Mutation of *luxS* affects motility and infectivity of *Helicobacter pylori* in gastric mucosa of a Mongolian gerbil model

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Helicobacter pylori is associated with gastric disorders in humans and some experimental animals, and possesses the *luxS*/type 2 autoinducer (AI-2) system. The effects of a specific *luxS* mutation on the characteristics of *H. pylori* were examined. On 0.3% agar medium, motility of *H. pylori* HPKY08 (*luxS*::cat) was significantly lower than that of wild-type *H. pylori* TK1402. The *luxS*-complemented strain HPKY21 exhibited motility comparable to that of *H. pylori* TK1402. It was shown that the *luxS*/AI-2 system plays an important role in *H. pylori* motility. The *luxS* mutant exhibited a reduced infection rate relative to the wild-type parent strain TK1402 in a Mongolian gerbil model. At 3 months after oral inoculation, lower numbers of *H. pylori* were detected by semi-quantitative real-time reverse transcription PCR (qRT-PCR) in *luxS*[−] mutant-infected gerbils than in TK1402-infected gerbils. Gastric inflammation and increased antibody titre for *H. pylori* were observed in TK1402-infected gerbils only.

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INTRODUCTION

Quorum sensing (QS) is a cell-to-cell communication system that regulates bacterial phenotypes, including the expression of virulence factor genes. The signalling molecules are known as autoinducers (AIs), and when these molecules reach a critical threshold concentration within a bacterial population, a signal transduction cascade is triggered, and this forms the basis for alterations in gene expression (Fuqua *et al.*, 1994). Many Gram-negative bacteria utilize *N*-acylhomoserine lactone molecules, AI-1, as signals, while Gram-positive bacteria actively export peptides as signalling molecules. There is a second signalling system involved in a wide range of bacterial species (Schauder *et al.*, 2001), and this system is utilized by both Gram-positive and Gram-negative bacteria. The signalling molecule known as type 2 autoinducer (AI-2) is a furanosyl borate diester (Chen *et al.*, 2002), and the enzyme responsible for its synthesis is encoded by the *luxS* gene (Surette &

Bassler, 1999). The genomes of many bacterial species, notably *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, *Proteus mirabilis*, *Vibrio cholerae*, *Vibrio vulnificus*, *Campylobacter jejuni*, *Porphyromonas gingivalis*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Clostridium perfringens* and *Clostridium difficile*, include *luxS* homologues. In several of these, *luxS*-related AI-2 signals are involved in bacterial characteristics such as biofilm formation (Balestrino *et al.*, 2005; Blehert *et al.*, 2003; Fong *et al.*, 2001; Wen & Burne 2004), flagella and motility (Jeon *et al.*, 2003; Schneider *et al.*, 2002; Stroehrer *et al.*, 2003), type III secretion systems (Sperandio *et al.*, 1999), toxin production (Ohtani *et al.*, 2002) and virulence (Lyon *et al.*, 2001; Parsonnet *et al.*, 1991; Stroehrer *et al.*, 2003).

Helicobacter pylori has been identified as the aetiological agent of chronic active gastritis, peptic ulcer disease (Blaser, 1992; Graham, 1989), gastric adenocarcinoma (Parsonnet *et al.*, 1991) and mucosal-associated lymphoid tissue (MALT) lymphoma (Wotherspoon *et al.*, 1993). *H. pylori* has flagellar motility and is able to move through the

Abbreviations: AI-2, type 2 autoinducer; qRT-PCR, semi-quantitative real-time reverse transcription PCR.

viscous mucus gel lining the epithelium of the human stomach, thus leading to chronic infection of the human gastric mucosa. *H. pylori* produces extracellular signalling molecules related to AI-2, and production of AI-2 is dependent on *luxS* function (Forsyth & Cover, 2000; Joyce *et al.*, 2000). These authors have reported that the production of AI-2 by *luxS* is growth-phase dependent, with maximal production occurring in the mid-exponential phase of growth. Furthermore, in their studies, maximal accumulation of AI-2 in the culture occurred in stationary phase. It has also been reported that the expression of *H. pylori flaA* is growth-phase dependent (Thompson *et al.*, 2003), and that *flaA* transcription increases with culture density (Loh *et al.*, 2004) at stationary phase. Mutating the *luxS* gene eliminates the growth-phase dependence of *flaA*, and growth-phase dependence is restored when the *luxS* mutant is complemented with wild-type *luxS*. Accordingly, we expected that the mutation of *luxS* of *H. pylori* might affect motility in the stationary phase, but not in exponential phase.

