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In Vivo Behavior of a *Helicobacter pylori* SS1 *nixA* Mutant with Reduced Urease Activity

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Helicobacter pylori **mutants devoid of urease activity fail to colonize the gastric mucosa of mice; however, the effect of decreased levels of urease on colonization has not been examined. The *nixA* gene, required for full urease activity, encodes a cytoplasmic membrane nickel transporter that imports nickel ions and leads to incorporation of nickel ions into apourease. A *nixA* mutant of the Sydney strain of *H. pylori* (SS1) was constructed by disruption of the *nixA* gene with a kanamycin resistance cassette. This mutant retained only half the urease activity of the wild-type (wild-type) SS1 strain. C57BL/6j ($n = 75$) and BALB/c ($n = 75$) mice were inoculated independently with the wild-type or the *nixA* strain. The level and distribution of colonization were assessed by bacterial colony counts and histological grading at 4, 12, and 24 weeks postinfection. Colonization levels of the *nixA* strain in BALB/c mice were significantly lower compared with SS1 ($P = 0.005$), while colonization in C57BL/6j mice was similar for both the wild-type and mutant strains. Subtle differences in colonization of the different regions of the stomach, determined by microscopic grading, were observed between wild-type SS1 and the *nixA* strain in BALB/c mice. On the contrary, when C57BL/6j ($n = 35$) and BALB/c ($n = 35$) mice were coinfected with the wild-type and *nixA* strains simultaneously, the *nixA* mutant failed to colonize and was outcompeted by the wild-type SS1 strain, which established normal levels of colonization. These results demonstrate the importance of the *nixA* gene for increasing the fitness of *H. pylori* for gastric colonization. Since *nixA* is required for full urease activity, the decreased fitness of the *nixA* mutant is likely due to reduced urease activity; however, pleiotropic effects of the mutation cannot be completely ruled out.**

Helicobacter pylori, a gram-negative, microaerophilic, motile, spiral-shaped bacterium, has been established as the etiological agent of chronic gastritis (9, 16, 17, 40) and a predisposing factor for peptic ulceration (9, 17) and gastric adenocarcinoma (28).

The ecological niche of *H. pylori* is the human stomach. To survive within the low pH of the gastric milieu, *H. pylori* has evolved unique mechanisms, including the production of high levels of urease. Urease (urea amidohydrolase; EC 3.5.1.5), a nickel-requiring metalloenzyme, hydrolyzes urea to ammonia and carbon dioxide. Ammonia reacts with water to form ammonium ions (27), which are thought to maintain the periplasmic pH at 6.2 (24, 29, 31, 32). This preserves the proton motive force across the cytoplasmic membrane, permitting generation of energy in the cell by the passage of protons through the F_1/F_0 enzyme complex, resulting in the production of ATP (22, 24).

Urease activity has been shown to be essential for colonization of the gastric mucosa by *H. pylori*; urease-negative mutants are unable to colonize nude mice (37), gnotobiotic piglets (6, 7), and *Cynomolgus* monkeys (34). In addition, urease-negative mutants of *Helicobacter mustelae* fail to colonize the ferret stomach (2).

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Recent investigation of the microecology of *Helicobacter* infection suggests that local acid and urease activity may also determine the areas within the stomach where optimal growth of the bacterium may occur (10, 11). This hypothesis is based on the results of in vitro studies which have shown that urease activity protects *H. pylori* in the presence of physiological urea in an acidic environment (pH 2 to 4) by neutralizing the local gastric acid. In contrast, when urea is present in a more neutral environment (pH 4.5 to 7), *H. pylori* does not survive and may indeed self-destruct due to the lack of protons which are required to neutralize excess ammonia (4). Hence, *H. pylori* survives over a narrow pH range at which it maintains the proton motive force.

These recent advances in the understanding of the *H. pylori* urease gene cluster provide the opportunity to further test the hypothesis that urease activity determines the location of bacterial colonization. Nickel ions required for urease activity are transported into *H. pylori* by a high-affinity cytoplasmic membrane nickel transport protein, NixA, encoded by the *nixA* gene (25). Once inside the cell, the nickel ions are incorporated into apourease, presumably by the urease accessory proteins UreE, UreF, UreG, and UreH to yield the catalytically active holoenzyme. A *nixA* mutant of *H. pylori* strain ATCC 43504 (American Type Culture Collection) has been shown to have significantly reduced nickel transport (approximately a threefold reduction) and reduced urease activity (42% reduction) compared with the wild-type (wild-type) strain (3).

Based on the observations by Lee et al. (14), who hypothe-

sized that local acid output impacts on the behavior and location of *H. pylori* within the stomach, one would predict that the NixA-deficient mutant exhibiting significantly reduced urease activity would demonstrate a different pattern of colonization than the wild-type *H. pylori* strain. The ability to test this hypothesis was aided by the development of an *H. pylori* Sydney strain (SS1) mouse model, which had provided basic information on the colonization patterns of *H. pylori* SS1 in several different strains of mice (13). Such studies showed that in C57BL/6j mice, *H. pylori* SS1 colonizes mainly the antrum and cardia, the areas without acid-secreting parietal cells, as well as the two transitional zones between body and antrum and between body and cardia. In contrast, in BALB/c mice, SS1 was found to mainly colonize the transitional zones of the stomach. Given the fact that acid suppression has been shown to alter *Helicobacter* colonization patterns within the mouse stomach (13), such differences in *Helicobacter* colonization patterns of SS1 in these two mouse strains suggest that this may be due to variations in local acid output within the gastric mucosa of the mouse stomach.

