Vaccination prevents *Helicobacter pylori*-induced alterations of the gastric flora in mice

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Abstract

Molecular analysis of the gastric microflora in mice revealed that *Helicobacter pylori* infection causes an increase in microbial diversity. The stomachs of *H. pylori*-infected animals were colonized by bacteria which are naturally restricted to the lower intestinal tract. Clostridia, *Bacteroides/Prevotella* spp., *Eubacterium* spp., *Ruminococcus* spp., streptococci and *Escherichia coli* were detected exclusively in the stomachs of infected animals, whereas lactobacilli dominated the gastric flora in noninfected mice. The *H. pylori*-induced shifts in the gastric microbiota were independent from histological pathology and from changes in the gastric pH but were prevented by immunization of mice with live *Salmonella* expressing *H. pylori* urease. Immunized mice displayed reduced *H. pylori* levels in the gastric epithelium and developed a normal gastric microflora, indicating that vaccination may be protective against *H. pylori*-induced changes in the gastric flora.

Introduction

Bacterial colonization of the human stomach is associated with a variety of gastrointestinal disorders. The noxious activity of N-nitroso compounds produced by bacteria colonizing the gastric mucosa under circumstances of hypochlorhydria or achlorhydria is considered a risk factor for gastric cancer (Correa, 1992), and this hypothesis agrees with epidemiological evidence linking nitrate-rich diets and low levels of vitamin C to increased gastric cancer incidence (Palli, 2000). *Helicobacter pylori* infection adds a further risk (Peek & Blaser, 2002) and can cause other severe gastric enteropathies such as severe gastritis, peptic ulcer disease and mucosa-associated lymphoid tissue (MALT) lymphoma (Ernst & Gold, 2000). It was recently reported that *H. pylori*-infected patients treated with proton pump inhibitors develop an increased gastric load with potentially nitrosating bacteria (Mowat *et al.*, 2000), suggesting that *H. pylori* infection can induce alterations in the gastric microflora, which might represent a possible risk factor for the development of the associated diseases. Because *H. pylori* infection is associated with severe gastrointestinal diseases, including gastric cancer, the overgrowth of the gastric mucosa by other potentially dangerous bacteria may act synergistically in carcinogenesis. *Helicobacter pylori*-infected patients presenting with clinical symptoms are currently treated with antibiotic therapy in combination with proton pump inhibitors. The therapy is costly and treatment can fail owing to a lack of compliance (Vakil, 2005). Furthermore, increasing antibiotic resistance amongst *H. pylori* strains has been observed (McLoughlin *et al.*, 2005), and these limitations make vaccination a desirable alternative for disease management (Kleanthous *et al.*, 1998; Del Giudice *et al.*, 2001). In animal models, oral vaccination with live but attenuated *Salmonella* expressing *H. pylori* urease subunits proved to be successful in reducing the *H. pylori* loads by two orders of magnitude. However, vaccination rarely eliminates the organisms completely (Ermak *et al.*, 1998; Gomez-Duarte *et al.*, 1998; Lucas *et al.*, 2001). To investigate whether *H. pylori* infection leads to changes in the gastric flora and whether vaccination could prevent possible *H. pylori*-induced overgrowth of the gastric mucosa by other bacteria, we studied the influence of *H. pylori* infection on the gastric microflora in the mouse model by comparing infected and noninfected as well as immunized and nonimmunized animals. The composition of the gastric microbiota was analyzed by comparative sequence analysis of bacterial 16S rRNA genes in clone libraries, which is currently one of the most powerful approaches to display complex microbial communities including fastidious and yet uncultured bacteria (von Wintzingerode *et al.*, 1997; Vaughan *et al.*,

2000). The results show that *H. pylori* infection fosters an increase in the gastric microbial diversity, which was prevented by vaccination.

Materials and methods

Study design

The gastric bacterial flora was analyzed in naïve mice, in *Helicobacter pylori*-infected and in infected nonimmune or immunized animals (3–5 per group). For immunization, mice were treated with *Salmonella* SL3261(pYZ97) expressing *H. pylori* urease A/B subunits. Mice treated with the empty carrier strain SL3261 served as controls. Four weeks later, the animals were challenged with *H. pylori*. Noninfected animals were treated with phosphate-buffered saline (PBS). Mice were sacrificed 8–9 weeks after the infection and their stomachs were removed for the molecular analysis of the gastric microflora. The investigations were completed by cultural determination of the *H. pylori* load, by histological investigation of the mucosal tissue integrity and by measuring the gastric pH.

