

Helicobacter pylori tissue tropism: mouse-colonizing strains can target different gastric niches

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Studies with the mouse-adapted *Helicobacter pylori* strain SS1 had supported an idea that infections by this pathogen start in the gastric antrum and spread to the corpus after extensive mucosal damage. This paper shows that the unrelated strain X47 colonizes the corpus preferentially. Differences between strains in preferred gastric region were detected by co-inoculating mice with a mixture of SS1 and X47, and genotyping *H. pylori* recovered after 2–8 weeks of infection by *vacA* s allele PCR and RAPD fingerprinting. Mixed infections were found in each of 59 co-inoculated young C57BL/6J mice. On average, however, SS1 was fourfold more abundant than X47 in the antrum and X47 was threefold more abundant than SS1 in the corpus. Similar results were obtained in mice inoculated first with one strain and then the other strain 2 weeks later. SS1 was even more abundant in the antrum of elderly (> 1 year old) mice (97 % of isolates). Qualitatively similar SS1 and X47 tissue distributions were seen using unrelated mouse lines (AKR/J, A/J, DBA/2J, BALB/cJ, LG/J, SM/J), but with significantly different SS1 : X47 ratios in some cases. These results suggest the existence of at least two distinct gastric niches whose characteristics may be affected by host genotype and age (physiology), and indicate that strains differ in how effectively they colonize each niche. Differences among gastric regions and the mixed infections that these allow may contribute to *H. pylori* diversity and genome evolution.

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INTRODUCTION

Helicobacter pylori chronically infects the gastric mucosa of more than half of all people worldwide, persists for years or decades once established, and constitutes a major cause of gastritis and peptic ulcer disease, and an early risk factor for gastric cancer (Cover *et al.*, 2001; Sipponen *et al.*, 1998). It is one of the most genetically diverse bacterial species: independent clinical isolates typically differ by some 2–5 % in sequences of essential genes and by some 5 % or more in gene content (Achtman *et al.*, 1999; Alm *et al.*, 1999; Israel *et al.*, 2001; Salama *et al.*, 2000). This diversity probably reflects a combination of factors including: (i) mutation (Bjorkholm *et al.*, 2001; Wang & Taylor, 1999); (ii) recombination among divergent lineages (Achtman *et al.*, 1999; Kersulyte *et al.*, 1999; Suerbaum *et al.*, 1998); (iii) gene transfer from unrelated species (Tomb *et al.*, 1997); (iv) preferential transmission among family members, and thus little chance of selection for any one or few potentially optimal genotypes (Mukhopadhyay *et al.*, 2003); and (v)

diversity among hosts in traits that are important to individual strains, and selection for adaptive changes after transmission to new hosts (Dubois *et al.*, 1999).

Certain differences among strains can impact on colonization or disease – among them: abilities to induce synthesis of cytokine IL-8 and thereby severe inflammatory responses (Censini *et al.*, 1996), and to form vacuoles in host tissues (Atherton *et al.*, 1995) (traits that depend on the *cag* pathogenicity island and *sI*-type alleles of the *vacA* toxin gene, respectively); adherence to carbohydrate Lewis B and other carbohydrate structures (Evans & Evans, 2000; Ilver *et al.*, 1998; Mahdavi *et al.*, 2002); survival after brief acid exposure, as in the gastric lumen (Karita & Blaser, 1998); and the genetically distinct ability to grow under mildly acidic conditions, as in the gastric mucin (pH ~ 5), where most *H. pylori* reside *in vivo* (Bijlsma *et al.*, 2000; Schade *et al.*, 1994).

The present study was begun to help examine inferences that human *H. pylori* infections start in the antrum (Fig. 1) and may spread later to the corpus (where acid-secreting parietal cells are located), if acid secretion is decreased by therapy or infection-induced mucosal damage (Dixon, 1994; Sipponen *et al.*, 1998). The interplay between local gastric acidity and

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Abbreviation: RAPD, random amplified polymorphic DNA.

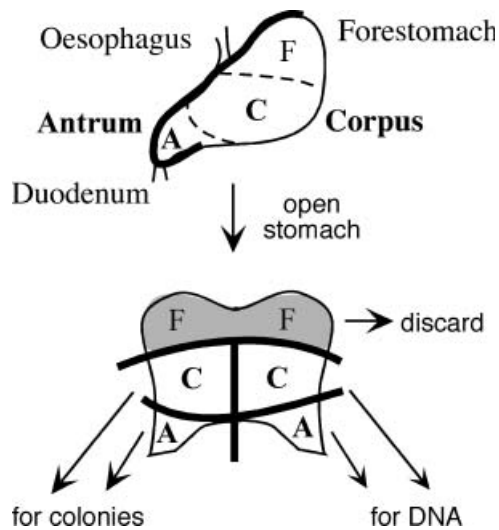


Fig. 1. Structure of stomach and design of experiments.

sites of *H. pylori* infection that this implies has been modelled in mice using SS1, a strain that is sometimes proposed as the standard for experimental infection studies (Lee *et al.*, 1997; Nolan *et al.*, 2002). Given *H. pylori*'s genetic diversity, it seemed that SS1 might not be fully representative of all *H. pylori* strains; and that strains differ in tissue tropism, with some colonizing the corpus preferentially.