Thus, the goal of the present study was to compare the magnitude and pattern of colonization of an isogenic *nixA* mutant of *H. pylori* SS1 (*nixA*) with that of the wild-type strain (wild-type SS1) in two different mouse strains, C57BL/6j and BALB/c, and to examine the outcome of coinfecting mice with a mixture of the *nixA* mutant and wild-type SS1 strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain DH5α (*supE44 ΔlavU169* φ80 *lacZΔM15/hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was the host for replicating recombinant plasmids, cloning, and mutagenesis experiments. *E. coli* strains were cultured either on solid or in liquid Luria-Bertani (LB) medium. For selection of *E. coli* strains containing plasmids, the growth medium was supplemented with kanamycin (50 μg/ml) and ampicillin (100 μg/ml) (Sigma Chemical Company, St. Louis, Mo.).

H. pylori Sydney strain (SS1) (13) was the parental strain used for transformation-mediated allelic exchange and for the assessment of gastric mucosa colonization in a mouse model. For the construction and in vitro characterization of the SS1 *nixA* mutant, bacteria were grown on Brucella agar (BA) (Becton Dickinson, Cockeysville, Md.) with 10% (wt/vol) defibrinated pooled sheep blood (Walz Farm, Smithsburg, Md.). The plates were incubated in a microaerobic environment generated either by an incubator set at 5% CO_2 and 100% humidity or in an anaerobic jar with the CampyPak Plus system (Becton Dickinson) for 48 h at 37°C. For selection of the SS1 *nixA* mutant strain, 20 μg of kanamycin (Sigma) per ml was added to the medium.

For the inoculation of mice, wild-type and *nixA* strains of *H. pylori* SS1 were grown on *Campylobacter* selective agar (CSA) as previously described (12, 13). Following 24 to 48 h of culture on CSA plates, bacterial cultures were harvested and inoculated into 300 ml of brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, England) containing 5% (vol/vol) sterile horse serum (Oxoid, Ltd.), 5 μg/ml amphotericin B (Fungizone; E. R. Squibb & Sons Inc., Princeton, N.J.), and Skirrow's (33) selective supplement: 10 μg of vancomycin (Sigma), 5 μg of trimethoprim lactate (Sigma), and 2,500 IU of polymyxin B (Sigma) per ml.

For selection of the *H. pylori nixA* isogenic mutant, the growth medium was supplemented with 20 μg of kanamycin (Sigma) per ml. Liquid *H. pylori* cultures were incubated in an anaerobic jar with a microaerobic gas generating kit (Oxoid Ltd.) for a period of 36 to 48 h at 37°C with gentle shaking. Following incubation, liquid cultures were centrifuged (5,000 rpm, 5 min, 4°C), and the cell pellets were resuspended in sterile BHI to obtain an approximate bacterial concentration of 10^9 CFU/ml.

For the coinfection experiment, SS1 and the mutant strain were grown separately and mixed in equal numbers to yield a final bacterial suspension of 10^9 CFU/ml before infecting mice. Enumeration of final cell concentrations was done using a hemacytometer. A retrospective plate count using a series of tenfold dilutions in BHI broth was also performed to confirm that the inoculation dose was 10^9 CFU/ml.

Wild-type and *nixA* mutant isolates of *H. pylori* SS1 passaged through mice were grown on Glaxo selective supplement agar (GSSA) (19) under microaerobic conditions in an incubator set at 10% CO_2 and a relative humidity of 95% at 37°C for a period of 5 to 6 days for initial isolation and 36 to 48 h for subsequent cultivation as previously described (12, 13). The supplements in GSSA medium were 5 μg of amphotericin (Fungizone; E. R. Squibb & Sons Inc.) and the Glaxo selective supplement (10 μg of vancomycin σ, 0.33 μg of polymyxin B σ, 20 μg of bacitracin σ, and 1.07 μg of nalidixic acid σ per ml). Kanamycin (20 μg/ml) was added to the medium to select for the *nixA* mutant strain when necessary.

To confirm the purity and identity of cultures before and after infecting mice, tests for catalase, oxidase, and urease were regularly performed (23). In addition, *H. pylori* cultures were regularly assessed by phase contrast microscopy for spiral morphology and motility.

Construction of the isogenic SS1 nixA mutant. A previously constructed plasmid, pUEF401, which contains a nonpolar kanamycin resistance cassette inserted into the *Ssp*I site of *nixA* (*nixA*::*aphA-3*) (3), was transformed into *H. pylori* strain SS1 by electroporation (800 ë, 2.5 kV, 25 μF). The nonpolarity of this kanamycin cassette was supported by sequence analysis, which shows that the cassette lacks a transcriptional terminator, thus permitting the transcription of downstream genes and avoiding or greatly reducing potential polar effects. In addition, the gene downstream of *nixA* (*hp1076* of strain 26695, also found in strain J99) is predicted to be transcribed in the opposite direction.

