *H. hepaticus* mutants**

The *hefA* (hh0224) gene of *H. hepaticus* strain ATCC 51449 was amplified using specific primers (Table 2) and cloned in pGEM-T easy (Promega), resulting in plasmid pCB20. The *hefA* gene was subsequently interrupted by insertion of the chloramphenicol resistance gene from pAV35 [24] in a unique EcoRV restriction site, resulting in plasmid pCB21. This plasmid was introduced into *E. coli* ER1793 and used for natural transformation of *H. hepaticus* ATCC 51449 as described before [19]. Colonies derived from two independent transformations were tested. Correct allelic replacement of the wild-type gene with the interrupted version was confirmed by polymerase chain reaction (PCR).

**qRT-PCR analyses**

*H. hepaticus* ATCC 51449 was grown in BBC medium without extra supplementation, or in BBC medium supplemented with cholic acid (874 mg/L), taurocholic acid (672 mg/L), deoxycholic acid (108 mg/L), tauro- deoxycholic acid (529 mg/L), or amoxicillin (8 mg/L). Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. cDNA was produced by reverse transcription by incubating 100 ng of RNA with gene-specific primers *hefA* RQ1 and 16S RQ1 (Table 2) for 10 minutes at 70 °C and rapid cooldown to 4 °C. Then 20 U RNasin (Promega), 10 mmol/L dNTP, and 5 units of avian myeloblastosis virus reverse transcriptase (Promega) were added. Elongation was performed for 10 minutes at room temperature followed by 1 hour at 42 °C. The reverse transcriptase enzyme was inactivated by 5 minutes incubation at 95 °C followed by 5 minutes at 4 °C. cDNA was used directly for Quantitative RT-PCR (qRT-PCR). PCR primer combinations *hefA* FQ1/*hefA* RQ1 and 16S FQ1/16S RQ1 were first used in standard PCRs, and the identity of the PCR product was confirmed by size estimation inferred after size separation on agarose gels. qRT-PCRs contained 5 µL of cDNA template, 25 pmol/L gene-specific primers (Table 2), 10 mmol/L dNTP, 1 unit Taq polymerase, and dH2O to a final volume of 25 µL. SYBR green (Bio-Rad, Veenendaal, the Netherlands) was added in a 10,000-fold dilution as described by the manufacturer of the qRT-PCR machine (IQ5 Cycler System, Bio-Rad). Fluorescence was detected at the end of each extension step. The specificity of the amplicons nucleic acid composition of the fragments as inferred from melting curve analysis. Transcript levels were normalized to the levels of 16S rRNA in each sample and gene expression was calculated using the 2(ΔΔC(T)) analysis method [25]. qRT-PCR assays were performed using RNA isolated from three independent growth experiments.

**Results**

**Amoxicillin susceptibility of gastric and enterohepatic *Helicobacter* species**

Several *Helicobacter* species were tested for amoxicillin resistance (Table 1), and the susceptibility breakpoint of amoxicillin for *H. pylori* (0.5 mg/L) was also used for other *Helicobacter* species. The reference strains of the gastric *Helicobacter* species *H. pylori*, *H. acinonychis*, and *H. mustelae* were all susceptible to amoxicillin with MIC values of 0.047, < 0.016, and 0.25 mg/L, respectively (Table 1).
whereas the amoxicillin-resistant *H. pylori* strain BH9802-108 had an MIC of amoxicillin of 4 mg/L, consistent with previous reports [22,26]. Interestingly, the enterohepatic *Helicobacter* species *H. hepaticus, H. bilis, and H. rappini* displayed high levels of amoxicillin resistance. The three tested *H. hepaticus* isolates had MIC values ranging from of 6–64 mg/L, whereas *H. bilis* and *H. rappini* had MIC values of amoxicillin of 16 and 8 mg/L, respectively (Table 1).

**Amoxicillin resistance in *H. hepaticus* is not associated with β-lactamase activity or a specific mutation in penicillin-binding protein 1A**