Several experimental animal models, such as monkeys (Drazek *et al.*, 1994; Fujioka *et al.*, 1997; Schauder *et al.*, 2001), gnotobiotic piglets (Eaton *et al.*, 1997), C57/BL6 mice (Lee *et al.*, 1997; Kim *et al.*, 2003) and Mongolian gerbils, are helpful in understanding the pathogenesis of human *H. pylori* infection. The Mongolian gerbil model, in which *H. pylori* is able to colonize long-term, is particularly easy to handle, and is useful as a small-animal model for the severe inflammation and obvious ulceration caused by *H. pylori* (Hirayama *et al.*, 1996; Matsumoto *et al.*, 1997; Yokota *et al.*, 1991). The aim of this study is to investigate the effects of a defined *luxS* mutation on the characteristics of *H. pylori* and to determine whether the absence of AI-2 production allows *H. pylori* to infect and/or maintain infection in the gerbil gastric mucosa.

METHODS

Strains and culture. *H. pylori* strain TK1402 was isolated from a gastric ulcer and duodenal ulcer patient. This strain exhibits infectivity in germ-free mice (Osaki *et al.*, 1998), C57/BL6 mice (Yamaguchi *et al.*, 2000) and Mongolian gerbils (Nakagawa *et al.*, 2005). In addition, TK1402 is *cagA* positive and produces vacuolating cytotoxin (VacA). *H. pylori* TK1402 and its mutant strains were cultured under microaerobic conditions at 37 °C either on a brain heart infusion agar plate containing 7% horse blood or in Brucella broth containing 7% horse serum (Brucella-serum broth). The reporter strains *Vibrio harveyi* BB170 (*luxN::Tn5*) and BB152 (*luxL::Tn5*) were kindly provided by Dr B. L. Bassler (Princeton University). *V. harveyi* strains were routinely cultured at 30 °C on Luria-marine (LM) agar.

Construction of *H. pylori luxS* mutant. Primers HP105F2 (5'-GCTATTGCCTTGCAACAAATCCCCGC-3') and HP105R2 (5'-ATACTAGCGGGCATAGCGATG-3') were used to PCR-amplify a 1752 bp fragment containing the *H. pylori luxS* gene allele (*luxS::cat*) with genomic DNA from strain TN2 (*luxS::cat*) as template. The primers and genomic DNA of TN2 were kindly provided by Drs Ogura and Berg (Washington University, St Louis, MO). The fragment was then introduced directly into *H. pylori* 1402 by natural

transformation via allelic exchange, and chloramphenicol-resistant colonies were isolated. Gene disruption in the mutant (designated HPKY08) was confirmed by PCR using the primer pairs HP105F2 and HP105R2, and HP02F (5'-CATACGAAAAAGCCGCC-3') and HP02R (5'-CTTGTCTGTGGGGATTGAG-3'), which yielded a PCR product of increased size. The mutation was also confirmed by Southern blot analysis with a probe containing the *luxS* gene. The mutant HPKY08 was used in all subsequent experiments.

Complementation of *luxS* mutant. The gene *luxS* along with its promoter region was amplified using primers 105F2 and 105R2. This fragment was then cloned into the *SspI* site adjacent to the *cat* gene in pGEM. The resultant plasmid, pTKY451, was then transformed into the *luxS*⁻ strain by natural transformation and was selected on Brucella-serum agar containing 5 µg kanamycin ml⁻¹, and strain HPKY21, with *luxS* inserted in the chromosome, was obtained. Mutation of *luxS* and restoration of *luxS* function in the mutant were confirmed by AI-2 assay and Southern blot analysis of genomic DNA digested with *SspI*, using probes for the *luxS* gene, the chloramphenicol acetyl transferase gene (*cat*) and the kanamycin-resistance gene (*aph*) (Fig. 1).

AI-2 assay. In order to confirm the production of AI-2 by *H. pylori* TK1402, *V. harveyi* AI-2 reporter strain BB170, which lacks the AI-1 sensor but contains an intact sensor for AI-2, was used in a bioluminescence assay (Table 1). *V. harveyi* was grown at 30 °C in autoinducer bioassay (AB) broth medium (Hardie *et al.*, 2003). Luminescence bioassay was performed as described previously (Forsyth & Cover, 2000). *H. pylori* or *V. harveyi* BB152 was grown in Brucella-serum broth, and culture supernatants were collected at various time points. Cell-free conditioned medium (CM) was prepared by centrifuging *H. pylori* or *V. harveyi* BB152 cultures in Brucella-serum broth at 8000 g for 15 min, followed by filtration (pore size 0.2 µm). CM preparations were routinely stored at -30 °C until use. The 18 h culture of *V. harveyi* BB170 was diluted 1:5000 in fresh AB medium. The experimental CM preparation

Fig. 1. (a) Construction of *luxS* mutant strain (HPKY08) and mutant complemented with *luxS* gene (HPKY21). WT, wild-type. (b) Southern blot analysis of wild-type TK1402 strain (lane 1) and its mutant strains (lane 2, HPKY08; lane 3, HPKY21), using *luxS*, *cat* and *aph* genes as probes.