Also motivating our study were reports that infections by two or more strains are common in some societies (Berg *et al.*, 1997; Brown *et al.*, 2002; Morales-Espinosa *et al.*, 1999), and interest in the dynamic bacterial–host interplay that may result. In particular, mixed infection should facilitate interstrain gene transfer, which in turn would speed adaptation to new or changing gastric mucosal environments (Achtman *et al.*, 1999; Kersulyte *et al.*, 1999; Suerbaum *et al.*, 1998). In addition, infections that seemed to be mixed were reported to be associated with higher risk of overt disease (Morales-Espinosa *et al.*, 1999). This might reflect: (i) a greater chance that at least one strain will be virulent; (ii) complementary ways of coping with host defences by different strains, and thereby more easily overwhelming these defences; or (iii) greater receptivity of the most disease-prone people to multiple strains. Finally, it seemed that experimental mixed infections could be used to gain new insights into *H. pylori* tissue tropisms; and to improve diagnostic strategies to link particular bacterial genotypes to disease or the failure of therapy, or that distinguish new infection after therapy vs re-emergence of earlier infections.

H. pylori tissue tropisms and the stability, underlying mechanisms, and possible disease and evolutionary consequences of mixed infections cannot be examined experimentally in human subjects for ethical reasons. Here we describe a mouse model for studying these phenomena, and report that co-inoculation of mice with strains SS1 and X47

generally results in persistent mixed infection; and that these two unrelated strains colonize different parts of the stomach preferentially.

METHODS

***H. pylori* strains and culture.** Two unrelated mouse-adapted *H. pylori* strains were used: SS1 (Lee *et al.*, 1997; Nolan *et al.*, 2002) and X47 (also known as X47-2AL; Ermak *et al.*, 1998). SS1 is much used as the standard mouse-adapted strain for experimental infection ('The Sydney Strain'). It carries the *cag* pathogenicity island, an *s2* (putatively non-toxicogenic) allele of the *vacA* vacuolating cytotoxin gene (GenBank accession no. AY049006), and an ON allele of the *oipA* gene (GenBank accession no. AF233683). X47 had been used primarily for studies of immune responses and for vaccine development (Ermak *et al.*, 1998; Kleanthous *et al.*, 2001; Londono-Arcila *et al.*, 2002), but is becoming increasingly popular for mutational analyses (Jeong *et al.*, 2001; O'Rourke *et al.*, 2003), in part because it is easier than SS1 to transform with mutated DNAs. Strain X47 lacks the *cag* pathogenicity island entirely, but contains an *s1* allele of *vacA* (GenBank accession no. AY049007), and an OFF allele of *oipA* (GenBank accession no. AF411912). Strains SS1 and X47 are easily distinguished by arbitrarily primed (RAPD) PCR or *vacA* allele-specific PCR, as illustrated below.

H. pylori was grown in a gas-controlled incubator under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 37 °C, usually on brain-heart infusion agar (Difco) supplemented with 7% horse blood, 0.4% Isovitalex and the antibiotics amphotericin B (8 µg ml⁻¹), trimethoprim (5 µg ml⁻¹) and vancomycin (6 µg ml⁻¹). Nalidixic acid (10 µg ml⁻¹), polymyxin B (10 µg ml⁻¹) and bacitracin (200 µg ml⁻¹) were added to this medium when culturing *H. pylori* from mouse stomachs. *H. pylori* was also sometimes grown on Brucella Agar (1.5%) (Difco) containing 5% horse serum and vancomycin (6 µg ml⁻¹), especially in studies of mild acid resistance (see below).

Acid resistance. Acid-shock resistance was scored using cultures that had been grown overnight on BHI agar, suspended in 150 mM NaCl at a density of 2 × 10⁹ c.f.u. ml⁻¹. Twenty-microlitre aliquots of bacterial suspension were then incubated with 180 µl pH-adjusted citrate buffer for 1 h, as recommended (Bijlsma *et al.*, 2000; Clyne *et al.*, 1995), diluted serially with phosphate-buffered saline, pH 7 (PBS), and spotted on BHI agar plates. Growth under mildly acidic conditions, which depends on different genetic factors (Bijlsma *et al.*, 2000), was scored by spotting 10 µl aliquots of a series of dilutions of equivalent cell suspensions on Brucella Agar (1.5%) whose pH had been adjusted before autoclaving. Viability was scored quantitatively, as the ability of single cells to form colonies after appropriate dilution, essentially as in Jeong *et al.* (2001).

Mice. All mice used here were from established inbred lines and were purchased from Jackson Laboratories; they were maintained in the Washington University Medical School Animal Quarters (Animal Welfare Assurance #A-3381-01) with water and standard mouse chow *ad libidum*, and used in protocols approved by the Washington University Animal Studies Committee (approval #20010039). Those mice designated as 'young adult' in the text were 7–18 weeks old; 'middle-aged' were 25–48 weeks old, and were obtained as retired breeders; 'elderly' were 53–76 weeks old, and had been used in a mouse genetics breeding programme (Cheverud *et al.*, 2001).