*H. hepaticus* strain ATCC 51449 was selected for further analysis of the mechanism of amoxicillin resistance in enterohepatic *Helicobacter* species, since this is the only enterohepatic *Helicobacter* species of which the complete genome sequence is available [27]. We first investigated whether β-lactamase activity is present in the amoxicillin-resistant *H. hepaticus* strain ATCC 51449 with the aid of the Dryslide Nitrocefin testing system. No β-lactamase activity could be detected in *H. hepaticus* ATCC 51449 when grown in standard conditions, nor was this induced by growth in the presence of amoxicillin, which is sometimes required in standard conditions, nor was this induced by growth in the presence of amoxicillin, which is sometimes required for induction of β-lactamase expression [28]. In addition to this, we determined the MIC values of amoxicillin of *H. hepaticus* in the presence of the β-lactamase-inactivating compound clavulanate (using augmentin for induction of β-lactamase activity) [22], although a contribution of other changes cannot be completely ruled out.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wild-type strain</th>
<th>hefA-mutant</th>
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<tbody>
<tr>
<td>Amoxicillin</td>
<td>64 (R)</td>
<td>0.25 (S)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Augmentin (amoxicillin + clavulanate)</td>
<td>32</td>
<td>ND</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.047</td>
<td>0.078</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.38</td>
<td>0.48</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.357</td>
<td>0.106</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.172</td>
<td>0.14</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0.121</td>
<td>0.033</td>
</tr>
<tr>
<td>Rifamycins</td>
<td>0.232</td>
<td>0.025</td>
</tr>
<tr>
<td>SDS</td>
<td>&gt;0.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>&gt;2</td>
<td>0.5</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>2150</td>
<td>860</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>216</td>
<td>21.6</td>
</tr>
<tr>
<td>Nickel chloride</td>
<td>119</td>
<td>119</td>
</tr>
</tbody>
</table>

*MIC values of *H. hepaticus* ATCC 51449 reference strain and its isogenic hefA mutant, determined by E-test, agar dilution or broth dilution assay (see Materials and Methods section). All values are means derived from three independent experiments.*

As neither β-lactamases nor PBP-1A mutations seemed to be the cause of the observed amoxicillin resistance of *H. hepaticus* ATCC 51449, we subsequently investigated the potential involvement of a third, commonly described mechanism for drug resistance in bacteria, i.e. efflux systems. The genome of *H. hepaticus* encodes three putative RND efflux systems homologous to several Gram-negative bacteria (Fig. 1). The *hh0224-hh0223-hh0222* cluster is annotated as the *hefABC* putative efflux system [27] with OMP, PMP, and IMP homologous to the *Campylobacter jejuni* cmeDEF operon and the *H. pylori* efflux system *hp0605-hp0606-hp0607* [32]. Both gene clusters are associated with multiple antibiotic resistance [33,34], and in *H. pylori* the orthologous efflux system is also involved in cholic acid resistance [34]. The HeIA putative OMP encoded by the *hefABC* system is orthologous to the TolC group of proteins, whereas the putative PMP and IMP are homologous to the *Salmonella enterica* serovar Typhimurium AcrA and AcrB proteins, respectively [35] (Fig. 1).

Insertional mutagenesis was used to create inactivated copies of the *hefA* (*hh0224*), *hefC* (*hh0222*), *hh0175*, and *hh0174* genes in *E. coli*. While the constructs for all four genes were readily obtained, we were unable to transfer the inactivated *hefC, hh0175*, and *hh0174* genes to *H. hepaticus* ATCC 51449, despite several independent attempts (data not available).

**Table 3 Antimicrobial susceptibility of *H. hepaticus* ATCC 51449 and hefA mutant**

**The TolC ortholog HefA is involved in antimicrobial resistance in *H. hepaticus***

As neither β-lactamases nor PBP-1A mutations seemed to be the cause of the observed amoxicillin resistance of *H. hepaticus* ATCC 51449, we subsequently investigated the potential involvement of a third, commonly described mechanism for drug resistance in bacteria, i.e. efflux systems. The genome of *H. hepaticus* encodes three putative RND efflux systems homologous to several Gram-negative bacteria (Fig. 1). The *hh0224-hh0223-hh0222* cluster is annotated as the *hefABC* putative efflux system [27] with OMP, PMP, and IMP homologous to the *Campylobacter jejuni* cmeDEF operon and the *H. pylori* efflux system *hp0605-hp0606-hp0607* [32]. Both gene clusters are associated with multiple antibiotic resistance [33,34], and in *H. pylori* the orthologous efflux system is also involved in cholic acid resistance [34]. The HeIA putative OMP encoded by the *hefABC* system is orthologous to the TolC group of proteins, whereas the putative PMP and IMP are homologous to the *Salmonella enterica* serovar Typhimurium AcrA and AcrB proteins, respectively [35] (Fig. 1).

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shown). In contrast, an isogenic hefA mutant was readily obtained in *H. hepaticus* ATCC 51449, and was used to assess the role of the hefA gene in antimicrobial resistance of *H. hepaticus*.

Inactivation of the hefA gene did not affect growth of *H. hepaticus* in standard laboratory growth conditions (data not shown). The *H. hepaticus* wild-type strain and hefA mutant cells were compared for antimicrobial susceptibility (Table 3). The most apparent difference between the wild-type strain and the hefA mutant was the sensitivity to amoxicillin on E-test, and this was confirmed by agar dilution. Wild-type *H. hepaticus* ATCC 51449 and its isogenic hefA mutant had MICs of amoxicillin of 64 mg/L and 0.25 mg/L, respectively (Table 3). Surprisingly, insertional inactivation of hefA did not alter susceptibility to the related β-lactam antibiotic cefotaxime (Table 3), suggesting that cefotaxime is not a substrate for efflux via the HefA-associated efflux system.

