Differential regulation of urease activity in *Helicobacter hepaticus* and *Helicobacter pylori*

Clara Belzer,1 Jeroen Stoof,1 Catherine S. Beckwith,2 Ernst J. Kuipers,1 Johannes G. Kusters1 and Arnoud H. M. van Vliet1

1Department of Gastroenterology and Hepatology, Erasmus MC – University Medical Center, Dr Molewaterplein 40, 3015 GD Rotterdam, The Netherlands
2Department of Comparative Medicine, Stanford University School of Medicine, Stanford, CA, USA

*Helicobacter hepaticus* is a pathogen of rodents, which causes diverse enteric and hepatic inflammatory diseases and malignancies. The urease enzyme is an important colonization factor of gastric *Helicobacter* species like *Helicobacter pylori*, but little is known about the role and regulation of urease in enterohepatic *Helicobacter* species. Here it is reported that urease activity of *H. hepaticus* does not contribute to acid resistance, and that it is nickel-responsive at the post-translational level. *H. hepaticus* strain ATCC 51449 did not grow or survive at pH 3·0, and supplementation with urea or NiCl2 did not abrogate this acid sensitivity. Furthermore, urease enzyme activity of *H. hepaticus* was acid-independent, which contrasts with the acid-induced urease system of *H. pylori*. Nickel supplementation of Brucella medium resulted in a tenfold increase in urease activity in both *H. hepaticus* and *H. pylori*, but the maximum level of urease activity in *H. hepaticus* was still three- to fivefold lower when compared to *H. pylori* in the same conditions. The increase in urease activity of *H. hepaticus* was not associated with elevation of urease mRNA or protein levels. Inhibition of protein synthesis by chloramphenicol did not affect nickel-responsive induction of urease activity in *H. hepaticus*, and confirmed that nickel induction occurs at the post-translational level, probably by activation of preformed apo-enzyme. In conclusion, both the role of the urease enzyme and the regulation of urease activity differ between the enterohepatic pathogen *H. hepaticus* and the gastric pathogen *H. pylori*.

**INTRODUCTION**

*Helicobacter hepaticus* is a pathogen of rodents, and colonization with *H. hepaticus* may result in enteric and hepatic inflammatory diseases and malignancies (Fox et al., 1994; Ward et al., 1994). *H. hepaticus* has recently also been implicated as participating in the formation of cholesterol gallstones (Maurer et al., 2005). Members of the genus *Helicobacter* are Gram-negative, microaerophilic bacteria. While the genus is best known for the human gastric pathogen *Helicobacter pylori*, a large number of other species are recognized (Solnick & Schauer, 2001). Based on their target organ, *Helicobacter* species are subdivided into gastric and enterohepatic lineages (Solnick & Schauer, 2001). Both enterohepatic and gastric *Helicobacter* species can colonize humans and a wide variety of animals, resulting in chronic infections and chronic inflammation. With gastric *Helicobacter* species, this can progress to gastric ulceration, atrophy, metaplasia and malignancies (Blaser & Atherton, 2004). Enterohepatic *Helicobacter* species like *H. hepaticus* have been less thoroughly studied, but depending upon the species are associated with inflammatory and proliferative bowel lesions, hepatitis, and in some cases, hepatic cancer (Franklin et al., 1998, 1999; Ward et al., 1994). There is increasing evidence linking infection with *Helicobacter* species to liver cancer in humans (Nilsson et al., 1999; Queiroz & Santos, 2001; Verhoef et al., 2003), and *H. hepaticus* infection of mice may prove to be an excellent model for the study of hepatic carcinogenesis (Suerbaum et al., 2003).