Animals, immunization and infections

Female BALB/c mice (6–8 weeks old) were from the Federal Institute for Health Protection of Consumers and Veterinary Medicine (Berlin, Germany). Mice were kept under specific pathogen-free conditions and were fed a diet of Altromin (Lage, Germany) and acidified water *ad libitum*. The streptomycin-resistant *H. pylori* strain P76 (Gomez-Duarte *et al.*, 1998; Lucas *et al.*, 2001) used for experimental infections was routinely grown on GC agar plates (Gibco, Eggenstein, Germany) containing 10% horse serum (serum plates, Gibco). For infection, a culture in brain heart infusion (BHI) (Difco, Becton Dickinson, Sparks, MD), supplemented with 10% fetal calf serum (FCS) (Gibco) and with 400 μg mL^{-1} of streptomycin of optical density ($OD_{590\,nm}$) 0.1, was incubated for 24 h at 37 °C under microaerobic conditions (5% O_2, 85% N_2 and 10% CO_2) with shaking. A secondary culture for the determination of bacterial viability was set up under the same conditions. Bacteria were harvested by centrifugation, washed twice in PBS and resuspended in PBS to a final concentration of $OD_{590\,nm}$ 4. The actual number of *H. pylori* CFU was determined by plating of aliquots and bacterial growth on solid media. Mice were infected with 100 μL of the *H. pylori* suspension (corresponding to 10^9 CFU) by gastric intubation. The *Salmonella enterica* serovar Typhimurium SL3261 *aroA* mutant expressing the *H. pylori* urease subunits UreA and UreB from plasmid pYZ97 was used as a live vaccine (Gomez-Duarte *et al.*, 1998; Lucas *et al.*, 2001). The nonrecombinant strain SL3261 served as a carrier control. Immunizations were performed by gastric intubation with 10^9 CFU stationary phase bacteria.

Sample collection and assessment of *Helicobacter pylori* colonization

Mice were sacrificed by cervical dislocation. Stomachs were removed and cut along the greater curvature into two tissue fragments encompassing antral and oxyntic mucosa as well as the nonsecretory epithelium. Half of the stomach was placed in BHI medium, weighed, and homogenized. Serial dilutions of the homogenate were plated on GC agar (see above) with serum and streptomycin. The average number of *H. pylori* was expressed as $\log_{10}$ CFU per 100 mg of tissue ± standard deviation.

Molecular analysis of the gastric microflora

Total DNA (extracted from one half of mouse stomachs with the QIAmp® DNA Mini Kit from Qiagen, Hilden, Germany) served as the template for amplification (95 °C for 3 min followed by 25 cycles of 95 °C for 45 s, 56 °C for 45 s and 72 °C for 3 min, with final elongation at 72 °C for 5 min) of the bacterial 16S rRNA genes by PCR with the consensus primer pair 8f (5′-AGAGTTTGATCMTGGCTCAG-3′, nucleotide (nt) positions 8–27 in *Escherichia coli* 16S rRNA) and 1513r (5′-ACGGCTACCTTGTTACGACTT-3′, nt positions 1513–1492 in *E. coli* 16S rRNA). The 16S rRNA genes were cloned into plasmid pCR® 2.1 using the TA Cloning® kit (Invitrogen, Karlsruhe, Germany). Plasmids were isolated and complete 16 S rRNA genes were amplified by PCR with vector-specific M13 forward (− 40) and reverse primers. Amplicons containing full-length 16S rRNA genes were silica-purified (GFX™ MicroPlasmidPrep Kit, Amersham Biosciences, Freiburg, Germany) and subjected to further analysis by sequencing (see below) or hybridization. For dot-blot hybridizations, the amplicons containing 16S rRNA genes from DNA libraries were denatured by heating (95 °C, 10 min) and were spotted on nylon membranes. Hybridizations and detection of digoxygenylated DNA probes (see below) were performed with the digoxigenin detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Therefore, the amplicons with 16S rRNA genes fixed on the nylon membranes by UV irradiation (250 nm, 5 min) were hybridized at 55 °C to the digoxigenin-(DIG)-labeled DNA probe Lac-141 (5′DIG-GACTGGGATAACACCTG-3′), which is specific for lactobacilli at the genus level. Specificity was confirmed by hybridization to 16S rRNA genes from reference strains (*Lactobacillus gasseri*, *Lactobacillus johnsonii* and other *Lactobacillus* species; data not shown). The probe did not hybridize to 16S rRNA genes from other eubacteria and non-*Lactobacillus* species listed in Table 1, which served as negative controls. Clones containing *H. pylori* 16S rRNA genes were detected by hybridization with the digoxigenylated *H. pylori*-specific probe Hpy-1 (5′G-CACACCTGACTGACTATCCCG-3′) as described (Trebesius *et al.*, 2000). The