Table 1. AI-2 production by *H. pylori* strains

All strains were incubated in Brucella-serum broth.

Strains	Characteristics	Time (h)	Number of <i>H. pylori</i> [log (c.f.u. ml ⁻¹)]	Activation of luminescence in <i>V. harveyi</i> BB170 (%)*
<i>H. pylori</i>				
HPKY08	<i>luxS::cat</i>	24	10 ^{8.78}	0.4
		33	10 ^{8.74}	1.6
		54	10 ^{8.56}	2.6
TK1402	Wild-type, clinical isolate	24	10 ^{8.60}	109.9
		33	10 ^{9.15}	326.5
		54	10 ^{9.15}	69.1
HPKY021	<i>luxS</i> complemented	33	10 ^{9.36}	94.3
<i>V. harveyi</i>				
BB152	<i>luxL::Tn5</i>	18	—	100

*At various time points, total luminescence was quantified. The luminescence of the culture supernatant of *V. harveyi* BB152 was taken as 100 %.

(15 µl) was added to the diluted *V. harveyi* culture (135 µl), which was then incubated at 30 °C with agitation (180 r.p.m.). Next, 100 µl of this culture was added to a 96-well plate at various time points, and total luminescence was quantified using a luminometer (Mithras LB940, Berthold Technologies). Viable cell counts of *V. harveyi* BB170 were determined by culture using LM agar, and relative light units were calculated.

Motility assay. Motility of *H. pylori* was assessed on 0.3 % agar Brucella medium containing 7 % horse serum. *H. pylori* strains grown in Brucella-serum broth for 24 h (OD₆₀₀ 0.6) were collected and centrifuged in 1.5 ml tubes. Pellets were inoculated into medium using a sterile picker. Several days after incubation under microaerobic conditions at 37 °C, the diameter of the halo was measured. At least three plates were used for each experiment. The mean diameter was analysed statistically.

The velocity of each *H. pylori* strain in Brucella broth containing 0.5 % glucose was also assessed using video systems. *H. pylori* was irradiated by the slanted dark-field light of a phase-difference condenser (c-c ph condenser, Nikon) and by observing scattered rays; *H. pylori* was detected as a bright point, which was not distinct in liquid medium against a light field. The obtained dark-field images were photographed at 30 frames per second sequentially by a 3CCD camera (DC-330, Dage-MTI), and transferred to computer in AVI file protocol through a capture card (SIM-PCI, DITECT). Motility was analysed using motility analysis software (DIPP-MOTION2D, DITECT). The movement of *H. pylori* on every frame was tracked for 1 s, and each coordinate was determined. The velocity of each *H. pylori* cell was calculated by the same software from coordinates using a frequency distribution chart. In one sample, 60 *H. pylori* bodies were tracked, and the mean velocities of wild-type and mutant strains were estimated and compared.

Ultrastructural study. For scanning electron microscopy, cultures of *H. pylori* strains in Brucella-serum broth were collected and attached to poly-L-lysine-coated cover slips (IWAKI) by centrifugation (1500 g, 5 min). Cells on the cover slips were washed twice in PBS and fixed with 2 % glutaraldehyde. Specimens were examined using a scanning electron microscope (JEOL JSM-5600LV).

Acid tolerance. Brucella broth supplemented with 10 % fetal calf

serum was adjusted to pH 3 or pH 1 with HCl, and precipitated proteins were removed by filtration [acid-precipitated (AP) Brucella broth]. Next, 1–3 × 10⁶ c.f.u. *H. pylori* ml⁻¹ was inoculated into AP Brucella broth, with or without 30 mM urea, at pH 1 or 3. After 1 or 3 h incubation at 37 °C, viable bacterial counts were determined.