*H. hepaticus* expresses a nickel co-factorable urease enzyme (Beckwith et al., 2001). Urease is a multimeric, nickel-containing enzyme produced by many pathogenic and non-pathogenic bacteria (Burne & Chen, 2000). In *Helicobacter* species, the urease enzyme consists of UreA and UreB subunits (Beckwith et al., 2001). These are encoded by the ureA and ureB genes and are located in an operon, also containing the ureI gene (encoding a urea channel), and the ureEFGH genes encoding accessory proteins involved in enzyme activation (Beckwith et al., 2001; Burne & Chen, 2000; Skouloubiris et al., 1998). Active urease converts urea into ammonia and bicarbonate; the ammonia produced is thought to mediate protection against acidic microenvironments, but may also serve as a nitrogen source (Williams et al., 1996).
In gastric Helicobacter species like H. pylori and Helicobacter mustelae, urease is an important virulence factor (Andrutis et al., 1995; Tsuda et al., 1994). The ammonia produced is thought to allow protection against acidic environments, and mutants lacking urease are unable to colonize the gastric environments in different animal models (Andrutis et al., 1995; Tsuda et al., 1994). In H. pylori, urease expression and enzyme activity are controlled by an intricate regulatory network, centred on the environmental pH and the availability of the cofactor nickel (Scott et al., 2002; van Vliet et al., 2004b). For H. hepaticus and other urease-positive enterohepatic Helicobacter species, the role of urease in metabolism or pathogenesis has not yet been established.

Recently, differences between the urease and nickel-transport genes of H. hepaticus and H. pylori were reported (Beckwith et al., 2001; Suerbaum et al., 2003), which suggested that there may be differences in regulation of urease expression and activity between these two Helicobacter species. Here it is demonstrated that urease activity in H. hepaticus is nickel-responsive, but solely at the post-translational level. In contrast to H. pylori, H. hepaticus urease activity is not induced at acidic pH, and H. hepaticus does not grow or survive at pH 3-0. This suggests that the urease system is not a universal acid-resistance factor of ureolytic Helicobacter species, and that its functions may differ between gastric and enterohepatic Helicobacter species.

METHODS

Bacterial strains, plasmids and growth conditions. H. hepaticus strain ATCC 51449 (Fox et al., 1994) and its isogenic ureB mutant (this study), and H. pylori strain 26695 (Tomb et al., 1997), were routinely cultured at 37°C in microaerobic conditions (10% CO₂, 5% O₂ and 85% N₂) on Dent agar (Biotrading) (van Vliet et al., 2001). Liquid growth was performed in Brucella broth (Difco) supplemented with 0.2% (w/v) β-cyclodextrins (Fluka) (BBC). Bacteria were inoculated at a starting OD₆₀₀ of 0.05. The pH of liquid media was adjusted from 7.0 to 6.0, 5.5 or 3.0 using HCl and did not change by more than 0.5 pH unit during the experiments. NiCl₂ was purchased from Sigma, filter-sterilized, and used at a final concentration of 100 μM. Escherichia coli ER1793 was grown aerobically in Luria–Bertani medium (Sambrook et al., 1989) at 37°C. When indicated, growth media were supplemented with chloramphenicol to a final concentration of 20 μg ml⁻¹.

Acid shock survival. H. hepaticus was grown for 24 h in BBC at pH 7.0 at a starting OD₆₀₀ of 0.05. Bacteria were then harvested by centrifugation for 10 min at 4000 g and resuspended to a final OD₆₀₀ of 0.2 in PBS adjusted to pH 7.0, 5.5 or 3.0. When indicated, PBS was supplemented with NiCl₂ or urea to a final concentration of 100 μM and 0.5 mM, respectively. Cells were incubated for 30 min at 37°C in microaerobic conditions. Subsequently 5 μl of tenfold dilutions were spotted on Dent agar. Plates were incubated for 2 days at 37°C in microaerobic conditions and the presence or absence of growth at the spots was assessed by visual inspection (Baillon et al., 1999). Differences in the highest dilution still containing growth between different conditions indicated differences in survival rates.

Urease enzyme assay. Urease activity was determined in freshly sonicated lysates by measuring ammonia production from hydrolysis of urea, as described previously (van Vliet et al., 2001). The concentration of ammonia in the samples was inferred from a standard NH₄Cl concentration curve. Enzyme activity was expressed as μmol urea substrate hydrolysed min⁻¹ (mg protein)⁻¹.