After 2 days of outgrowth, bacteria were recovered and plated on BA plates containing 10% sheep blood plus kanamycin (20 μg/ml). Mock transformations of wild-type SS1 with water or buffer yielded no kanamycin-resistant colonies under the same transformation conditions used for transforming pUEF401. Genomic DNA was isolated by the method of McAllister et al. (18) from two kanamycin-resistant transformants. Southern blotting was used to confirm a double-crossover mutation in the *nixA* gene within the chromosome of the parental SS1 strain (data not shown). Genomic DNA was digested to completion with *Bgl*II and *Bam*HI restriction enzymes, then electrophoresed on an agarose gel, and transferred by Southern blotting to a positively charged nylon membrane using standard techniques (30) and the conditions described previously (21). The Southern blot was probed with a 1.2-kb PCR-amplified *nixA* fragment from pUEF201, containing the wild-type *nixA* gene (25), and a 1.2-kb *Eco*RI fragment encompassing the *aphA-3* gene encoding kanamycin resistance from pHP1 (obtained from the H. Kleanthous laboratory, Oravax, Boston, Mass.). One of two confirmed mutants was designated the SS1 *nixA* mutant and used for this study (see below).

In vitro characterization of the SS1 nixA mutant strain: urease extract preparations, protein determinations, and urease activity. Urease extracts were prepared by harvesting BA-grown *H. pylori* into 750 μl of 50 mM HEPES buffer (pH 7.5). Following two washes in HEPES buffer, bacteria were resuspended in 300 μl of fresh HEPES buffer. Cells were water bath sonicated (40% intensity, three pulses for 30 s each) (Cell Disruptor; Ultrasonics, Inc.) on ice to release cytosolic proteins, including urease. Viable CFU counts showed that >99% of the bacteria were lysed by this method. Following centrifugation (12,000 × *g*, 2 min, 4°C), supernatants containing urease were transferred to microcentrifuge tubes and placed on ice. Protein determinations of the urease extracts were conducted by the bicinchoninic acid assay method (Pierce Chemical Company, Rockford, Ill.), according to the manufacturer's 30-min protocol. Bovine serum albumin was used as the standard.

Urease activity was determined by the phenol-hypochlorite urease assay method as previously described (20, 21, 41), using ammonium chloride as the standard and urease extracts diluted tenfold in 50 mM HEPES buffer (pH 7.5) containing 25 mM urea. Addition of exogenous nickel to this buffer did not significantly alter urease activity. The alternative Welch's *t* test was used to compare the means, using InStat 2.03 software (GraphPad Software, San Diego, Calif.).

RAPD PCR analysis of the nixA mutant and parental SS1 strain. Genomic DNA for randomly amplified polymorphic DNA (RAPD) PCR analysis was extracted from *H. pylori* cultures (35). A RAPD PCR method (1) was used to confirm that the *nixA* strain originated from the *H. pylori* wild-type SS1 parental strain both before and after in vivo passage through mice. As the stringency of RAPD PCR is low, two arbitrary primers were used to ensure consistent and reproducible results. The two primers used were known as 1281 (5'-AACGCG CAAC-3') and 1290 (5'-GTGGATGCGA-3') (1).

Animal procedures. (i) Strains of mice. Female, specific-pathogen-free C57BL/6j ($n = 110$) and BALB/c ($n = 110$) mice aged 6 to 8 weeks obtained from the Biological Resources Centre (University of New South Wales, Sydney, New South Wales, Australia) were housed under conventional conditions in the Microbiology Animal Facility, University of New South Wales. The Animal Care

and Ethics Committee, University of New South Wales, approved all procedures involving animals.

(ii) Infection of mice by orogastric gavage. Bacterial suspensions of wild-type SS1 and the *nixA* strain were prepared to equal concentrations of 10^9 CFU/ml as described above. Mice were orogastrically dosed with either a bacterial inoculum or a negative BHI broth control three separate times over a 5-day period using a polyethylene stomach catheter (Dural Plastics, Auburn, New South Wales, Australia). In the first experiment, which examined the ability of the *nixA* strain to colonize C57BL/6j and BALB/c mice, a volume of 0.1 ml of either strain (SS1 or *nixA*) was administered to separate groups of mice (approximately 10^8 CFU/mouse). Negative-control mice received the same volume of sterile BHI broth.

The second experiment examined coinfection dynamics of SS1 and *nixA* strains in C57BL/6j and BALB/c mice. A volume of 0.1 ml of each bacterial suspension was administered to the coinfection group of mice (approximately 2×10^8 CFU/mouse). Two separate groups of positive-infection-control mice received a volume of 0.2 ml from either SS1 or the *nixA* strain culture, which were derived from the same cultures as those combined for coinfection. Negative-control mice received 0.2 ml of sterile BHI broth alone.