Animal experiments. Mongolian gerbils (MGS/sea; age 8 weeks; male) purchased from Seac Yoshitomi were fasted for 2 days and inoculated per os with 1–2 × 10⁹ c.f.u. *H. pylori* TK1402 or HPKY08 (*luxS*⁻ mutant) in Hanks' solution (Sigma) on two consecutive days. At each time point, four to five gerbils per group were sacrificed, and one half of the stomach was fixed in Carno's solution for histological analysis and the mucus layer of the other half was scraped off with a spatula and collected into 1 ml Hanks' solution. Of this 1 ml, 0.1 ml was inoculated onto M-BHM medium (Nikken Bio Medical Laboratory) and cultured at 37 °C for 7 days under microaerobic conditions. After the incubation period, gold colonies that exhibited urease activity were counted. Another 0.1 ml was treated with RNA protect (Qiagen) and stored at -80 °C until used for RNA preparation.

The infective rate was determined by semi-quantitative real-time reverse transcription PCR (qRT-PCR), which detects expression of 16S rRNA (Nakagawa *et al.*, 2005). Total RNA was extracted from samples by RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Contaminating chromosomal DNA was digested with DNase I (DNA-free, Ambion) [1 U (µg RNA)⁻¹] for 20 min at 37 °C. One microgram of DNase I-treated total RNA was used with avian myeloblastosis virus (AMV) reverse transcriptase and random primers in a commercial reaction mixture (20 µl; AMV Reverse Transcription System, Promega) for first-strand complementary DNA synthesis.

Quantitative analysis was performed using the SYBR Green method. The generation of quantitative data was based on the different PCR kinetics of samples with various levels of target-gene expression. cDNA was amplified using PCR primers for *H. pylori* 16S rRNA, 16S2-F (5'-CGCTAAGAGATCAGCCTATGTCC-3') and 16SB2-R (5'-CCGTGTCTCAGTTCAGTGTGT-3'). Amplification of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene on cDNA derived from Mongolian gerbils using the primers G3PDH-F (5'-ACCACAGTCCATGCCATCAC-3') and G3PDH-R (5'-TCCAC-

CACCCTGTTGCTGTA-3') (RT-PCR Primer Set, TOYOBO) was used as a control and for standardization of target gene transcriptional activity data. For each primer set, PCR was performed in triplicate. Quantitative data were calculated from a standard curve generated by amplifying serial dilutions of a known quantity of amplicon. For this approach, the specificity of the PCR product was confirmed by dissociation curve analysis (7500 quantification program, Applied Biosystems).

ELISA. Serum antibodies from gerbils infected with *H. pylori* were assessed by ELISA at 3 months after infection, as previously reported (Nakagawa *et al.*, 2005). A 96-well plate was coated with sonicated *H. pylori* TK1402 antigen (3 µg per well). Each serum sample was diluted 300-fold with PBS and added to the plates. Bound immunoglobulins were detected with protein G-conjugated horseradish peroxidase (Sigma) and were developed using 0.1% *o*-phenylenediamine and 0.035% H₂O₂ in developing buffer (0.1 M citric acid, 0.07 M sodium phosphate dibasic). After incubation at room temperature for 5 min, the reaction was stopped by adding 50 µl 2(NH₂)SO₄. A₄₉₀ was measured using a microplate reader (model 550, Bio-Rad).

Cell culture and characterization of *H. pylori*. Adhesion activity of *H. pylori* strains was assessed by flow cytometry, as previously reported (Osaki *et al.*, 1997). AGS cells, originating from a gastric adenocarcinoma cell, were cultured in RPMI 1640 medium containing 10% fetal calf serum under 5% CO₂ at 37°C. Cells (5 × 10⁵) were cultured with PKH-2-labelled HPKY08 (*luxS*[−] mutant) or the wild-type strain for 1 h at room temperature. The fluorescence intensity of the cells, which reflects adhesion activity, was analysed by flow cytometry (FACSVantage, Becton Dickinson).

Statistical analysis. Statistically significant differences were examined by Student's or Welch's *t* test.

RESULTS AND DISCUSSION

AI-2 production by *luxS*[−] mutant

It was shown that AI-2 production by wild-type TK1402 was maximal at 33 h after inoculation, after which it gradually decreased (Table 1). At 24, 33 and 54 h after inoculation, a cell-free culture of HPKY08 (*luxS*[−] mutant) did not yield bioluminescence in the assay, indicating no significant production of AI-2. However, cell-free culture of *luxS*-complemented HPKY21 showed AI-2 activity. These results clearly indicate that the *luxS* gene is responsible for the production of AI-2 in *H. pylori*. These data were in agreement with the results obtained by Forsyth & Cover (2000) and Joyce *et al.* (2000). In other words, *H. pylori luxS* plays an essential role in the production of extracellular signalling molecules.