The hefA mutant was also more sensitive to ethidium bromide and the bile acids cholic acid and deoxycholic acid, when compared to the wild-type strain, suggesting that absence of HefA results in a more generalized defect in efflux properties in *H. hepaticus* (Table 3). To confirm that amoxicillin susceptibility of the hefA mutant was due to decreased efflux, we determined the susceptibility of wild-type *H. hepaticus* ATCC 51449 in the presence of the protonophore CCCP, which is known to block efflux via RND pumps. Unfortunately, CCCP was toxic at the commonly used concentrations of 10 and 100 µmol/L [36], resulting in total absence of growth, and lower concentrations of CCCP (1 and 0.1 µmol/L) did not change the MIC of amoxicillin (data not shown).

**Expression of the hefA gene is bile acid-induced, but amoxicillin-independent**

To investigate if hefA expression was responsive to the presence of antimicrobials in the growth medium, the levels of hefA mRNA were monitored in response to incubation with different antimicrobials. Levels of hefA mRNA were twofold increased in the presence of the bile acid cholic acid and threefold increased in the presence of deoxycholic acid, whereas the presence of amoxicillin or two other bile acids had no significant effect on hefA mRNA levels in *H. hepaticus* (Fig. 2).

**Discussion**

A major stress factor for pathogenic bacteria during infection of a mammalian host is the presence of antimicrobial agents, either produced by the host, the resident microbial flora, or by antibiotic treatment of the infection. Multidrug efflux pumps have been shown to participate in antimicrobial resistance in gram-negative bacteria.
bacteria, by removing the toxic components that pass through the membranes [37, 38]. These pumps thus contribute to persistence of the infection. The carriage of efflux pump genes on the chromosome may give these pathogens an intrinsic mechanism that allows survival in hostile environments. Enterohepatic Helicobacter species like H. hepaticus are very successful colonizers of the intestinal tract, and hence can be predicted to be able to deal with such stresses [1, 2].

In this study we describe the involvement of the TolC ortholog-encoding hefA gene in H. hepaticus resistance to amoxicillin and bile acids. Efflux systems that contribute to antibiotic resistance have been described from a number of clinically important bacteria, including H. pylori [34] and C. jejuni [23, 39]. Especially the RND type pumps can transport several structurally unrelated drugs [15, 40]. While penicillin type antibiotics such as amoxicillin are usually not transported by RND pumps [41], a relationship between outer membrane transporters of the RND systems and resistance to β-lactam antibiotics has been described before [42–44]. However, this relationship is mostly limited to artificially induced overexpression and adaptation of the recognition sites of TolC-like outer membrane transporters in a limited number of bacterial species [44, 45]. Resistance to amoxicillin and other β-lactam antibiotics has been intensively studied in H. pylori, where it is independent of β-lactamase activity, but has been attributed to mutations in penicillin-binding proteins [22, 26, 29, 31]. Other mechanisms like active and passive efflux, and decreased membrane permeability have also been suggested [29, 46, 47]. Interestingly, after submission of this manuscript, the H. pylori HeF-A ortholog (hp0605) was suggested to function in multidrug resistance [32], but its absence did not change susceptibility of H. pylori to amoxicillin, indicating that there are differences between the H. hepaticus and the H. pylori RND systems.