(iii) Collection of gastric tissue. Mice were sacrificed by cervical dislocation at weeks 4, 12, and 24 postinfection, and gastric tissue was collected for assessment of gastric colonization and histopathological changes. Ten mice from each group inoculated with a bacterial suspension and five mice from the negative control group were sacrificed at each time point. All mice included in the coinfection experiment were sacrificed by cervical dislocation at 5 weeks postinfection, and gastric tissue was collected for assessment of the level of *H. pylori* colonization. The stomach from each animal was removed, one half was placed in BHI for the determination of viable-cell counts, and the other half was fixed in 10% neutral buffered Formalin (Fisher Scientific) for histological examination to determine the distribution of *H. pylori* in the different anatomical regions of the stomach (13).

Assessment of bacterial colonization and distribution. *H. pylori* colonization was determined by viable-colony count. Immediately after collection of the gastric tissue, one half of each stomach was weighed and placed in 2 ml of BHI broth. The gastric tissue was homogenized using an Ultra-Turrax homogenizer (John Morris Scientific Pty. Ltd., Chatswood, New South Wales, Australia). Serial tenfold dilutions of the homogenate in BHI were plated onto GSSA plates to determine the numbers of both wild-type SS1 and mutant bacteria as previously described (13) or onto GSSA plates supplemented with 20 μg of kanamycin per ml to detect the *nixA* strain only.

H. pylori distribution in the different anatomical regions of the stomach was assessed using the system originally developed for *H. felis* (5) and later adapted for *H. pylori* SS1 (13). Briefly, the distribution of bacterial colonization in May-Grunwald-Giemsa stained "blinded" tissue sections was graded on a scale of 0 to 4 in four regions of the mouse stomach: antrum (A), antrum-body transitional zone (AB), body (B), and body-cardia transitional zone and cardia (BC/C). The grading system was as follows: 0, no bacteria detected in any crypts; 1, low level of colonization and bacteria not detected in every crypt; 2, low level of colonization with bacteria detected in the majority of crypts; 3, moderate to heavy colonization in all crypts; and 4, severe colonization, with all crypts densely packed with bacteria. All the grade scores for a section were averaged to give a mean colonization grade, and standard deviations (SD) were calculated. Animals were considered uninfected if no bacteria were visible and infected if even one bacterium was visible. The lowest level of detection of bacteria determined by viable-colony counts has been reported to be 10^3 CFU/g (21, 38) in two different studies using mice. For this study we have estimated the detection limit for colony counts to be 10^2 to 10^3 CFU, whereas for microscopic analysis, which is less sensitive, the limit is estimated to be 10^3 to 10^4 CFU.

Assessment of gastric histopathology. The histopathology of blinded 5-μm sections stained with hematoxylin and eosin was assessed under light microscopy to examine the degree of gastric inflammation and histopathological changes in the gastric tissue. Each stomach was graded for the degree of neutrophil and mononuclear cell infiltration in the antrum and body as follows: 1, mild multifocal; 2, mild widespread or moderate multifocal; 3, mild widespread and moderate multifocal or severe multifocal; 4, moderate widespread; 5, moderate widespread and severe multifocal; and 6, severe widespread (8). The total number of gland abscesses and lymphoid aggregates was recorded for each stomach section. Parietal cell atrophy (loss of parietal cells) or mucous cell hyperplasia was graded using a three-point scale: 0, no glands affected; 1, 1 to 10 glands affected; 2, 10 to 20 glands affected; and 3, >20 glands affected.

Statistical analysis. Differences were determined between the viable-colony count levels of bacterial colonization by wild-type SS1 and the *nixA* strain in the gastric mucosa of mice using a set of planned contrasts in a repeated-measures analysis of variance (ANOVA). Statistical analysis of the results was undertaken using the computer package Contrast, an add-in macro program for Microsoft Excel (Microsoft Corporation, Redmond, Wash.) version 4 for the Apple Macintosh (Apple Computer Inc., Cupertino, Calif.), which was developed in the School of Psychology, University of New South Wales, Sydney, Australia. Contrast uses a multivariant approach in performing the ANOVA, allowing analysis even if the number of subjects in each group is not equal (unlike standard univariant ANOVA). Furthermore, the Contrast program allows testing of specific differences between groups by specific differences across repeats without the uncontrolled risk of type I error rate inflation that can occur using methods such as the protected *t* test. Colonization levels were found to be significantly different when $F(x,y) = z$, $P < 0.05$, where x is the numerator (degrees of freedom effect), y is the denominator (degrees of freedom error), and z is the F value.

RESULTS

Growth rate and urease activity of the nixA strain. An isogenic *nixA* mutant of *H. pylori* SS1 was constructed and found to have the same growth rate as the parental SS1 strain (data not shown). The RAPD PCR profiles generated for the *nixA* and wild-type SS1 strains were identical in vitro and after in vivo colonization of both C57BL/6j and BALB/c mice (data not shown). Therefore, no genetic changes detectable by this method occurred during colonization in vivo for up to 6 months postinfection of mice.

Urease activity differed significantly ($P < 0.0001$) between the isogenic strains with 22,859 ± 4,828 nmol of ammonium (NH_{4^+}) min^{-1} (mg of protein)$^{-1}$ for the *nixA* strain ($n = 10$) compared with 45,704 ± 4,182 nmol of NH_{4^+} min^{-1} (mg of protein)$^{-1}$ for wild-type SS1 ($n = 5$).