Growth of *luxS* mutant

The microaerobic growth of HPKY08 (*luxS*[−] mutant) in Brucella-serum broth medium was compared to that of TK1402. In the exponential phase (0–16 h), the generation times for TK1402 and HPKY08 were 3.1 ± 0.3 h and 4.0 ± 0.8 h, respectively (*P* = 0.1789, *n* = 3). From the late-exponential to the stationary phase (24–64 h), the mean bacterial number (10^{7.65} ± 10^{0.82} c.f.u. ml^{−1}) for HPKY08 was slightly lower than that for TK1402 (10^{8.33} ± 10^{0.68} c.f.u. ml^{−1}) (*P* = 0.1035, *n* = 3). These results indicate a slight difference in growth characteristics

between TK1402 and HPKY08 during the late-exponential phase. The generation time and mean bacterial number for HPKY21 complemented with *luxS* were 3.3 ± 0.2 h and 10^{8.23} ± 10^{0.81} c.f.u. ml^{−1}, respectively, which were not significantly different from those of the TK1402 strain (*P* = 0.6617 and 0.7886, respectively). These results suggest that the growth delay in *H. pylori* may be due to the effects of *luxS* mutation.

Motility on semi-solid agar and flagella formation of the HPKY08 (*luxS*[−] mutant) strain

Three strains of *H. pylori* were cultured on Brucella-serum medium containing 0.3% agar. After 5 days of culture, halo diameters were measured. The HPKY08 (*luxS*[−] mutant) was motile, but the halo diameter (8.0 ± 1.4 mm; *n* = 4) after 5 days of culture was significantly smaller than that of TK1402 (12.3 ± 2.0 mm; *n* = 8) (*P* < 0.05) (Fig. 2). The mean halo diameter of HPKY21 complemented with *luxS* was 14.0 ± 1.2 mm (*n* = 4), which was not significantly different from that of TK1402. These results confirmed that the reduction in *H. pylori* motility may be due to the effects of *luxS* mutation.

In both the wild-type and *luxS*[−] strains, three to five polar flagella were observed by electron microscopy after 15 h incubation (Fig. 3). No morphological difference was observed between the two strains harvested after 15, 24 and 48 h cultivation.

On the 0.3% agar-containing medium, the motility of *H. pylori* HPKY08 (*luxS*[−] mutant) was significantly lower than that of the wild-type TK1402 strain. In contrast, the *luxS*-complemented strain HPKY21 exhibited motility comparable to that of *H. pylori* TK1402. These results suggest that the *luxS*/AI-2 system plays an important role in *H. pylori* motility. There have been similar reports in other bacteria. Jeon *et al.* (2003) have reported that mutation of *luxS* in *C. jejuni* reduces the transcription of *flaA* and results in

Fig. 2. Motility of HPKY08 (*luxS* mutant), HPKY21 (*luxS* complemented) and TK1402 (wild-type) strains on Brucella-serum semi-solid agar after 5 days culture.

Fig. 3. Flagella formation of HPKY08 (a) and TK1402 (b). Both strains were inoculated in Brucella-serum broth, and the morphology of the strains was analysed by scanning electron microscopy after 15 h incubation. Bar, 2 µm.

reduced motility. In the *luxS* mutant of enteropathogenic *E. coli* (EPEC), less flagellin is detected on Western blotting, and motility is decreased on semi-solid agar (Sircili *et al.*, 2004). The present study is the first to show significantly reduced motility of the *H. pylori luxS* mutant. Recently, it has been shown that mutating the *H. pylori luxS* gene eliminates growth-phase-dependent control of *flaA* (Loh *et al.*, 2004).

Effects of glucose on *H. pylori* motility

To evaluate the influence of glucose on the motility of *H. pylori*, HPKY08 (*luxS*[−] mutant) was cultured on Brucella semi-solid medium containing various concentrations of glucose (Fig. 4). On the medium containing 0.13–2 % glucose, HPKY08 motility was significantly inhibited. On the other hand, the motility of both wild-type and *luxS*-complemented strains was enhanced on medium containing 0.13–1 % glucose. At each concentration from 0 to 2 % glucose, the motility of the mutant was significantly lower

Fig. 4. Effect of glucose on motility of HPKY08 (*luxS* mutant; □, dashed line), HPKY21 (*luxS* complemented; △, dashed line) and TK1402 (wild-type; ●, solid line) strains. The strains were cultured on Brucella-serum semi-solid agar for 4 days, and the diameter of the halo was measured. **The mean diameter of halo was significantly lower than that of TK1402 ($P < 0.01$).

than that of the wild-type and *luxS*-complemented strains ($P < 0.05$) (Fig. 4). These results indicate that the motility of the *luxS* mutant is more sensitive to glucose.