Experimental infection. Bacteria were grown overnight on BHI agar, and suspended in PBS at densities of approximately 2 × 10⁹ c.f.u. ml⁻¹. Mice were then inoculated with 0.4 ml of suspension (SS1, X47, or a mixture of both strains). To score colonization, mice were killed by CO₂ asphyxiation. Immediately after death, the mice were cut open with clean and sterile scissors; their stomachs

were removed and cut longitudinally along the lesser curvature (Fig. 1); and any food was removed with clean and sterile forceps. The forestomach (not a major site of *H. pylori* colonization) was identified as a rather thin structure, separated from the corpus by a white line, and was removed and discarded. The antrum and corpus from each longitudinal section were then separated at the transition between a thick brownish wall (corpus) and a thinner, paler and smoother wall (antrum), essentially as described by Lee *et al.* (1982). Half of the antrum and half of the corpus were used for *H. pylori* culture, and the other half of each was used for DNA extraction for PCR. For quantitative culture, the corpus and antrum tissues were diced and homogenized in 200 μ l PBS using a disposable Pellet Pestle (Kontes), and aliquots of each suspension were spread on BHI agar medium and incubated. *H. pylori* densities in the antrum and corpus were estimated by quantitative culture.

RAPD typing. To type *H. pylori* strains by arbitrarily primed PCR (RAPD) fingerprinting (Akopyanz *et al.*, 1992), DNA was isolated from bacteria that had been grown from single colonies as 1 cm² confluent patches on BHI agar medium. Bacterial cells were suspended in 50 μ l TE, and DNA was extracted by a standard phenol/chloroform method. One microlitre of DNA solution was used in 25 μ l for RAPD fingerprinting with primer 1254 (Akopyanz *et al.*, 1992; Kersulyte *et al.*, 1999), with the following cycling conditions: 94 °C, 1 min; 36 °C, 1 min; 72 °C, 2 min; 45 cycles. The PCR mixture also contained 1.75 units of Biolase (thermostable DNA polymerase; MidWest Scientific) and 4 mM MgCl₂. Samples (7 μ l) were analysed by electrophoresis in 2% agarose/TAE gels.

vacA allele typing. To type strains by their *vacA* s alleles, PCR was carried out using DNA extracted by phenol/chloroform (above), or obtained by suspending cells from a fully-grown colony in 10 μ l TE and heating to 99 °C for 5 min. PCR was carried out with primers VA1-F and VA1-R (Atherton *et al.*, 1995), 1 unit of Biolase, and 1 μ l of DNA extract, in 10 or 20 μ l final volume. PCR conditions were: 94 °C, 50 s; 50 °C, 80 s; 72 °C, 50 s; 30 cycles.

Statistical analyses. *H. pylori* densities in the antrum and corpus in each group of mice were compared statistically using the paired *t*-test. The densities in mice of different age groups were compared using the unpaired *t*-test. The relative abundance of SS1 vs X47 in the antrum and corpus (paired samples) in various mouse groups was compared using the Wilcoxon signed-rank test. Differences in SS1:X47 ratios in the antrum or corpus between mice of two age groups or lines (unpaired samples) were evaluated using the Mann-Whitney U test. Data from mice from which 10–30 colonies from each gastric region had been typed were used in these statistical analyses. In each kind of test, differences with *P* values <0.05 were considered significant.

PCR directly from infected gastric tissue. DNA was extracted from the halves of the antrum and of the corpus of each mouse that had not been used for *H. pylori* culture, using the QIAamp DNA Mini Kit (Qiagen), as recommended by the manufacturer, except that tissue lysis was completed with overnight proteinase K digestion at 56 °C, and DNA was eluted in the final step in 100 μ l of the elution buffer provided by the manufacturer. PCR analyses of *vacA* alleles in DNA from infected stomach tissue were performed as above, except that 4 μ l of the DNA solution was used with cycling conditions of 94 °C, 40 s; 52 °C, 40 s; 72 °C, 40 s; 28–32 cycles.

RESULTS

H. pylori strains can differ in preferred sites of colonization

Initially C57BL/6J mice were inoculated with strain SS1 alone or strain X47 alone, and *H. pylori* densities in the

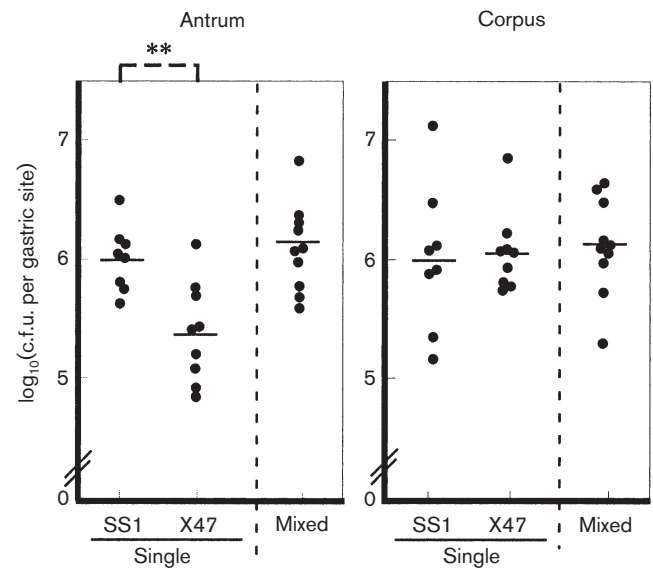


Fig. 2. *H. pylori* titres in antrum and corpus of singly- and mixed-inoculated C57BL/6J mice. SS1 and X47 densities were significantly different in the antrum (*t*-test, *P*=0.0018, as indicated by the asterisks at the top of the figure), but not in the corpus (*P*=0.82). Bars indicate means of each group.