Colonization in the mouse stomach. *H. pylori* SS1 and the *nixA* strain were able to colonize both BALB/c and C57BL/6j mice when each strain was inoculated separately. Regardless of the infecting bacterial strain, colony counts were generally 10-fold lower in colonization level for BALB/c mice (10^5 to 10^6 CFU/g) (Table 1) compared with C57BL/6j mice (10^6 to 10^7 CFU/g) (Table 2).

Histological assessment of colonization in the stomachs of BALB/c mice showed that there were negligible levels of bacteria in the antrum and low levels in the body. Colonization mainly occurred in the two transitional zones (Table 1). By comparison, in C57BL/6j mice, both SS1 and the *nixA* strain were distributed throughout the entire glandular stomach, with lower levels of colonization observed in the body (Table 2). The patterns of *H. pylori* SS1 colonization in both strains of mice are identical to those observed in previous studies (13).

Colonization in BALB/c mice. Overall, the colony count assessment indicated that the *nixA* strain colonized BALB/c mice at significantly lower levels than SS1 regardless of time ($F[1, 47] = 11.707$, $P = 0.005$). The largest colony count difference compared with wild-type SS1 ($\approx 10^6$ CFU/g) was approximately a 5- to 10-fold decrease in mice infected with the *nixA* strain ($\approx 10^5$ CFU/g), observed at 12 and 24 weeks (Table 1). Consistent with the colony count data, the grades of colonization showed that the *nixA* strain colonized BALB/c mice at a lower or similar level in all four regions of the stomach at all time points compared with the parental SS1 strain (Table 1).

When the actual proportion of mice colonized by either SS1 or the *nixA* strain within the four specific regions was analyzed, a lower level of *nixA* colonization compared with SS1 was noted in the body-cardia transitional zone at 4 and 12 weeks (Table 1). Specifically, in the body-cardia transitional zone at 4 weeks, only 22% (two of nine) of the mice infected with the

TABLE 1. Gastric colonization of BALB/c ($n = 75$) mice infected with wild-type and *nixA* strains of *H. pylori* SS1

Group[a]	Time (wk postinfection)	Colony counts		Mean grade[d] ± SD (no. of mice colonized/no. tested)			
		Log_{10} CFU/g (mean ± SD)[b]	No. of mice colonized/total[c]	A	AB	B	BC/C
Control	4	0 ± 0	0/5	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)
	12	0 ± 0	0/4	0 ± 0 (0/4)	0 ± 0 (0/4)	0 ± 0 (0/4)	0 ± 0 (0/4)
	24	0 ± 0	0/5	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)
SS1	4	5.7 ± 0.4	10/10	0 ± 0 (0/10)	1.2 ± 0.5 (10/10)	0.5 ± 0 (2/10)	1.3 ± 0.7 (8/10)
	12	6.1 ± 0.5	10/10	0 ± 0 (0/10)	1.0 ± 0.6 (10/10)	1.0 ± 0.7 (2/10)	1.2 ± 0.4 (9/9)
	24	6.0 ± 0.5	8/8	0 ± 0 (0/8)	1.7 ± 0.5 (8/8)	0.8 ± 0.5 (8/10)	1.3 ± 0.5 (9/9)
nixA	4	5.5 ± 0.6	8/10	0 ± 0 (0/10)	1.4 ± 0.5 (6/10)	0 ± 0 (0/10)	0.8 ± 0.4 (2/9)
	12	5.2 ± 0.4*	7/7	0 ± 0 (0/9)	0.9 ± 0.2 (5/8)	0 ± 0 (0/9)	0.5 ± 0 (2/9)
	24	5.4 ± 0.9*	8/8	0.5 ± 0 (1/6)	1.3 ± 0.4 (7/8)	0.6 ± 0.2 (5/8)	1.1 ± 0.4 (5/7)

[a] BALB/c mice were inoculated with sterile BHI broth (negative control) or ~10^8 CFU of either SS1 or the *nixA* strain.
[b] Means and standard deviations calculated for infected mice only. *, colony count values for the *nixA* strain were significantly lower ($P = 0.005$) than those for the parental WT SS1 strain.
[c] Proportion of mice colonized was determined by viable-colony counts.
[d] Histological sections from the antrum (A), antrum-body transitional zone (AB), body (B), and body-cardia transitional zone and cardia (BC/C) were graded from 0 to 4 as defined in the text. (Means and standard deviations were calculated for infected mice only.) The proportion of mice colonized was determined by the histological grading system.

nixA strain were colonized compared with 80% (8 of 10) of SS1-infected mice. Similarly, in the body-cardia transitional zone at 12 weeks, only 22% (two of nine) of the *nixA* strain-infected mice were colonized compared with 100% (nine of nine) of SS1-infected mice.