Table 1. Molecular identification of bacterial species detected in the mouse stomach

Mouse group	Accession number	Database match	Sequence identity (%)	Identical clones	Closely related group/ genus/species/strain
Infected,non-immunized, mock-treated*	AF371582	Uncultured bacterium clone p-57-a5	92	8	*Clostridium coccoides* group OTU-70
	AF371633	Uncultured bacterium clone p-2186-s959-3	95	2	*Clostridium coccoides* group OTU-61
	AF371583	Uncultured bacterium clone p-389-o3	93	1	*Clostridium coccoides* group OTU-215
	AF371673	Uncultured bacterium clone p-1321-a5	91	1	*Clostridium coccoides* group OTU-293
	AJ418949	Uncultured bacterium clone k010.	98	1	*Clostridium coccoides* group OTU-390
	AF371579	Uncultured bacterium clone p-4162-6Wa5	93	1	*Clostridium coccoides* group OTU-343
	AJ419018	Uncultured bacterium clone kl046	99	4	*Clostridium coccoides* group OTU-235 *Eubacterium hallii*
	AB062773	Uncultured bacterium BCf1-16	90	1	*Ruminococcus* sp. OUT-227
	AF371888	Uncultured bacterium clone p-828-a5	93	1	*Prevotella* sp. OTU-19
	AJ400242	Uncultured bacterium clone S30-5	95	2	*Bacteroides distasonis* S30-5
	AJ400236	Uncultured bacterium clone F3.	90	3	*Bacteroides forsyth*us F3
	AJ419055	Uncultured bacterium clone kl115	98	1	*Bacteroides forsythus* F3
	AJ408963	Uncultured bacterium clone HuCA7.	96	2	*Bacteroides putredinis* adhufec73
	AJ408997	Uncultured bacterium clone HuCB23.	95	2	*Bacteroides putredinis* adhufec73
	AB094160	Uncultured bacterium clone HuCA19	96	1	*Ruminococcus productus*
	Aj408969	Uncultured bacterium clone HuCA17	90	1	*Clostridium indolis*
	AF157054	*Eubacterium plexicaudatum* ASF492	92	1	*Eubacterium plexicaudatum* ASF492
	AF157051	Bacterium ASF500 16S ribosomal RNA	98	1	*Clostridium* sp. ASF500
	AF157052	*Clostridium* sp. ASF356	91	1	*Clostridium* sp. ASF356
	AJ011522	*Eubacterium ramulus*	92	3	*Eubacterium ramulus*
	AF202259	*Eubacterium oxidoreducens* strain G2-2	90	1	*Eubacterium oxidoreducens* strain G2-2
	Y10584	*Clostridium* sp. 16S rRNA	89	1	*Clostridium* sp. 16S rRNA
	X76163	*Clostridium aerotolerans* 16S	92	1	*Clostridium aerotolerans*
	AF127912	*Helicobacter* sp.	99	2	*Helicobacter* sp.
Infected, immunized	AJ308392	Uncultured bacterium clone S25-5	99	1	*Lactobacillus reuteri* S25-5
	X76328	*Lactobacillus reuteri* (DSM 20016T)	98	1	*Lactobacillus reuteri* S25-5
	AF371506	Uncultured bacterium clone p-29-a5	94	1	*Streptococcus alactolyticus* OTU-180
	AY017059	*Lactobacillus* sp. CLE-4	99	1	*Lactobacillus* sp.
Noninfected, immunized	AF157049	*Lactobacillus murinus* 16S	99	1	*Lactobacillus murinus*
	AJ308392	Uncultured bacterium clone S25-5	96	1	*Lactobacillus reuteri*
Infected	AF352166	*Lactococcus garvieae* strain FLG12 16S	97	2	*Lactococcus garvieae*
	AF233451	*Escherichia coli* 16S rRNA gene	98	5	*Escherichia coli*
	U01331	*Helicobacter pylori* isolate MC937	99	2	*Helicobacter pylori*