antrum and corpus of the stomach (Fig. 1) were estimated by quantitative culture after several weeks of infection (Fig. 2). The number of c.f.u. was, on average, about four-fold higher in the antrum of SS1-infected than in X47-infected mice (1.0×10^6 vs 2.4×10^5 , respectively; *P*<0.01), whereas the number of c.f.u. was about the same in the corpus of SS1- and X47-infected mice (1.0×10^6 and 1.2×10^6 , respectively).

The antrum was about 0.41 as large as the corpus in these mice (31 ± 4 μ g vs 75 ± 9 μ g, respectively; *n*=27); the SS1 densities per μ g were therefore estimated to be 2.5-fold higher in the antrum than in the corpus, in accord with an earlier report (Lee *et al.*, 1997), and X47 densities were estimated to be 2.1-fold higher in the corpus than in the antrum.

The possibility that SS1 and X47 differ in their ability to colonize particular gastric regions was studied further by co-inoculating mice with 1:1 mixtures of these two strains, and characterizing the *H. pylori* recovered a few weeks later by RAPD and *vacA* s allele PCR typing. Initial tests showed that each of 70 isolates from seven co-inoculated mice was matched to either SS1 or X47 in RAPD pattern, and contained the expected *vacA* s1 and *vacA* s2 alleles, respectively (illustrated in Fig. 3). The two strain types were non-randomly distributed, however: of 35 antrum isolates typed, 27 were SS1 and eight were X47; whereas among 35 corpus isolates, only seven were SS1 but 28 were X47. Tests of *vacA* s allele types of another 370 isolates from 12 additional co-inoculated C57BL/6J mice again showed SS1 to be about

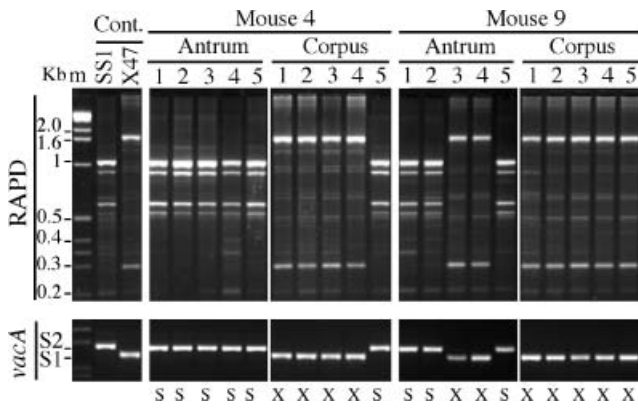


Fig. 3. Analysis of single-colony isolates from representative mice recovered 2 weeks after onset of mixed infection. Upper panels: RAPD fingerprints of isolates using primer 1254. Lower panels: *vacA s* (signal sequence) allele PCR typing from the same isolates as above. Inferred strain type (S, SS1; X, X47) is indicated below the figure.

threefold more abundant than X47 in the antrum but only one-fourth as abundant as X47 in the corpus (Table 1). Similar results were obtained with single colonies from mice after 9 weeks, rather than just 2–4 weeks, of infection.

Changes in SS1 phenotype after long-term infection

With six of nine mice that had been co-infected with SS1 and X47 for 9 weeks, minute barely visible colonies (~0.1–0.3 mm in diameter after 4–5 days of incubation) were formed by some 20–80% of *H. pylori* from the antrum and 20–60% of those from the corpus. In contrast, only normal-sized (~1 mm diameter) colonies were formed by *H. pylori* after just 2–4 weeks of infection. RAPD tests of minute-colony isolates using primer 1254 yielded profiles equivalent to those of SS1 in Fig. 3 in the cases of 45 of 46 randomly selected isolates from the antrum and 20 of 30 from the corpus. The minute-colony phenotype of each of 37 such SS1-like isolates persisted through two sequential restreakings, suggesting a genetic basis, whereas each of four

Table 1. Strain distribution in young adult (7–18 week old) C57BL/6J mice inoculated with 1:1 mixtures of strains SS1 and X47

Single colonies were typed by RAPD fingerprinting and/or *vacA s* allele PCR, as in Fig. 3. All isolates matched either SS1 or X47.

Period (weeks)	No. of mice	Percentage SS1 (no. of SS1/total)	
		Antrum	Corpus
2–4	19	77 (166/216)	21 (46/224)
9	9	73 (103/141)	13 (18/142)

X47-like isolates tested formed normal-sized colonies on restreaking. Further RAPD tests of 13 representative SS1-like minute colony isolates with three additional RAPD primers (1281, 1283, 1290) also yielded profiles that were indistinguishable from those of the parental SS1 strain (data not shown). Thus, the small-colony phenotype may reflect selection for SS1 derivatives that are better adapted to mice, and that might have arisen either by mutation or by DNA transfer from the co-infecting X47 strain.