Colonization in C57BL/6j mice. No significant difference between *nixA* and SS1 colony counts was seen at any time point (Table 2). The mean grades of colonization supported the colony count results, showing that SS1 and the *nixA* strain established similar levels of colonization specifically within all four regions of the mouse stomach at all time points (Table 2). In addition, the SS1 strain was found to colonize at a slightly higher level within the antrum and antrum-body transitional zone at 4 weeks compared with the *nixA* strain (Table 2).

Statistical analyses indicate that the colony counts in C57BL/6j mice decreased significantly between 12 and 24 weeks postinfection regardless of the strain of bacteria ($F[1, 52] = 4.603, P = 0.037$). This decrease in the colony counts was also reflected in the grades of colonization for C57BL/6j mice, where the grades decreased between 12 and 24 weeks in almost all regions of the stomach for both bacterial strains, the decrease being most prominent for SS1-infected mice (Table 2).

Histopathology in the mouse stomach. Infection of C57BL/6j mice with either *H. pylori* wild-type SS1 or the *nixA* strain of SS1 induced mild, multifocal to widespread infiltration of neutrophils (grade 1 to 2) after 4 weeks of infection in 20 to 56% of animals. This infiltration increased in severity over the course of the experiment. By week 24, many animals (50 to 80%) infected with either the wild-type SS1 or *nixA* strain had neutrophil infiltration that ranged from a slight scattering of cells across the antrum or body to a moderate to severe multifocal neutrophilic gastritis which centered around the antrum-body transitional zone (grade 3 to 4).

A similar scenario was observed with the infiltration of

TABLE 2. Gastric colonization of C57BL/6j ($n = 75$) mice infected with wild-type and *nixA* strains of *H. pylori* SS1[a]

Group[a]	Time (wk postinfection)	Colony counts		Mean grade[d] ± SD (no. of mice colonized/no. tested)			
		Log_{10} CFU/g (mean ± SD)[b]	No. of mice colonized/total[c]	A	AB	B	BC/C
Control	4	0 ± 0	0/5	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)
	12	0 ± 0	0/5	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/4)
	24	0 ± 0	0/5	0 ± 0 (0/5)	0 ± 0 (0/4)	0 ± 0 (0/5)	0 ± 0 (0/4)
SS1	4	6.9 ± 0.2	10/10	2.7 ± 0.5 (10/10)	2.2 ± 0.3 (10/10)	0.5 ± 0 (4/10)	1.4 ± 0.6 (9/10)
	12	6.8 ± 0.5	10/10	2.9 ± 0.8 (8/10)	2.5 ± 0.7 (10/10)	1.9 ± 0.5 (10/10)	2.0 ± 0.3 (10/10)
	24	6.3 ± 0.7	10/10	2.2 ± 1.3 (7/10)	2.0 ± 0.9 (7/10)	1.4 ± 0.9 (8/10)	1.9 ± 0.6 (8/10)
nixA	4	6.5 ± 0.2	9/9	2.2 ± 0.4 (9/9)	1.6 ± 0.5 (9/9)	0 ± 0 (0/9)	1.6 ± 0.6 (7/8)
	12	6.9 ± 0.4	10/10	2.6 ± 0.9 (9/10)	2.3 ± 0.4 (9/10)	1.6 ± 0.6 (10/10)	1.9 ± 0.3 (9/9)
	24	6.4 ± 0.7	9/9	2.5 ± 1.1 (10/10)	2.4 ± 0.6 (8/9)	1.6 ± 0.6 (7/10)	1.7 ± 0.6 (9/9)

[a] BALB/c mice were inoculated with sterile BHI broth (negative control) or ~10^8 CFU of either SS1 or the *nixA* strain.
[b] Means and standard deviations calculated for infected mice only. *, colony count values for the *nixA* strain were significantly lower ($P = 0.005$) than those for the parental WT SS1 strain.
[c] Proportion of mice colonized was determined by viable-colony counts.
[d] Histological sections from the antrum (A), antrum-body transitional zone (AB), body (B), and body-cardia transitional zone and cardia (BC/C) were graded from 0 to 4 as defined in the text. (Means and standard deviations were calculated for infected mice only.) The proportion of mice colonized was determined by the histological grading system.

TABLE 3. Coinfection of C57BL/6j and BALB/c mice with wild-type and *nixA* strains of *H. pylori* SS1 at 5 weeks postinfection

Mouse strain	Group[a]	Colony counts[b] on plates without kanamycin		Colony counts[b] on plates with kanamycin	
		Log_{10} CFU/g (mean ± SD)	No. of mice colonized/total[c]	Log_{10} CFU/g (mean ± SD)	No. of mice colonized/total
C57BL/6j	Control	0*	0/5	0*	0/5
	SS1	6.7 ± 0.2	10/10	0*	0/10
	nixA	NA	NA	6.4 ± 0.4	10/10
	SS1 + *nixA*	6.5 ± 0.3	9/9	0*	0/9
BALB/c	Control	0*	0/5	0*	0/5
	SS1	5.8 ± 0.3	10/10	0*	0/0
	nixA	NA	NA	4.8 ± 0.7	10/10
	SS1 + *nixA*	5.4 ± 0.7	10/10	0*	0/0