There is still a lack of genetic tools for use in Helicobacter species. Complementation of mutations has only been described once in H. hepaticus [48], and used the antibiotic resistance cassette to insert a wild-type copy of the ahpC gene. While this is possible with mono-cistronic genes like the ahpC gene, its use is limited in operons like the hefABC genes, which are cotranscribed and usually expressed at similar levels. Although HeF-A plays a key role in H. hepaticus antimicrobial resistance, a role of the putative inner membrane and periplasmic components of the hefABC operon cannot be excluded. Mutation of the hefA gene may also lead to polar effects on the expression of other genes in the operon. Furthermore, two other RND efflux systems were identified in the H. hepaticus genome sequence (Fig. 1): the hh0175-hh0174 genes encode homologs of the C. jejuni CmeAB periplasmic and inner membrane fusion proteins. In C. jejuni this system is part of the CmeABC cluster and is involved in resistance to bile, macrolides, and tetracycline antibiotics [23, 39], and the hefFED (hh0623-hh0624-hh0625) operon is annotated as an efflux system homologous to the H. pylori CznCBA efflux system (Figure 1), which mediates antimicrobial and metal resistance in H. pylori [34, 49]. The contribution of these systems to H. hepaticus antimicrobial resistance is yet to be determined. It has been described before that a outer membrane component can be encoded separately and can thus be functional with more than one efflux system, acrAB of E. coli for example [15]. Bile resistance of H. hepaticus, as shown to be modulated by hefA, may well be the result of the combined function of the CmeAB-orthologs HH0175 and HH0174 PMP IMP and the HefA OMP. Bile acids induced transcription of the hefA gene two- to threefold (Figure 2), and increased expression of hefA mRNA can lead to higher HeF-A protein levels. Such an increase in the levels of RND transporters along the membrane could result in increased resistance of H. hepaticus to diverse antimicrobials in the intestinal tract. Interestingly, hefA transcription was not responsive to amoxicillin (Fig. 2), which suggests that responses to bile acids are differentially governed than responses to antibiotics. Evaluation of the contribution of the other yet uncharacterized RND systems in H. hepaticus should improve our understanding of its antimicrobial resistance, but was not possible in our study as we were unable to isolate mutants in these genes. This could indicate that these systems are essential in laboratory growth conditions, but this would require further investigation.

Nowadays, triple therapy (proton pump inhibitor, amoxicillin, and clarithromycin or metronidazole) is often described for the eradication of H. pylori in humans [30], and has also been used for treatment of H. hepaticus in laboratory mice [11, 50]. Antibiotic resistance to components of the multiple antibiotic therapy is known to negatively affect efficacy of the treatment [30]. However, not much was known about amoxicillin susceptibility of H. hepaticus or other enterohepatic Helicobacter species. The data presented here show that all tested isolates of the three enterohepatic Helicobacter species (H. hepaticus, H. bilis, and H. rappini) are resistant to amoxicillin. This implies that the currently used eradication therapy regimens may possibly not meet up to the expectations for complete eradication of enterohepatic Helicobacter species. A 2-week triple treatment (metronidazole, amoxicillin/tetracycline, and bismuth) of A/JCr mice was sufficient for eradication of H. hepaticus [12]. However, similar therapy administered in drinking water was less effective than oral gavage: this study group isolated H. hepaticus from livers a month after treatment [12, 13, 50]. Amoxicillin-containing triple therapy was not effective for eradicating H. bilis and H. rodentium or for preventive treatment of rats; in the
latter case the treatment is only sufficient when three cycles of a 2-week treatment is used and even than the animals will test negative by PCR testing for a period of only 8 weeks [11]. In contrast, treatment with a quadruple therapy including amoxicillin, metronidazole, clarithromycin, and omeprazole does seem to eradicate _H. hepaticus_ [10], but it is unclear what the contribution of amoxicillin in this regimen is, as it has not been tested without amoxicillin.

Rodents are the most widely used experimental animals in biomedical research, including research on intestinal pathogenesis and inflammation. Mice are among the natural hosts of enterohepatic _Helicobacter_ species, and many mouse colonies worldwide are colonized by enterohepatic _Helicobacter_ species [9,11]. Since the presence of _Helicobacter_ species results in chronic inflammation, this may well influence results obtained using infection experiments with microbial pathogens, and could potentially be responsible for lack of reproducibility between studies. This is why animal facilities have developed eradication programs against _Helicobacter_ species, but these may need to be revised and optimized in view of the increased knowledge on antibiotic resistance of enterohepatic _Helicobacter_ species. Resistance to one of the antibiotics included in combination therapy usually does negatively affect efficacy of the treatment [30], and the fact that enterohepatic _Helicobacter_ species can be amoxicillin-resistant should be considered in the adaptation of eradication programs in animal facilities worldwide.

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Competing interests: The authors have no competing interests.

References


Belzer et al. Helicobacter hepaticus Amoxicillin Resistance

Supporting information

Additional Supporting Information may be found in the online version of this article:

Alignment of the penicillin-binding protein 1A (PBP-1A) sequence of the amoxicillin-sensitive Helicobacter pylori strain 26695 (indicated by HP0597_AMXS) and of the amoxicillin resistant Hardenberg strain [1] (indicated by HP0597_AMXR) with that of the amoxicillin-resistant Helicobacter hepaticus strain ATCC 51449 (indicated as HH0890). The alignments was made with the ClustalX2 program [2], and asterisks represent residues conserved in all sequences, colons represent conserved substitutions, and dots indicate semi-conserved substitutions. The residue listed in yellow background is the Ser414 residue, of which mutation to Arg414 (indicated in green background) has been described to mediate amoxicillin resistance in H. pylori [1,3]. Residues listed in black background are residues which are potentially associated with amoxicillin resistance in H. pylori [4].

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