Strain distribution does not depend on order of inoculation

Sets of mice were inoculated with either SS1 or X47, and then with the other strain 2 weeks later; the distribution of strain types recovered 2 or 7 weeks after the second inoculation was scored by PCR tests of single colonies. The results (Fig. 4A) showed that SS1 predominated in the antrum, and that X47 was generally as or more abundant than SS1 in the corpus, independent of the order of inoculation. Similar results were obtained by *vacA* PCR using DNAs from infected stomachs (Fig. 4B). Thus, the overall distribution of these two strains was not affected strongly by which strain became established first, nor by the duration of infection (Fig. 4A).

The differences among mice in SS1:X47 ratios found by testing single colonies were larger than expected from sampling error alone. A few cases of significantly different SS1:X47 ratios in different halves of the antrum or corpus of the same mouse were also seen when data from PCR tests of single-colony isolates and total gastric mucosal DNAs were compared [e.g. mouse 5, left panels in Fig. 4: SS1 predominated in antrum mucosal DNA (B), but not among single-colony isolates (A)]. Such differences may stem from use of different halves of the stomach in these two tests and the patchiness of many *H. pylori* infections (Bayerdorffer *et al.*, 1989; Anonymous, 1986).

Mouse age affects strain distribution

To test for possible effects of age of host on receptivity to *H. pylori*, elderly (>1 year old) and middle-aged (34–38 weeks old) C57BL/6J mice were inoculated with 1:1 mixtures of SS1 and X47, and the *H. pylori* recovered 2–4 weeks later were analysed as above. No major differences in *H. pylori* densities in elderly vs middle-aged vs young adult mice were found (approx. $1-2 \times 10^7$ c.f.u. g^{-1} , on average, in antrum and $2-4 \times 10^7$ c.f.u. g^{-1} in corpus in each age group). With elderly mice, however, 97.5% (115 of 118) of antrum isolates were SS1, in contrast to 143 of 186 isolates (76.9%) from young adult mice ($P=0.004$; Mann–Whitney U test). An intermediate SS1 level (92.0%, 218 of 237) was found in the antrum of middle-aged mice (Fig. 5). SS1 was also more abundant in the corpus of elderly than of young mice (50/116, 43.1% vs 42/194, 21.6%; $P=0.014$), and again an intermediate value was seen in the corpus of middle-aged mice (97/234; 41.5%).

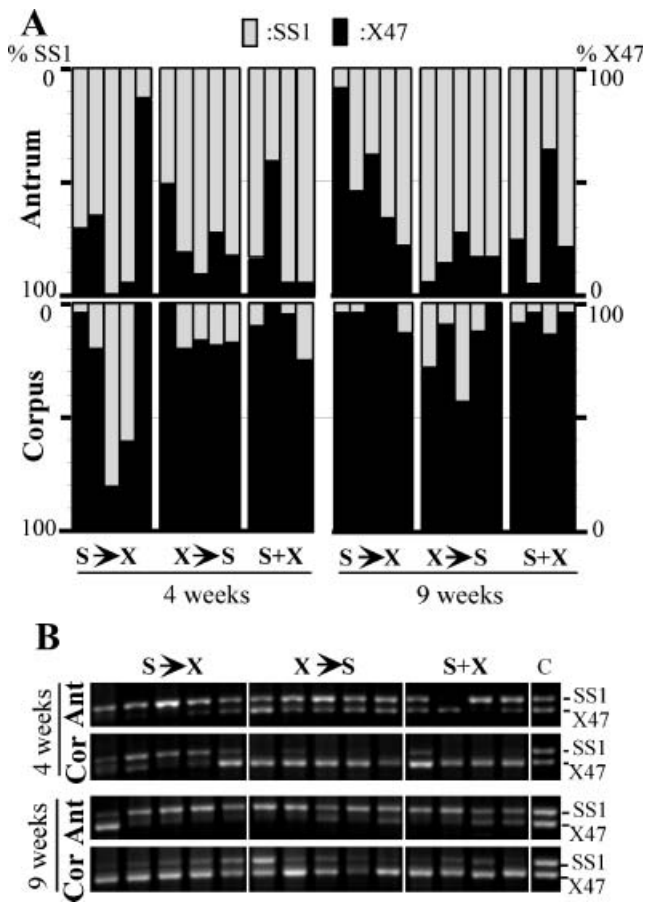


Fig. 4. Sequential infection analysis of SS1 and X47 tropisms. Each column in the bar graph (A) and lane (B) represents data from different halves of the longitudinally split stomach (Fig. 1) of an individual mouse. The mice in the set marked S→X were first infected with SS1, superinfected with X47 2 weeks later, and then maintained for either another 2 or another 7 weeks before being killed for analysis of resident bacteria. Conversely, the set marked X→S were inoculated with these two strains in the reverse order. The set marked S+X were inoculated with a mixture of the two strains, as in the experiments shown in Fig. 3. (A) Data from *vacA* s typing of 20–30 single-colony isolates per site per mouse; (B) data from *vacA* s allele typing using gastric mucosal DNAs (without *H. pylori* culturing). Cor, corpus; Ant, antrum.