Table 1. Continued.

Mouse group	Accession number	Database match	Sequence identity (%)	Identical clones	Closely related group/ genus/species/strain
	AF127912	*Helicobacter* sp.	98	1	*Helicobacter* sp.
	AF157049	*Lactobacillus murinus* 16S	99	5	*Lactobacillus murinus*
	AF371575	Uncultured bacterium clone p-1921-s962-3	91	1	*Clostridium coccoides* group OTU-40
	AF371875	Uncultured bacterium clone p-2190-s959-3	92	1	*Clostridium coccoides* group OTU-61
	AJ400242	Uncultured bacterium clone S30-5	95	1	*Bacteroides distasonis* S30-5
	AJ400241	Uncultured bacterium clone S30-4	95	1	*Bacteroides distasonis* S30-4
	AJ400236	Uncultured bacterium clone F3	91	1	*Bacteroides forsythus* F3

*Nonimmunized animals were treated with *Salmonella* carrying the empty plasmid without *Helicobacter pylori* genes for urease subunits.

16S rRNA genes from clones that hybridized neither to the *Lactobacillus*-specific nor to the *H. pylori*-specific probes were sequenced on a CEQ^{TM} 8000 Genetic Analysis System (Beckman Coulter, Krefeld, Germany) with the CEQ^{TM} DTCS Quick Start Kit. Primers 1513r (see above) and RTU5 (5′-CCGTCAATTCMTTTRAGTTT-3′, nt positions 926–906 in *E. coli* 16S rRNA) were used to obtain sequences that include the variable regions 6–8 (V6–V8) in bacterial 16S rRNA. All sequences were compared with entries in public databases using the BLASTN2 tool of the HUSAR software package (DKFZ, Heidelberg, Germany). The closest match(es) found in the database or literature were reported. Changes in the relative amount of *Lactobacillus* rRNA genes in the total DNA preparations from gastric specimens were further determined by real time PCR on a LightCycler instrument (Roche) with SYBR Green. Therefore, the 16S rRNA genes from *Lactobacillus* species were amplified with primer 8f (see above) in combination with the *Lactobacillus*-specific primer 16S-Lac138+(5′-AGAGATCGGGATAACACCTG-3′, nt positions 138–157 in *E. coli* 16S rRNA). The total eubacterial DNA content was determined by amplification with primers 8f (see above) and RTU2b (5′-CTGCCTCCCGTAGGAGT-3′, nt positions 354–338 in *E. coli* 16S rRNA). The relative abundance of *Lactobacillus* rRNA genes in the total DNA preparations was calculated by comparing the signal intensities of the respective amplicons. The signal intensities of the amplicons containing eubacterial 16S rRNA genes were set at 100%.

Histological analysis of pathological processes in the gastric mucosa

Stomach tissue (half of the organ, as described in the Materials and methods section) was washed with ice-cold PBS and fixed at 4 °C in 2.0% paraformaldehyde overnight. After fixation, the tissue was washed overnight in PBS with 6.8% sucrose, then dehydrated in 100% acetone (2 h) before being infiltrated with and embedded in Technovit 8100 resin (Hereus Kulzer, Hanau, Germany). Thin sections (2 μm) were stained with periodic acid Schiff stain for mucins using protocols recommended for resin embedded tissues (Energy Beam Sciences, East Granby, MA) and counterstained with hematoxylin or were stained conventionally with hematoxylin/eosin.