To confirm the inhibitory effect of glucose on the motility of *H. pylori luxS*[−] mutant strains, the velocity of *H. pylori* strains was determined by a video system. In Brucella medium containing 0.5 % glucose and 7 % horse serum, the mean velocity of *H. pylori* strain HPKY08 (OD_{600} 0.9) was $6.57 \pm 2.62 \mu\text{m s}^{-1}$, significantly slower than that of the wild-type ($12.02 \pm 3.88 \mu\text{m s}^{-1}$; OD_{600} 1.2) and *luxS*-complemented ($10.32 \pm 3.20 \mu\text{m s}^{-1}$; OD_{600} 1.0) strains ($P < 0.05$) at 20 h after inoculation. These results indicate that the reduced motility in *H. pylori* in the early stationary phase of culture was due to the effects of *luxS* mutation.

We demonstrated that the presence of glucose (0.13–2 %) strongly inhibited the motility of the *luxS*[−] mutant strain, although its growth was not inhibited by glucose (0.13–1 %) (data not shown). Kim *et al.* (2003) have reported that *V. vulnificus* shows increased signalling activity when cultured in the presence of 0.5 % glucose without growth enhancement. It has been reported that AI-2 production by virulent *E. coli* and *S. Typhimurium* is affected by glucose and pH level (Surette & Bassler, 1999). Similarly, in the present study, AI-2 production by the *H. pylori* wild-type TK1402 strain was significantly enhanced in the presence of 0.5 % glucose (data not shown). We also checked directly the movement of *H. pylori* strains in the Brucella-broth medium containing glucose, and showed that the velocity of the *H. pylori luxS* mutant strain was significantly lower than that of the wild-type and *luxS*-complemented strains.

Acid tolerance of the HPKY08 strain

Both wild-type and HPKY08 strains were prepared from stationary-phase cultures. In AP Brucella broth lacking urea, the number of both wild-type and HPKY08 (*luxS*[−] mutant) *H. pylori* decreased rapidly at pH 1, and no cells were viable after 1 h (data not shown). In medium containing 30 mM urea at pH 1, both the HPKY08 and wild-type strains recovered to 10^4 c.f.u. ml^{-1} at 1 h after inoculation of approximately 10^6 c.f.u. *H. pylori* ml^{-1} . In the presence of 30 mM urea, both strains were maintained at 10^4 c.f.u. ml^{-1} for 3 h of incubation at pH 3. Similar results were obtained when the strains were in exponential phase (data not shown). These results show that there are no differences in survival between the wild-type and *luxS*[−] mutant strains under acidic conditions in the presence of urea.

Adhesion of HPKY08 *H. pylori* strain to MKN45 cells

The adhesion activities of HPKY08 (*luxS*[−]) and wild-type *H. pylori*, which were obtained from 3-day cultures on BHI agar supplemented with 7 % horse blood, were assessed by flow cytometry. The mean fluorescence intensities of the wild-type strain and HPKY08 (*luxS*[−]) strains were

2622.3 ± 1400.8 and 1934.7 ± 932.6 , respectively, at m.o.i.=600, and there was no significant difference in adhesion activity between the two strains in any of the experiments (m.o.i.=60 or 6000). These results indicate that mutation of the *luxS* gene does not affect the adherence activity of *H. pylori* to AGS cells.

Colonization of Mongolian gerbils with HPKY08

In the Mongolian gerbil experiment, the detectable frequency of HPKY08 (*luxS*⁻) by culture was lower than that of the wild-type strain. At 1, 4 and 12 weeks after infection with TK1402, the rates of *H. pylori*-positive gerbils to total gerbils were 4/4, 4/5 and 3/5, and mean colonization numbers were $10^{2.61} \pm 10^{1.56}$, $10^{3.70} \pm 10^{0.91}$ and $10^{5.05} \pm 10^{2.51}$ c.f.u. (g gastric mucus)⁻¹, respectively, upon culture using M-BHM agar plates (Fig. 5). In this culture method, the detection limit of *H. pylori* was $10^{2.3}$ c.f.u. (g gastric mucus)⁻¹. However, at 1, 4 and 12 weeks after infection with HPKY08, no *H. pylori* was isolated from the gastric mucus.