Mouse genotype affects strain distribution

To test for possible effects of host genotype on tissue tropism, mice of six other inbred lineages (AKR/J, A/J, DBA/2J, BALB/cJ, SM/J and LG/J) were inoculated with mixtures of SS1 and X47, and bacterial densities and the distribution of strain types were estimated (Table 2). Co-colonization was observed in 130 of 142 infected mice. SS1 was more abundant in the antrum and X47 was more abundant in the corpus in all mouse lineages except AKR/J, where SS1 predominated in both gastric regions. More generally, the

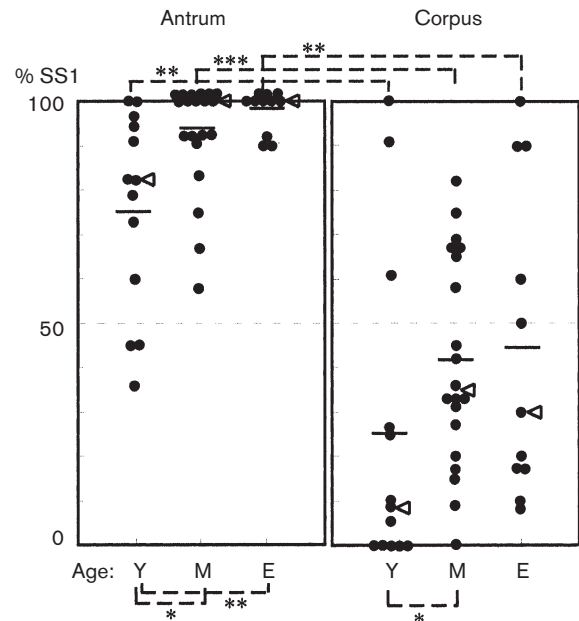


Fig. 5. Effect of mouse age on distribution of strains SS1 and X47 after mixed inoculation. Y, young adult; M, middle-aged; E, elderly, as defined in Methods. Data from *vacA* s allele typing of 10–30 single-colony isolates per site per mouse were used. Open triangles and bars indicate the median and mean, respectively, of each group. The asterisks at the top of the figure indicate that differences in percentage SS1 in antrum vs corpus were significant by Wilcoxon signed-rank test in each group (paired samples): Y, ** $P=0.005$; M, *** $P=0.0001$; E, ** $P=0.008$. The asterisks at the bottom of the figure identify differences in percentage SS1 in a tissue that differed significantly with age [Mann–Whitney U test, ** $P=0.004$, * $P=0.014$ (antrum) and 0.031 (corpus)].

SS1:X47 ratios in antrum and corpus seemed to differ among lineages. These differences were statistically significant in several cases, as indicated by asterisks next to the values in Table 2 (e.g. medians of 17% vs 68% SS1 in corpus of young C57BL/6J vs young AKR/J mice, respectively). Some differences among lineages in overall bacterial densities were also evident in the quantitative culture data (Table 3). The SM mouse strain, in particular, harboured significantly fewer *H. pylori* than did C57BL/6J, especially in the antrum (Table 3).

Acid tolerance of strains SS1 and X47

The acid resistance of SS1 and X47 was compared because of differences in preferred sites of colonization by these two strains (see above), and because gastric acidity is higher in the corpus than in the antrum (Dixon, 1994). Two measures of acid resistance were used: (i) incubation in acidic buffer (Karita & Blaser, 1998); and (ii) formation of colonies on mildly acidic (pH ~ 5) culture medium (Bijlsma *et al.*, 2000; Schade *et al.*, 1994). The first test (Fig. 6A) showed that

Table 2. Effect of host age and genotype on relative abundance of SS1 and X47 after mixed inoculation

Data from *vacA s* typing of 10–30 single-colony isolates per site per mouse were used. All isolates that were not SS1-like were X47-like in *vacA s* type. Bacterial densities in young adult mice were quantified (see Table 3).

Mouse strain	Young adult (7–18 wk)				Middle-aged (25–48 wk)			
	No. of mice	Percentage SS1 [Median (lower–upper quartiles)]†			No. of mice	Percentage SS1 [Median (lower–upper quartiles)]†		
		Antrum‡	Corpus‡	WS§		Antrum‡	Corpus‡	WS§
C57BL/6J	23	82 (63–93)	17 (0–26)	**	20	100 (92–100)	35 (26–65)	***
AKR/J	10	92 (84–92)	68 (42–93)*	**	12	92 (91–100)	72 (41–94)*	*
DBA/2J	16	49 (10–93)	31 (10–42)		10	81 (55–98)	50 (21–60)	**
BALB/cJ	15	77 (68–100)	25 (17–33)		17	91 (50–100)*	25 (8–50)	**
A/J	11	70 (33–92)	38 (31–57)*	*	14	69 (20–98)*	29 (8–79)	
SM/J	13	61 (9–94)	42 (17–100)		12	84 (39–100)	35 (20–77)	*
LG/J	–	–	–		13	100 (75–100)	40 (18–64)	

†Percentage SS1 = (no. of SS1-like colonies/total no. of colonies typed) × 100.

‡Asterisks in these columns identify statistically significant differences ($0.01 < P < 0.05$) in the same age group between C57BL/6J and other mouse strains, based on the Mann–Whitney U test.