RNA analysis. RNA was isolated from H. hepaticus using Trizol reagent (Invitrogen), according to the manufacturer’s instructions. Gel electrophoresis of RNA, transfer to positively charged nylon membranes (Roche), cross-linking, hybridization to DIG-labelled specific RNA probes and detection of bound probe were performed as described previously (van Vliet et al., 2001). Probes specific for H. hepaticus ureA and ureB were synthesized by in vitro transcription using T7 RNA polymerase (Roche) and PCR products obtained with primers HhUrea-F1 (5’-TGCATATGGCGGCACTA-3’) and HhUreaR1-T7 (5’-ctaatcagctacaatggagaATAGGTCTATCGCCCTATTG-3’), and HhUreB-F1 (5’-TTGGTAAACGCGGAAATCCAG-3’) and HhUreB-R1 T7 (5’-ctaatcagctacaatggagaGTGTGCAGTGATGAGTTTG-3’). Lower-case letters indicate the T7 promoter used for in vitro transcription.

Construction of an H. hepaticus ureB mutant. The ureB gene of H. hepaticus ATCC 51449 was amplified using primers HhUreaB-mutF1 (5’-TTCCTGTGCGCTCCACCAAT-3’) and HhUreaB-mutR1 (5’-GCATTATGCGGCGACT-3’), and cloned in pGEM-T easy (Promega), resulting in plasmid pJS10. The ureB gene was subsequently interrupted by insertion of the chloramphenicol-resistance gene from pAV35 (van Vliet et al., 1998) in the unique BsmI site, resulting in plasmid pJS11. This plasmid was introduced into E. coli ER1793 and subsequently used for natural transformation of H. hepaticus ATCC 51449. Chloramphenicol-resistant colonies isolated were designated 51449ureB. Two colonies derived from independent transformations were tested, and both colonies gave identical results in all experiments. Correct allelic replacement of the wild-type ureB gene with the interrupted version was confirmed by PCR.

Protein analysis. H. hepaticus wild-type and ureB mutant cells were grown for 24 h in unsupplemented BBC medium or BBC medium supplemented with 100 μM NiCl₂, centrifuged at 4000 g for 10 min at room temperature and resuspended in PBS pH 7-4 to a final OD₆₀₀ of 10. The cells were subsequently lysed by sonication for 15 s on ice, using an MSE Soniprep 150 at amplitude 6. The protein concentration of lysates was determined using the bicinchoninic acid method (Fierce) using bovine serum albumin as standard. Proteins were separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel and stained with Coomassie brilliant blue. Western blotting was performed by electrotransfer of proteins onto nitrocellulose membrane (Roche). The blot was probed with antibodies raised in rabbits to Helicobacter felis UreA or UreB (Intervet International). Bound antibodies were visualized with swine anti-rabbit antibodies labelled with alkaline phosphatase (Promega), using 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) (Promega) as substrate.

RESULTS

H. hepaticus is acid-sensitive and does not grow at acidic pH

In gastric Helicobacter species, urease activity is required for growth in acidic conditions and survival of acid shock. To determine whether H. hepaticus is able to grow under acidic conditions, cells were resuspended in BBC of pH 7-0, 5-5 and 3-0 in the presence or absence of 100 μM NiCl₂ (Fig. 1). There was no increase in OD₆₀₀ at pH 3-0, indicating absence of growth (Fig. 1). H. hepaticus was able to grow at pH 5-5, although the final OD₆₀₀ values were slightly lower.
H. pylori with were selected on the basis of their use in previous studies or presence (black bars) of 100 μM NiCl₂ adjusted to pH 5. H. hepaticus urease activity, to determine the effect of medium pH on growth and acid-independent, but nickel-induced H. hepaticus is acid-sensitive, and that urease is not involved in its acid shock, cells were resuspended in PBS of pH 7. Viability was not affected by incubation at pH 5 (Fig. S1). H. hepaticus is acid-sensitive and does not grow at pH 7.0, 5.0, or 3.0 in the absence (white bars) or presence (black bars) of 100 μM NiCl₂. Data points shown are the mean of three independent experiments; error bars denote SD. These pH values were selected on the basis of their use in previous studies with H. pylori, as pH 5.5 and 6.0 are thought to resemble the pH of the mucus layer (van Vliet et al., 2004b). pH 5.5 was the lowest pH value allowing growth of H. hepaticus (Fig. 1). The pH of the medium did not change significantly during 24 h growth. Urease activity in H. hepaticus was not significantly affected by the pH of the medium (Fig. 2a), while urease activity in the positive control H. pylori increased at pH 6.0 and pH 5.5 (Fig. 2b), consistent with earlier data (Scott et al., 2002; van Vliet et al., 2004b).