Determination of gastric pH

The gastric pH was measured in the gastric lumen under inhalation anesthesia (Schreiber *et al*., 1999) with halothane in 60% O_2, 2% CO_2 and 38% N_2O in a semiclosed system. During anesthesia, blood supply to the stomach (monitored by observing the diameter of the vena gastrica dextra), heart rate and respiratory frequency were continuously monitored, and the arterial oxygen saturation and acid–base status were measured. All parameters were normal at the end of the experiment. A small incision was made in the ventral abdominal wall and in the antrum wall of the stomach. The pH electrode (InLab 423, Mettler-Toledo, Giessen, Germany) was inserted into the lumen to measure the pH of the gastric juice without touching the mucosa.

Statistical analysis

If not otherwise mentioned, statistical analysis was performed with the Student's *t*-test, or with the χ^2 test.

Results

Alterations of the gastric flora in *Helicobacter pylori*-infected mice

To investigate whether *Helicobacter pylori* colonization induces changes in the endogenous gastric microflora, we infected BALB/c mice intragastrically with *H. pylori*. Noninfected controls were mock-treated with PBS. After 2 months, when *H. pylori* infection was fully established, mice

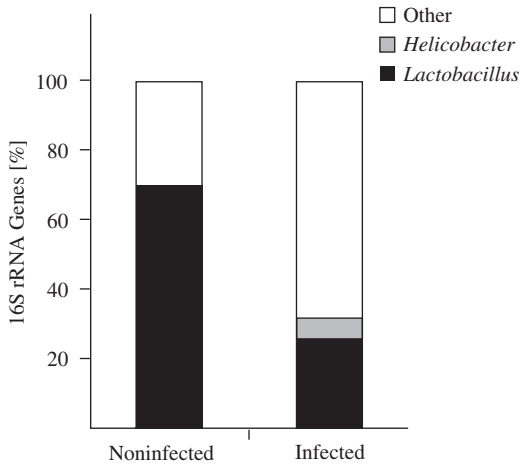

Fig. 1. *Helicobacter pylori* infection increases microbial diversity in the stomach. Randomly selected clones of 16S rRNA gene libraries from *H. pylori*-infected mice ($n = 35$) and control animals treated with phosphate-buffered saline ($n = 30$) were hybridized to species-specific DNA probes as described in the Materials and methods section. The percentage of clones detected by the *Lactobacillus*- and *H. pylori*-specific probes is indicated by black and grey bars, respectively. The number of clones containing 16S rRNA genes from other species, which did not hybridize with both probes, is indicated by white bars.

were sacrificed and the total gastric tissue DNA was used to establish 16S rRNA gene libraries from one representative animal in each experimental group. Because lactobacilli are dominant in the mouse stomach, their relative abundance can be exploited to monitor changes in the gastric microflora. The analysis of randomly selected, individual 16S rRNA genes from DNA libraries of each experimental group by dot-blot hybridization with probes specific for lactobacilli revealed that *H. pylori* infection does significantly increase the bacterial diversity in the stomach (Fig. 1). Lactobacilli predominant in the gastric microflora of noninfected mice ($>$ 60% of 30 clones; Fig. 1) were significantly reduced in *H. pylori*-infected animals (33% of the 35 clones analyzed). The fact that the number of other bacteria was increased (Fig. 1) in the infected animal indicated that *H. pylori* induces alterations in the composition of the mouse gastric flora.

Immunization prevents *Helicobacter pylori*-induced changes in the gastric microflora