qRT-PCR was applied to determine the relative number of *H. pylori* in the gerbil stomach samples, inoculated with both strains (Fig. 5). At 1 week after infection with *H. pylori*, the mean relative number of the *luxS* mutant [$10^{1.16} \pm 10^{0.88}$ (g mucus)⁻¹] was significantly lower than that of TK1402 [$10^{2.94} \pm 10^{1.18}$ (g mucus)⁻¹] ($P=0.046$). At 12 weeks after infection, the mean relative number of the *luxS* mutant was $10^{2.24} \pm 10^{0.28}$ (g mucus)⁻¹, which was significantly lower than that of the wild-type strain [$10^{3.86} \pm 10^{0.43}$ (g mucus)⁻¹] ($P<0.01$). These data indicate that the colonization rate of the *luxS* mutant was significantly lower than that of TK1402 in gerbil stomachs, and that the *luxS* gene plays an important role in *H. pylori* infectivity in gerbils.

For comparison of survival rates between the HPKY08 and TK1402 strains in the gerbil stomachs, we assessed the bacterial number in stomachs at 1 h after inoculation. The mean colonization number of HPKY08 [$10^{3.0} \pm 10^{1.3}$ c.f.u. (g mucus)⁻¹] was not significantly different from that of TK1402 ($10^{3.3} \pm 10^{0.8}$ c.f.u. (g mucus)⁻¹). This suggests that there are no differences in survival in gerbil stomachs between the HPKY08 and TK1402 strains at this early time point.

Serum antibody titres from gerbils infected with *H. pylori* were assessed by ELISA at 12 weeks after infection. The ELISA value of serum antibodies from gerbils infected with HPKY08 ($A_{490} 0.24 \pm 0.90$) was significantly lower than that of gerbils infected with TK1402 ($A_{490} 0.992 \pm 0.474$; $P<0.042$). However, the antibody titre of gerbils infected with HPKY08 was higher than that of non-infected gerbils ($A_{490} 0.042 \pm 0.043$; $P<0.01$).

On histopathological examination, no pathological changes were observed in the stomachs of gerbils infected with HPKY08 (*luxS*⁻) at 1 and 3 months after inoculation

Fig. 5. Effect of *luxS* mutation on the infectivity of *H. pylori* to Mongolian gerbils, estimated by culture (a) and qRT-PCR (b). (a) A total of $1-2 \times 10^9$ c.f.u. of HPKY08 (black bars) and TK1402 (grey bars) strains were inoculated into Mongolian gerbils per os in Hanks' solution on two consecutive days. Error bars indicate standard deviation ($n=4$, 1 h and 1 week; $n=5$, 4 and 12 weeks). (b) The 16S rRNA gene of *H. pylori* (black bars, HPKY08; grey bars, TK1402) was detected in gerbil stomachs by SYBR Green real-time RT-PCR. A single PCR amplicon from cDNA prepared from total RNA extracted from stomach samples at each of the times is shown. *Significantly different from TK1402-infected gerbils ($P<0.05$); **significantly different from TK1402-infected gerbils ($P<0.01$).

(Fig. 6a). However, severe gastritis was observed in gerbil stomachs infected with TK1402 at 3 months after infection (Fig. 6b). This suggests that the infectivity of the mutant strain is insufficient for induction of pathogenic changes in the gerbil stomach.

Implications of experimental data

In Mongolian gerbil experiments, it was shown that the total bacterial number of *H. pylori* HPKY08 *luxS*⁻ cells in the mucus of the stomach was significantly lower than that of *H. pylori* TK1402. *H. pylori* possesses several putative colonization factors, including flagella motility (Marshall & Warren, 1984), various adhesins (Evans *et al.*, 1993; Falk *et al.*, 1993; O'Toole *et al.*, 1995), *vacA* (Salama *et al.*, 2001) and urease (Marshall & Warren, 1984; Tsuda *et al.*, 1994), some of which have been shown to be necessary for gastric

Fig. 6. Histopathological findings in gastric mucosa of Mongolian gerbils 12 weeks after *H. pylori* HPKY08 (a) and TK1402 (b) infection (haematoxylin and eosin stain). Bar, 200 μ m.

colonization. Isogenic *flaA*⁻, *flaB*⁻ and *flaA flaB*⁻ mutant strains do not show full motility, and are unable to form effective colonies in the stomachs of germ-free piglets (Eaton *et al.*, 1996). The experimental data indicate that both flagellin species are necessary for full colonization by *H. pylori*. Iwao *et al.* (1999) have recently reported that phenotypic variants of *H. pylori* strains lacking either motility or urease activity lose their ability to colonize. In our study, there were no differences in flagella formation between *H. pylori* HPKY08 (*luxS*⁻ mutant) and wild-type strains. However, *H. pylori* HPKY08 exhibited both lower motility on soft agar medium and lower colonization potential in gerbil stomach when compared with TK1402.