[a] Mice were inoculated with sterile BHI broth (negative control) or ~10^8 CFU of either SS1 or the *nixA* strain or both SS1 and the *nixA* strain.
[b] Values are means and standard deviations calculated for infected mice only. NA, not applicable. *, no colonies were recovered; below limit of detection (10^2 to 10^3 CFU/g).
[c] Proportion of mice colonized was determined by viable-colony counts.

mononuclear cells. From 20 to 56% of C57BL/6j mice infected with the wild-type SS1 or the *nixA* strain exhibited a mild infiltration of mononuclear cells after 4 weeks of infection (grade 1 to 3). The severity of the mononuclear cell infiltration gradually increased over time to a severe multifocal and in some cases moderate widespread infiltration of cells at 24 weeks (grade 3 and 4), which, like the neutrophils, congregated around the vicinity of the antrum-body transitional zone.

In comparison, the neutrophilic gastritis in BALB/c mice produced by infection with either the wild-type SS1 or the *nixA* strain was less severe than that seen in C57BL/6j mice. After 4 weeks of infection with either the wild-type or the *nixA* strain, 20 to 40% of animals had a mild scattering of neutrophils (grade 1 to 2), which increased in severity over the 24-week experiment to a moderate multifocal neutrophilic gastritis (grade 2 to 3), which, as for C57BL/6j mice, was most severe around the antrum-body transitional zone.

Like C57BL/6j mice, BALB/c mice infected with either of the *H. pylori* strains produced a mild infiltration of mononuclear cells after 4 weeks of infection (grade 1 to 2). The mononuclear infiltration gradually increased over time, generating a moderate to severe multifocal mononuclear inflammation (grade 2 and 3) centered around the antrum-body transitional zone or the cardia equivalent at 24 weeks.

In summary, in both C57BL/6j and BALB/c mice, no major differences were found between the inflammatory cell responses induced by wild-type SS1 and the *nixA* strain. Uninfected control mice showed a few scattered neutrophils and mononuclear cells; however, this infiltration was less severe than that observed in *H. pylori*-infected animals at all time points. Gland abscesses and atrophy were observed in some animals at each time point in both the SS1- and *nixA*-infected mice but not in any of the control animals.

Coinfection with SS1 and nixA strains. In both C57BL/6j and BALB/c mice coinfected with equal numbers of SS1 and the *nixA* strain, the *nixA* strain was outcompeted by the wild-type strain and failed to colonize a single mouse, whereas the coinfected SS1 strain colonized to a similar level compared with infection with SS1 alone (Table 3). However, in mice singly infected with either SS1 or the *nixA* strain, the colonization levels ranged from 10^6 to 10^7 CFU/g (SS1 and *nixA* strain) in C57BL/6j mice, while in BALB/c mice the levels ranged from 10^5 to 10^6 CFU/g (SS1) and 10^4 to 10^5 CFU/g (*nixA* strain). These colonization levels were consistent with the colonization data for the single-infection experiment presented in Tables 1 and 2.

DISCUSSION

In this study a *nixA* mutant of the *H. pylori* Sydney strain (SS1) was used to examine the impact of decreased urease activity on the levels and distribution of colonization and pathology in C57BL/6j and BALB/c mice. The most striking result of this study was noted during experiments in which the *nixA* strain was completely outcompeted by the wild-type parental strain (SS1). The fact that no residual *nixA* strain bacteria remained in the mouse stomach after 5 weeks of coinfection suggests that the wild-type strain alters the gastric environment in a manner that prevents the *nixA* strain from colonizing. Exclusion of the *nixA* strain by the wild-type strain was unexpected and highlights the subtleties of gastric colonization. This result suggests that the level of urease activity is important for colonization.

At least two mechanisms for reduction of urease activity are possible. Mutation of *nixA* significantly reduces the rate of nickel ion transport (3). If the intracellular nickel concentration is lowered, urease accessory proteins could fail to load all active sites of the apoenzyme with the requisite metal ion. The result would be lowered catalytic activity. It has also recently been demonstrated that increased extracellular nickel concentrations induce transcription of urease genes 3.5-fold (39). It follows that prevention of nickel uptake by mutation of *nixA* would lower the rate of transcription of urease genes and thus lower overall urease activity. Either scenario would result in lower urease activity as a function of the *nixA* mutation.

Although it could also be argued that the lack of colonization by the *nixA* mutant in the coinfection experiment may be due to a polar mutation on a neighboring gene, we would consider this unlikely because the neighboring gene, *hp1076*, is transcribed in the opposite orientation from *nixA* in the *H. pylori* genome (36) and thus is unlikely to be affected by polar effects. Given the additional role of the *nixA* gene in transport-

ing nickel and other divalent cations (26), one possibility is that the *nixA* mutation causes defects in other enzyme activities in addition to the urease enzyme. Currently the only other known nickel-requiring enzyme of *H. pylori* is hydrogenase (15), and thus, if the *nixA* gene does affect the activity of other metalloenzymes, it is likely that hydrogenase activity would be affected by the *nixA* mutation. Comparison of the *nixA* mutant with the wild-type strain, however, has not shown any differences in hydrogenase activity (J. W. Olson and R. J. Maier, University of Georgia, Athens, personal communication). Since the *nixA* mutation does not appear to have effects on other enzymes, we hypothesize that the reduced urease activity of the *nixA* mutant is the major reason for the attenuation during coinfection in vivo.