To determine the effect of nickel supplementation of BBC medium on H. hepaticus urease activity, cells were grown for 24 h in BBC medium of pH 7.0 supplemented with NiCl₂ to a final concentration of 100 μM. Urease activity of H. hepaticus increased tenfold in nickel-supplemented BBC medium when compared to unsupplemented BBC medium [from 1.35 ± 0.56 to 17.07 ± 6.21 μmol urea min⁻¹ (mg protein)⁻¹; mean ± SD, n = 3] (Fig. S2a). Likewise, urease activity of H. pylori grown with nickel supplementation was also increased tenfold [from 6.04 ± 4.47 to 58.63 ± 16.14 μmol urea min⁻¹ (mg protein)⁻¹; mean ± SD, n = 3] (Fig. S2b), as was reported previously (van Vliet et al., 2001). Overall, H. hepaticus urease activity was three- to fivefold lower than that of H. pylori in comparable conditions.

Nickel-responsive induction of urease activity in H. hepaticus is mediated at the post-translational level

To identify at which level the nickel-responsive induction of urease activity in H. hepaticus was mediated, the effect of nickel on transcription of H. hepaticus urease genes and expression of urease protein was determined (Fig. 3). Nickel supplementation of BBC medium did not affect transcription of either the ureA or ureB genes (Fig. 3a). Likewise, expression of the UreA and UreB proteins was not altered by nickel supplementation, as was shown for both UreA and UreB by Western immunoblotting using antibodies to H. felis UreA and UreB protein (Fig. 3b). The Western blot data were independently confirmed using an isogenic ureB mutant of H. hepaticus ATCC 51449 (Fig. 3c). This ureB mutant showed an absence of UreB expression, and did not have any detectable urease activity; it did not show any

![Fig. 1](image1)

**Fig. 1.** H. hepaticus is acid-sensitive and does not grow at pH 3.0. H. hepaticus ATCC 51449 was grown for 24 h in BBC with a pH of 7.0, 5.5 or 3.0 in the absence (white bars) or presence (black bars) of 100 μM NiCl₂. Data points shown are the mean of three independent experiments; error bars denote SD.

![Fig. 2](image2)

**Fig. 2.** Urease activity is acid-independent but nickel-induced in H. hepaticus. Urease activity of H. hepaticus strain ATCC 51449 (a) and H. pylori strain 26695 (b) was assessed after 24 h growth in BBC medium with a pH of 7.0, pH 6.0 or pH 5.5. Note the difference in the scale of the y-axis. Each bar represents data from three to five independent experiments for each strain; error bars denote SD. Asterisks indicate a significant increase of urease activity in the test condition (pH 6.0 or 5.5) when compared to the standard growth conditions (pH 7.0) (P<0.05, Mann–Whitney U test).
significant changes in growth under the tested conditions (data not shown).

The bacteriostatic antibiotic chloramphenicol, which blocks protein synthesis (Kusters et al., 1997), was used to confirm that the nickel-responsive induction of urease activity in H. hepaticus is indeed mediated solely at the post-translational (enzyme activity) level. Supplementation of BBC medium with chloramphenicol to a final concentration of 20 μg ml⁻¹ resulted in cessation of growth of H. hepaticus, but did not significantly affect cell viability as determined by colony counts (not shown). Addition of chloramphenicol also did not affect nickel-responsive induction of urease activity of H. hepaticus when compared to cultures grown in BBC medium without chloramphenicol (Fig. 3d), demonstrating that this induction does not require protein synthesis. In