§The results of Wilcoxon signed-rank test for antrum vs corpus (paired samples) in each mouse strain in each age group. Significant differences are shown as *** ($P < 0.001$), ** ($0.001 < P < 0.01$), * ($0.01 < P < 0.05$).

strains SS1 and X47 each survived well during 1 h incubation in buffers ranging from pH 5.7 to 4.4, and that each strain was killed by incubation at pH 3.2 (survival $\leq 10^{-5}$). At the critical threshold pH of 3.8, however, there was 120-fold more killing of SS1 than of X47 ($S/S_0 = 0.0041$ vs 0.50, respectively). SS1 is *cag*⁺ and X47 is *cag*⁻. This difference in susceptibilities is in accord with studies of other strains that had indicated a relationship between *cag* pathogenicity island carriage and acid-shock susceptibility (Karita &

Blaser, 1998). The second test (Fig. 6B), however, showed that SS1 formed colonies more efficiently than X47 on mildly acidic medium. This was most evident at pH 5.7, a condition in which SS1 formed colonies 67-fold more efficiently than did X47 (Fig. 6B). To test for genetic heterogeneity or acid adaptation in these populations, SS1 and X47 cells recovered after growth on pH 5.7 medium were cultured overnight on normal medium and then retested for ability to grow at pH 5.7 or pH 5.6. SS1 again

Table 3. Effect of host genotype on bacterial densities after inoculation with a mixture of strains SS1 and X47

All mice were young adults (7–18 weeks). Infections were allowed to proceed for 2–4 weeks before animals were killed, and bacterial densities (this table) and distribution of strain types (Table 2) were determined.

Mouse strain	No. of mice	$\log_{10}(\text{c.f.u. g}^{-1})$, mean \pm SD (c.f.u. g^{-1} , mean)			Estimated c.f.u. g^{-1} of SS1 and X47§			
		Antrum†	Corpus†	PT‡	Antrum		Corpus	
					SS1	X47	SS1	X47
C57BL/6J	14	7.34 ± 0.33 (2.2×10^7)	7.03 ± 0.33 (1.1×10^7)	**	1.8×10^7	4.0×10^6	1.9×10^6	9.1×10^6
AKR/J	10	$6.93 \pm 0.45^*$ (8.5×10^6)	$6.29 \pm 0.24^{***}$ (1.9×10^6)	***	7.8×10^6	6.8×10^5	1.3×10^6	6.1×10^5
DBA/2J	16	7.25 ± 0.27 (1.8×10^7)	6.88 ± 0.50 (7.6×10^6)	*	8.8×10^6	9.2×10^6	2.4×10^6	5.2×10^6
BALB/cJ	15	6.78 ± 1.05 (7.4×10^6)	6.70 ± 0.65 (5.0×10^6)		5.7×10^6	1.7×10^6	1.3×10^6	3.8×10^6
A/J	11	$6.83 \pm 0.33^{**}$ (6.8×10^6)	7.01 ± 0.38 (1.0×10^7)		4.8×10^6	2.0×10^6	3.8×10^6	6.2×10^6
SM/J	13	$5.45 \pm 0.73^{***}$ (2.8×10^5)	$6.39 \pm 0.38^{***}$ (2.5×10^6)	**	1.7×10^5	1.1×10^5	1.1×10^6	1.5×10^6

†The statistical significance of differences in $\log_{10}(\text{c.f.u. g}^{-1})$ in antrum or corpus between C57BL/6J and other strains was tested by the unpaired *t*-test. Significant differences are indicated as *** ($P < 0.001$), ** ($0.001 < P < 0.01$) and * ($0.01 < P < 0.05$).

‡The significance of differences in $\log_{10}(\text{c.f.u. g}^{-1})$ between antrum and corpus in each mouse strain was tested by the paired *t*-test and significant differences are identified as above.

§The colony number of each strain was estimated from the mean c.f.u. g^{-1} in this table and mean percentage SS1 in Table 2.

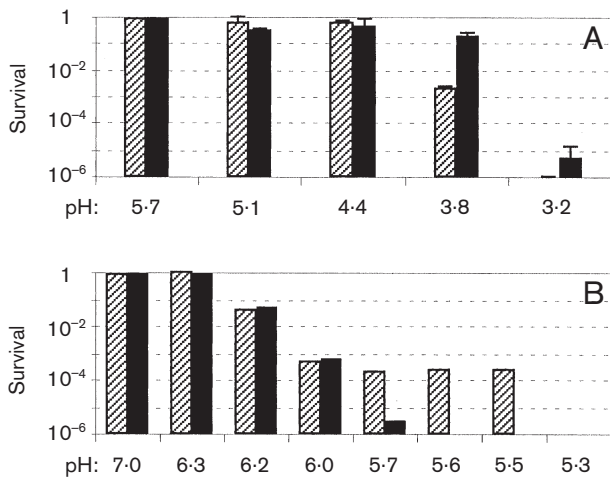


Fig. 6. Acid resistance of SS1 (striated bars) and X47 (solid bars) as determined by efficiency of colony formation. (A) Survival after acid shock: number of c.f.u. on standard BHI agar after 1 h in buffer at the indicated pH, relative to that of control cells held for 1 h in saline. (B) Efficiency of colony formation on acidic Brucella Agar medium of the indicated pH. Survival at pH less than 7 is calculated relative to survival at pH 7, taken as 1.0.

formed colonies more efficiently than did X47 on mildly acidic medium (150-fold and 400-fold higher c.f.u. at pH 5.7 and pH 5.6, respectively) (data not shown).