To confirm the effect of *H. pylori* infection on the gastric microflora diversity, we analyzed *H. pylori*-infected mice immunized with the *Salmonella enterica* Typhimurium strain SL3261(pYZ97) expressing *H. pylori* urease A and B subunits, in which the *H. pylori* load is significantly reduced (Gomez-Duarte *et al.*, 1998; Lucas *et al.*, 2001). Cultural analysis revealed that the *H. pylori* colonization levels in our immunized mice were reduced by two orders of magnitude (10^3–10^4 CFU per 100 mg tissue 8 weeks after challenge; Fig. 2) compared with infected animals pretreated with the nonrecombinant *Salmonella* strain SL3261. To determine the effect of vaccination on the diversity of the gastric microflora, 16S rRNA gene libraries from one animal each in the respective animal groups were constructed and the abundance of lactobacilli was determined by dot-blot hybridizations with a *Lactobacillus*-specific probe (Fig. 3a). The analysis of 60–77 randomly chosen clones per library showed that lactobacilli dominate the flora in noninfected immunized mice and constitute 97% of the 16S rRNA genes analyzed (Fig. 3a; compare Fig. 1). As expected, and corroborating the previous finding (Fig. 1) in infected mice, the proportion of lactobacilli was reduced to less than 30%, whereas *H. pylori* and other bacteria were detected more frequently (32 and 46%, respectively). This independent experiment demonstrated that *H. pylori* infection reproducibly increased bacterial gastric diversity. In contrast, in the 16S rRNA gene library from the infected and immunized mice, the frequency of lactobacilli was similar to noninfected animals, indicating that vaccination prevents the *H. pylori*-induced alterations in the gastric microflora ($P < 0.001$; Fig. 3a). Because rRNA gene sequences were amplified by PCR before library construction, the results from clone libraries may be biased in favor of abundant species (von Wintzingerode *et al.*, 1997). Therefore, we determined the *Lactobacillus* 16S rRNA gene contents directly in gastric total DNA extracts from two additional mice in each experimental group by real-time PCR (Fig. 3b). The relative abundance

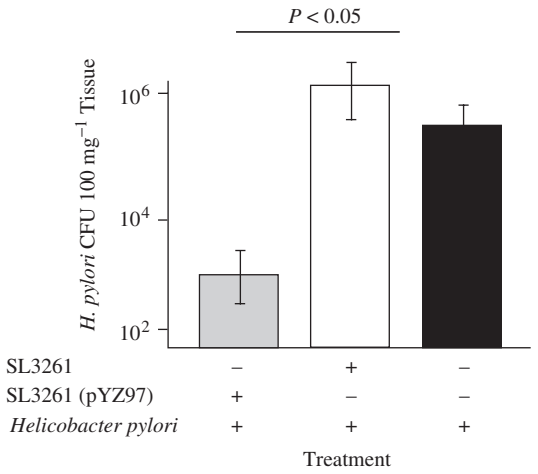

Fig. 2. Vaccinated mice control *Helicobacter pylori* infections at low levels. Mice were immunized by gastric inoculation of 10^9 CFU of either SL3261(pYZ97) expressing *H. pylori* UreA/B, or with the carrier strain SL3261. Controls were mock treated with phospate-buffered saline. Four weeks later, mice were challenged orally with *H. pylori* P76. At day 56 postinfection, mice were sacrificed and *H. pylori* burdens were determined by plating serial dilutions of gastric tissue homogenates. Data represent geometric mean values and standard deviations of three to five mice per group (*P*-values were determined by Mann–Whitney *U*-test).