![Fig. 3. Nickel-responsive induction of urease activity in H. hepaticus is mediated at the post-translational level. (a) Analysis of ureA and ureB transcription by Northern hybridization. Top panel, staining of total RNA with methylene blue; middle panel, visualization of ureB mRNA; bottom panel, visualization of ureA mRNA. Relevant marker sizes are indicated on the left. (b) Comparison of UreB (middle panel) and UreA (lower panel) protein expression levels using Western immunoblots with antibodies raised against H. felis UreA and UreB protein. The top panel displays the total protein profile of the lysates used for immunoblotting, stained with Coomassie brilliant blue. Relevant marker sizes are indicated on the left. (c) Comparison of UreB protein expression levels using SDS-PAGE followed by Coomassie brilliant blue staining. Total protein of H. hepaticus ATCC 51449 wild-type strain and isogenic ureB mutant, respectively, grown in unsupplemented BBC medium (lanes 1 and 3) and BBC medium supplemented with 100 μM NiCl₂ (lanes 2 and 4). Lanes 3 and 4 are included to facilitate identification of the UreB protein (indicated by an arrow). Relevant marker sizes are indicated on the right. (d) Effect of the protein synthesis inhibitor chloramphenicol on nickel-responsive induction of urease activity in H. hepaticus (left panel) and H. pylori (right panel). Activity of urease was assessed after 24 h growth in unsupplemented BBC medium (black bars) and BBC medium supplemented with NiCl₂ to a final concentration of 100 μM (white bars). Cells were either grown in the absence of chloramphenicol (–Cm) or in the presence of 20 μg ml⁻¹ chloramphenicol (+Cm). Data are from three to five independent experiments; error bars denote SD. Asterisks indicate a statistically significant increase in urease activity in nickel-supplemented versus unsupplemented medium (P ≤ 0.05, Mann–Whitney U test).
contrast, chloramphenicol supplementation almost completely abolished nickel-responsive induction of urease activity in *H. pylori* (Fig. 3d), consistent with the requirement for additional urease protein synthesis in nickel-responsive induction of urease activity (van Vliet et al., 2001).

**Nickel-responsive induction of urease activity in *H. hepaticus* is growth-phase-independent**

The effect of growth phase on nickel-responsive induction of urease was determined for *H. hepaticus* and *H. pylori* (Fig. 4). Nickel supplementation did not significantly affect the growth of *H. hepaticus* or *H. pylori*, and both species reached late-exponential phase after 24 h (Fig. 4a). *H. hepaticus* already reached maximum urease activity at approximately 12 h of growth in nickel-supplemented medium (Fig. 4b). In contrast, urease activity in *H. pylori* increased steadily in nickel-supplemented cultures, and reached its maximum at approximately 18 h of growth, consistent with the requirement for transcription and translation of extra urease protein for increased urease activity during this period of time (Fig. 4b). This indicates that in *H. hepaticus* urease activity peaks during early exponential phase, further supporting the hypothesis that nickel induction of urease activity is mediated by enzyme activation rather than increased expression of urease enzyme.

**DISCUSSION**

The nickel-containing enzyme urease is an important virulence factor of many bacterial pathogens (Burne & Chen, 2000). All gastric *Helicobacter* species known to date are urease-positive, and this urease activity is thought to be essential for their ability to colonize the gastric environment. In contrast, only a third of enterohepatic *Helicobacter* species are urease-positive, but are still unable to colonize the gastric environment (Solnick & Schauer, 2001), with the exception of *Helicobacter muridarum* (Lee et al., 1993). Thus it is likely that not just the presence of urease activity, but also the absolute level of urease activity or its regulation may be required to allow gastric colonization. While the operon encoding the *H. hepaticus* urease enzyme was previously described (Beckwith et al., 2001), to our knowledge the role of urease in *H. hepaticus* and other enterohepatic *Helicobacter* species has not yet been reported.

In this study it has been demonstrated that *H. hepaticus* is acid-sensitive, and that addition of urea does not increase its survival at pH 3-0 (Fig. S1). In contrast, addition of urea does allow gastric *Helicobacter* species like *H. pylori* to survive such acidic conditions. Taken together, these findings suggest that the urease enzyme of *H. hepaticus* is not involved in acid resistance. Consistent with this was the lack of acid-responsive induction of urease activity in *H. hepaticus* (Fig. 2a). However, urease activity in *H. hepaticus* is nickel-responsive, but this regulation is mediated solely at the post-translational level, probably through the activation of preformed apo-enzyme (Figs 3 and 4). During revision of this paper, nickel-responsive induction of *H. hepaticus* urease activity was confirmed in an independent study (Mehta et al., 2005), indicating that the observed nickel induction of *H. hepaticus* urease activity is not due to the conditions employed in our study.