Interestingly, the wild-type Sydney strain of *H. pylori* was found to have a particularly high urease activity (45,704 units) compared with other wild-type *H. pylori* strains that have an average activity of between 10,000 (strain 26695) and 22,500 (ATCC 43504) units (D. J. McGee and H. L. T. Mobley, unpublished observations). Initially we considered that this property might explain the ability of the Sydney strain to colonize mice, especially since urease is one of a limited number of factors previously shown to be critical for colonization of the gastric mucosa. However, not only did the *nixA* strain colonize and persist up to 24 weeks in the gastric mucosa of both C57BL/6j and BALB/c mice, but only minor differences in colonization levels and pathology were observed between the *nixA* strain and the wild-type SS1 strain.

The greatest difference in colonization between mice infected separately with either wild-type SS1 or the *nixA* strain was observed in BALB/c mice. This difference was significant, with approximately a 5- to 10-fold reduction in bacterial colony counts in BALB/c mice at 12 and 24 weeks of infection for the *nixA* strain compared with the wild-type strain. Despite this difference in colonization levels, no difference in the inflammatory response or pathological changes was observed in BALB/c mice infected with the *nixA* strain compared with the parental SS1 strain. In contrast to BALB/c mice, no absolute differences in total bacterial numbers were observed between wild-type SS1 and the *nixA* strain in C57BL/6j mice. In addition, there were no pathological differences observed in C57BL/6j mice infected with either the wild-type or the *nixA* strain. The high levels of colonization observed in C57BL/6j mice infected with either the wild-type or *nixA* strain supports previous findings that the *H. pylori* SS1 strain has a higher colonizing ability in C57BL/6j mice than any other strain of mouse (13).

Histological assessment showed subtle differences in the patterns of colonization in BALB/c mice. In this mouse strain, the *nixA* strain did not colonize the body-cardia transitional zone and cardia to the same level as the wild-type strain. In addition, the percentage of BALB/c mice colonized by the *nixA* strain in the body-cardia transitional zone and cardia was lower than the percentage colonized by wild-type SS1. Considering that the body-cardia transitional zone and cardia is one of the preferred niches for colonization in BALB/c mice, the finding that colonization by the *nixA* strain is lower in this region suggests that wild-type levels of urease activity are important for establishing colonization in particular gastric niches. This may relate to differences in the local acid environment. In comparison, in C57BL/6j mice there was no major difference in the colonization pattern observed between the wild-type SS1 and the *nixA* strain.

The results of the present study show that the 50% reduction in urease activity in the *nixA* strain does not dramatically affect the ability of *H. pylori* to establish colonization in mice. In contrast, coinfection of mice with the wild-type strain and the *nixA* mutant showed that the increased urease activity exhibited by the wild-type strain prevented colonization by the *nixA* strain. The finding that wild-type *H. pylori* outcompetes *nixA* mutant bacteria in the mouse stomach shows that NixA plays an important role during colonization in a mouse model of *H. pylori* infection. Hence, urease activity as modulated by *nixA* function may be a key factor in determining the colonization fitness of *H. pylori*. Clearly these conclusions would be strengthened if it were possible to complement the *nixA* mutant in vivo.

Further elucidation of the role of the urease enzyme in determining the location and pattern of colonization could be facilitated by the construction of a series of mutants with different levels of urease activity. These experiments are currently under way within our laboratories, with the intention of investigating their potential to establish colonization within the SS1 mouse models presented in this study. It will be interesting to determine the minimum amount of urease activity required for *H. pylori* to establish colonization.

In addition, competition experiments in mice under various levels of acid suppression may provide insight into the interaction between acid and urease levels. Acid suppression studies in the *H. felis* mouse model have shown that, following acid suppression, the level of *H. felis* decreases within the less acidic antral gastric mucosa, most probably due to the increase in localized pH. In contrast, in the acidic body gastric mucosa, *H. felis* levels increase due to the establishment of a more optimal pH following acid suppression (10). Given these observations, it is possible that the *nixA* strain may have an advantage over wild-type SS1 in a coinfected acid-suppressed animal.

The acid hypothesis, which highlights the link between the levels of local acid output with the localization and behavior of *H. pylori* within the gastric mucosa of the stomach, has provided a logical explanation for one of the great enigmas of *H. pylori*-associated disease, that is, how the same bacterium causes such different diseases, and how the diseases vary in different populations as a society develops. Gaining evidence to support these ideas is difficult in humans. Animal studies, using isogenic mutants such as those presented here, provide us with the opportunity to gain a better understanding of the subtleties of *H. pylori* behavior.

It is likely that such differences in colonization patterns may play an important role in the outcome of *H. pylori*-related disease in humans. The level of urease activity and the level of local acid are two important factors in colonization. The interaction of these two factors results in that final pathway to duodenal ulceration or gastric ulceration and gastric cancer. Improved understanding of these interactions may significantly improve our ability to treat these gastroduodenal diseases.

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