In addition, it has been reported that motile strains are able to colonize regardless of their vacuolating cytotoxin activities, thus indicating that vacuolating cytotoxin is not associated with epithelial damage in the gastric mucosa (Iwao *et al.*, 1999). In this study, it was shown that the *luxS* mutant had various virulence factors, such as vacuolating cytotoxin activity and hummingbird phenotype, caused by CagA protein injected into epithelial cells through type IV secretion machinery (data not shown). On histopathological examination, severe damage was observed in the gastric mucosa of gerbils infected with TK1402, but no damage was detected in the gastric mucosa of gerbils infected with

HPKY08. It is therefore possible that induction of gastric damage depends on bacterial numbers in the stomach, and that the colonization potential is not associated with expression of these virulence factors.

H. pylori possesses numerous adhesive molecules, which are important for colonization in animal models. Yamaoka *et al.* (2002) have reported that the switch status of several genes, such as *oipA*, *hopZ*, *hopO* and *hopP*, encoding outer-membrane proteins influences the colonization of *H. pylori* in the stomachs of C57/BL mice. In their study, when two or more of these genes were 'off', colonization rates were markedly reduced compared with conditions under which all genes were 'on'. However, in this study, it was shown that the adhesion of HPKY08 cells to MKN45 cells was almost identical to that of the wild-type TK1402 strain. In addition, there was no significant difference in the bacterial number in gastric mucosa at 1 h after inoculation between TK1402- and HPKY08-infected Mongolian gerbils. These results indicate that the decreased infectivity of the HPKY08 *luxS* mutant strain was not due to decreased adhesion activity.

Urease activity in *H. pylori* is required for its acid resistance, and thus for survival under the low-pH conditions of the stomach. In the present study, the urease activity of HPKY08 was as strong as that of TK1402 (data not shown). Both the wild-type TK1402 and the mutant HPKY08 strains survived at pH 3 in the presence of 30 mM urea. Therefore, the lower infectivity of HPKY08 is unlikely to be due to lower urease activity. In fact, there were no differences in the survival of the two strains in the stomachs of gerbils immediately after inoculation.

As shown by the qRT-PCR results (Fig. 5b) at 12 weeks after inoculation with HPKY08 (*luxS*⁻), considerable amounts of mRNA for *H. pylori* 16S rRNA were detected in the mucus of gerbil stomach, but isolation of *H. pylori* from gastric mucus was negative. We performed an animal experiment using the *luxS*-complemented strain HPKY21 in the Mongolian gerbil model. In the qRT-PCR assay, the number of colonizing *H. pylori* *luxS*-complemented HPKY21 strain cells was more than that of the *luxS*⁻ mutant strain. In addition, there was no significant difference in the number of colonizing *H. pylori* between TK1402- and HPKY21-infected Mongolian gerbils (data not shown). These results support a hypothesis that the *luxS* mutation is involved in the colonization and persistent infection of *H. pylori* in the gastric mucosa of the Mongolian gerbil.

Serum antibody titres in gerbils infected with HPKY08 were slightly higher than those in non-infected gerbils. These data imply that HPKY08 colonized the gastric mucus of the gerbils, although the number of colonizing *H. pylori* cells was below the detection limit, 2×10^2 c.f.u. (g mucus)⁻¹, for the culture-based assay. In gerbil stomachs, numerous other types of bacteria form colonies, and some of these may possess the *luxS*/AI-2 system. It is possible that the AI-2 produced by these bacteria affected the *luxS* mutant strain, thus allowing the mutant to survive in the gerbil stomach.

The biosynthetic pathway of AI-2 has recently been clarified (Schauder *et al.*, 2001). AI-2 is generated from S-adenosylmethionine (SAM) in three enzymic steps. SAM is an essential metabolite used in central metabolism. Following methyl transfer from SAM to its various substrates, S-adenosylhomocysteine (SAH) is formed. SAH is a potent inhibitor of SAM-dependent methyl transferase, and thus must be eliminated. However, the *luxS* mutant is unable to convert the second product, S-ribosylhomocysteine (SRH), which is the substrate of LuxS, to AI-2 and homocysteine (third product). The accumulation of SAH in the mutant may inhibit bacterial cell growth. In fact, the viable cell number of HPKY08 in the stationary phase was lower than that of TK1402.

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