DISCUSSION

We found that simultaneous or sequential inoculation of mice with the unrelated *H. pylori* strains SS1 and X47 resulted in persistent mixed infections. In general SS1 was more abundant than X47 in the gastric antrum, and conversely X47 was more abundant than SS1 in the corpus. These general patterns were seen in seven different inbred mouse lines, and in elderly as well as young adult mice. The SS1 : X47 ratio increased with age in C57BL/6J mice, one of the standard inbred mouse lines often used for experimental infection. However, the ratio also varied significantly among mouse lines. That is, preferential colonization of the antrum by SS1 and of the corpus by X47 seems to be host-independent in a qualitative sense. These results indicate that strains SS1 and X47 do not compete effectively with one another, and suggest that they, in effect, occupy different gastric mucosal niches. Significant differences are nevertheless seen, however, when the data are considered quantitatively (relative densities of antrum vs corpus colonization by SS1 and by X47 during mixed infection) in relation to lineage (genotype) or age of mice used as hosts. Such quantitative differences suggest that the size or availability of these gastric mucosal niches is affected by host genotype and age or physiology. We propose that mixed infections in humans may also often involve strains that differ in tropism for various gastric regions, and that the distribution of

strains in such cases will similarly be affected by human genotype or gastric physiology.

H. pylori's great genetic diversity has been ascribed variously to mutation (Bjorkholm *et al.*, 2001; Wang & Taylor, 1999); recombination among divergent lineages and species (Achtman *et al.*, 1999; Kersulyte *et al.*, 1999; Suerbaum *et al.*, 1998; Tomb *et al.*, 1997); a highly fragmented genetic population structure, and consequently relatively few of the selective sweeps that might promote emergence of any one or few most-fit genotypes (Mukhopadhyay *et al.*, 2003); and diversity among hosts in traits that are important to individual strains, and consequently potentially new selection for adaptive changes with each infection of a new host (Dubois *et al.*, 1999). To this list we would now add physiological differences between regions of a given host's gastric mucosa. Each chance infection with a strain that previously had been best adapted for just one gastric niche (e.g. with X47, the corpus) would tend to select for derivatives that grew better in other available locations (with X47, the antrum, especially in elderly mice). Such changes would be selected, even if they also made these derivatives less suited for their originally preferred niche (the corpus, in the case of X47). The minute-colony SS1 variants that were found after 2 months, but not after 2 weeks, of mixed infection may have resulted from selection for improved growth in the host gastric mucosal milieu. As in any adaptation to complex environments (Lenski & Travisano, 1994; Wright, 1982), different early chance mutational events may lead a given strain to evolve along different trajectories in different individual hosts. Because established human infections may persist for decades, even subtle selection pressures or differences in fitness could have dramatic effects on the distribution of genotypes that may ultimately emerge.

The nature of the niches to which SS1 and X47 are best adapted, e.g. whether discrete physical compartments, or positions in gradients of metabolites or macromolecules, is not known. A compartmental model is suggested by occasional findings of intracellular *H. pylori* (Allen, 2000; Amieva *et al.*, 2002), if it is assumed that SS1 and X47 tend to occupy different cell types (one predominant in the gastric antrum, the other in the corpus). One attractive molecular gradient model was suggested by pH differences in the antrum vs corpus. However, although X47 was more resistant than SS1 to acid shock, it grew less well on mildly acidic medium, a condition thought to mimic *H. pylori*'s mucosal milieu *in vivo* (Biljsma *et al.*, 2000; Schade *et al.*, 1994). Such results encourage consideration of other gradient models. For example, an ability to adhere to particular glycan moieties on the gastric epithelium is considered adaptive, although whether the optimal affinity is high or low may depend on the particular glycan used as receptor (Mahdavi *et al.*, 2002). *H. pylori* strains can differ markedly in adherence specificity, and a given strain can carry more than one adhesin (Evans & Evans, 2000; Ilver *et al.*, 1998; Mahdavi *et al.*, 2002). Hence the two niches or habitats

implied by our present results might reflect distributions of molecules that SS1 and X47 can each use as receptors. Or, two niches might be created by the joint distributions of inhibitors of *H. pylori* growth (e.g. reactive oxygen metabolites, antibacterial peptides, macrophages, lymphocytes, etc.), and/or local concentrations of exudates that *H. pylori* uses for nutrition (Blaser, 1993; Blaser & Berg, 2001), if SS1 and X47 differ in resistance to host defences and/or effectiveness of nutrient scavenging. Finally, the two strains might differ in chemotactic behaviour with respect to attractants and/or repellents that themselves differ in concentration in antrum and corpus. Regardless of actual mechanism, these considerations emphasize the potential complexity of the gastric ecosystem, and how local differences within it may promote *H. pylori* genetic divergence during chronic infection – evolutionary changes that may affect the vigour or specificity of colonization of new hosts and the spectrum of diseases that can sometimes ensue.

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