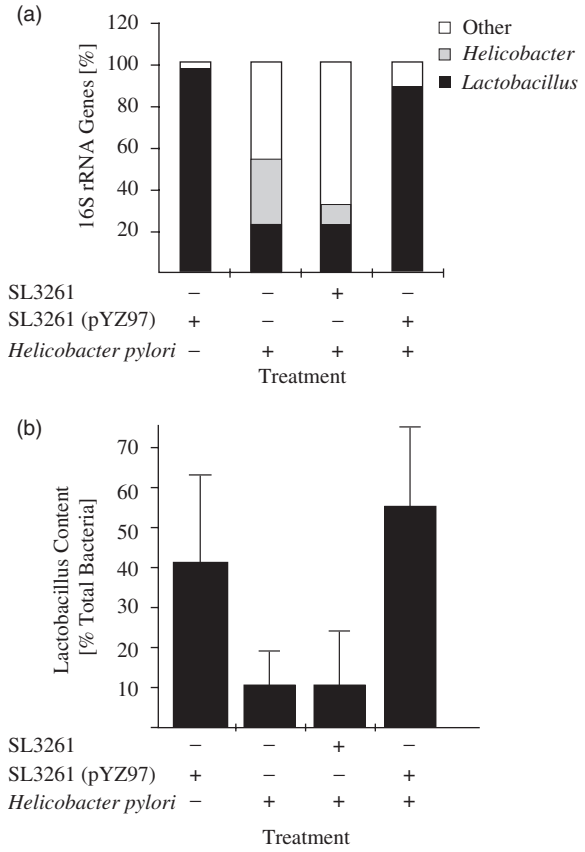

Fig. 3. Vaccination prevents *Helicobacter pylori*-induced changes of the gastric microflora. (a) Mice vaccinated and challenged with *H. pylori*, as described before, were sacrificed 8 weeks postinfection. Representative 16S rRNA gene libraries from individual animals per group were analyzed for the presence of lactobacilli, *H. pylori* and other bacteria by dot-blot hybridization, as described in the legend to Fig. 1. The relative proportions of lactobacilli, *H. pylori* and other bacterial species are indicated. The numbers of clones analyzed were $n = 67$, 60, 77 and 75 for 16S rRNA gene libraries from vaccinated only (control), *H. pylori* infected, SL3261 carrier treated and SL3261(pYZ97) vaccinated and challenged mice, respectively. The differences between nonprotected and protected (or uninfected) groups were significant (Student's *t*-test, $P < 0.001$). (b) Estimation of *Lactobacillus* spp. contents in gastric DNA by real-time PCR. The abundance of 16S rRNA genes from lactobacilli in the gastric flora is expressed as the percentage of total eubacteria (signals obtained for detection of eubacterial 16S rRNA genes were set 100%). Real-time PCR was performed with total DNA isolated from stomachs of two individual mice from the different experimental groups. Data represent mean values from two DNA samples from two mice per experimental group and standard deviations of $n = 5$ determinations from each of two mice per group. Differences between the groups were significant ($P < 0.001$ in two-way ANOVA).

of *Lactobacillus* rRNA genes normalized to the total rRNA gene contents from eubacteria confirmed that vaccination does effectively prevent the *H. pylori*-induced changes in the gastric flora, because reduced lactobacillus contents were detected exclusively in the *H. pylori*-infected mice and in the infected but nonprotected animals ($P < 0.001$).

Identification of bacteria that colonize the gastric microbiota in *Helicobacter pylori* infected mice

To identify the bacteria that colonize the stomach in *H. pylori*-infected mice, we sequenced all cloned 16S rRNA genes in the DNA libraries from the different mouse groups (see Fig. 3) that were detected by neither the *Lactobacillus*-specific nor by the *H. pylori*-specific probe (91 of 279 clones matched these criteria). Unambiguous sequences were obtained for 69 of these and a comparison with existing database entries revealed that the gastric flora of *H. pylori*-infected mice contains bacteria of the *Clostridium coccoides* group, *Clostridium* spp., *Bacteroides/Prevotella* spp., *Eubacterium* spp., *Ruminococcus* spp., *E. coli*, *Streptococcus* spp. and *Lactococcus* spp. (Table 1). Interestingly, 59% of the sequences matched those from yet uncultured bacteria, underscoring the appropriateness of the molecular approach. Single sequences in DNA libraries from nonimmunized mice and from infected mice treated with the carrier *Salmonella* matched (98% and 99% identity) with that of a recently described novel *Helicobacter* sp. capable of inducing colitis and typhlitis in IL-10 deficient mice (Fox *et al.*, 1999). Finally, sequence analysis of 16S rRNA genes in DNA libraries from vaccinated or noninfected mice identified lactobacilli which did not hybridize with our *Lactobacillus*-specific probe (Table 1) because the corresponding 16S rRNA genes carry single nucleotide variations in the binding site of our DNA probe. These results demonstrate that the *H. pylori*-induced increase in microbial diversity in the stomach is mainly reflecting the presence of bacteria, which are naturally restricted to the lower intestinal tract.

Helicobacter pylori-induced alterations in the gastric flora are not caused by immunopathology or by variations in gastric acid secretion

To investigate further whether the *H. pylori*-induced microflora changes are caused by pathological alterations in the infected gastric mucosa or by a modification in gastric acidity, we determined the degree of inflammation and measured gastric pH. In our mice, *H. pylori*-induced immunopathology occurred after a period of 2–6 months (Lee *et al.*, 1997). Because alterations of the gastric microflora may occur before pathological processes can be recorded, we assessed the degree of immunopathology in the gastric mucosa of mice from all treatment groups ($n = 3$–5 per group) 2 months after *H. pylori* infection, when the alterations in the microflora were already established. The findings that the number of inflammatory cells, the size of the mucus layer, the length of gastric glands as well as the