Despite overall similarities of the *H. hepaticus* and *H. pylori* urease gene clusters, there are notable differences. Both urease gene clusters consist of two structural genes (*ureAB*) and five accessory genes (*ureIEFGH*) (Beckwith et al., 2001), but the *ureB–ureI* intergenic distance of *H. hepaticus* is much shorter (9 bp) than that of *H. pylori* (200 bp). This indicates that in *H. hepaticus* there is probably no promoter directly upstream of the *ureI* gene, thus limiting the possibilities for transcriptional and post-transcriptional regulation (Akada et al., 2000; van Vliet et al., 2001). Furthermore, the overall amino acid sequence of UreI in both species is well conserved, but several amino acid residues which were shown to be required for acid activation of the *H. pylori* UreI urea channel are absent in the *H. hepaticus* UreI protein (Weeks et al., 2004). This is consistent with the extragastric lifestyle of *H. hepaticus* and its inability to grow or survive at
pH 3–0 (Figs 1 and S1). Whether this phenotype is due to the lack of acid-responsive regulation of urease activity, absence of a ureI promoter or absence of the acid activation of the UreI urea channel remains to be established.

Enzymic degradation of urea by urease results in the production of ammonia and bicarbonate, and both reaction products are thought to play an important part in bacterial pathogenesis. Ammonia serves as nitrogen source and may function in acid resistance (Burne & Chen, 2000; Scott et al., 2002; Williams et al., 1996), whereas bicarbonate may also function in acid resistance as well as in modulating the immune system of the host (Kuwahara et al., 2000; Marcus et al., 2005). Expression of urease in bacteria is controlled by different stimuli, such as urea availability, environmental pH, nitrogen status of the cell, or growth phase (Burne & Chen, 2000).

In this study it has been demonstrated that urease activity in *H. hepaticus* is nickel-responsive. This form of urease regulation has to date only been identified for two other urease systems, of *H. pylori* (van Vliet et al., 2001) and *Streptococcus salivarius* (Chen & Burne, 2003). The enzymic activity of *H. hepaticus* urease is induced by nickel supplementation of the growth medium, but this induction is mediated solely at the post-translational level (Fig. 3), probably by activation of urease apo-enzyme, as was previously described for *S. salivarius* (Chen & Burne, 2003). In contrast, the *H. pylori* urease system is nickel-induced at the enzyme activity and transcriptional level (van Vliet et al., 2001). The nickel- and acid-responsive transcriptional regulation of urease observed in *H. pylori* may be a specific adaptation to the gastric lifestyle. This suggests that the proposed link in *H. pylori* between intracellular nickel availability and environmental pH (van Vliet et al., 2004a) may not be universal in the genus *Helicobacter*.

In *H. pylori*, regulation of urease activity is dependent on nickel and the NikR regulatory protein (van Vliet et al., 2002). Mutants lacking the NikR regulators are unable to colonize the murine gastric mucosa (Bury-Mone et al., 2004). A NikR homologue is also present in *H. hepaticus* (Suerbaum et al., 2003), and it can be envisaged that NikR may indirectly affect urease activity via regulation of nickel uptake. In *H. pylori*, NikR regulates NixA-mediated nickel uptake (Ernst et al., 2005), and while the *H. hepaticus* genome sequence does not contain a nixA orthologue (Suerbaum et al., 2003), it does contain a putative nickel-uptake ABC transporter located adjacent to the urease operon (Beckwith et al., 2001). However, up to now the *H. hepaticus* nikR gene has proven refractory to insertion mutagenesis (C. Belzer & A. H. M. van Vliet, unpublished results), and thus a role of NikR in regulation of urease expression of *H. hepaticus* cannot be established yet.

In conclusion, *H. hepaticus* is acid-sensitive and lacks regulatory mechanisms mediating acid- and nickel-responsive regulation of urease expression. These characteristics may reflect its extragastric niche, and may be directly linked with the animal host colonized and/or the respective target organ. We hypothesize that enterohepatic *Helicobacter* species do not require high levels of urease activity in the rodent gut. The high levels of urease activity observed in gastric *Helicobacter* species (Bury-Mone et al., 2003; Scott et al., 2000) are likely to be an adaptation that allows them to thrive in the gastric environment, albeit at a high metabolic cost. Regulation of urease expression and activity will allow them to adapt to changes in acidity observed during fasting or feeding. In contrast, urease-positive enterohepatic *Helicobacter* species might use their urease system for nitrogen metabolism, securing a constant supply of ammonia. The diversity in urease function and regulatory responses in *Helicobacter* species is a prime example of the adaptation required for chronic colonization of host tissues.

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