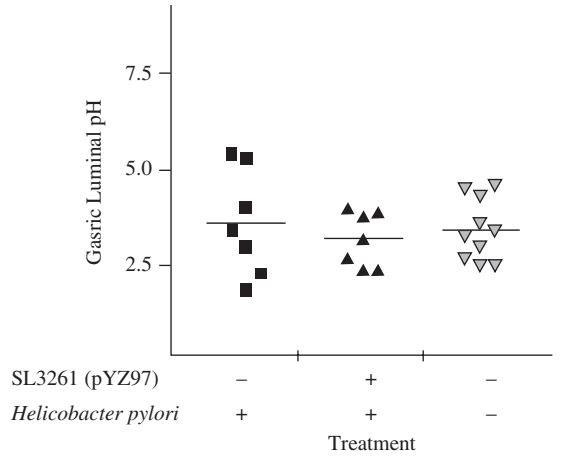

Fig. 4. Gastric pH is not affected by *Helicobacter pylori* infection after 9 weeks. The gastric pH was measured in the gastric lumen under general inhalation anesthesia (Vaughan *et al.*, 2000), but otherwise normal physiology was maintained. The pH was determined by inserting an electrode into the gastric lumen. Values indicate pH readings of individual mice. Differences between groups were not significant (Student's *t*-test, $P > 0.5$).

number of parietal cells (Lee *et al.*, 1997) were not different between the animal groups ($P > 0.05$ for all parameters), indicated that the *H. pylori*-induced alterations in the gastric microflora precede the onset of detectable pathology. Of note, mice infected with *H. pylori* strain P76 for longer time periods developed increased inflammation and immunopathology (Gomez-Duarte *et al.*, 1998; Lucas *et al.*, 2001). In addition, *H. pylori*-induced gastric inflammation has been correlated with increased gastric pH (Feldman *et al.*, 1996; El Omar *et al.*, 1997; Zavros *et al.*, 2002b) and less acidic conditions would offer a straightforward explanation for gastric colonization with gut bacteria. Thus, we determined the luminal gastric pH in naïve mice, infected mice, and immunized infected mice (Fig. 4). The fact that the acidity did not differ significantly among the experimental groups ($P > 0.5$) indicates that the *H. pylori*-induced gastric microflora changes are caused by other factors, which are more complex and need to be identified in further studies.

Discussion

Helicobacter pylori infection induces profound changes in the gastric microflora of mice, possibly creating novel niches for bacterial species that normally are on transient passage of the stomach. This extends reports on the gastric bacterial flora in *H. pylori*-infected Mongolian gerbils (Sun *et al.*, 2003) and in patients with gastrointestinal disorders (Monstein *et al.*, 2000; Mowat *et al.*, 2000). The latter studies revealed that in infected humans *H. pylori* lives in contact with other bacteria, which mainly originate form the oral flora but show altered representation of individual species (Monstein *et al.*, 2000). Thus, the increased bacterial diversity observed in the gastric mucosa of *H. pylori*-infected mice possibly correlates with observations made in infected humans. It is interesting to note that *H. pylori*-infected patients treated with the proton pump inhibitor omeprazole developed 100-fold increased total bacterial loads in gastric juice compared with noninfected controls (Mowat *et al.*, 2000), and this is probably related to different conditions in the absence of the proton pump inhibitor (Monstein *et al.*, 2000). Furthermore, elevated bacterial concentrations were correlated with increased nitrite and lower vitamin C levels, suggesting that nitrite-metabolizing bacteria may encounter high nitrite levels to form potentially dangerous nitrosamines, because under normal physiological conditions vitamin C promotes generation of nitric oxide from nitrite, thus reducing the hazardous nitrite levels (Mowat *et al.*, 2000; Williams, 2001). Therefore, in *H. pylori*-infected mice bacteria from the mouse lower intestinal tract could indeed contribute to gastric pathology, because lactobacilli, which do not reduce nitrate to nitrite, are replaced by nitrosating bacteria such as Clostridia (Table 1). The pathogenic potential of bacteria in the altered stomach flora of infected mice is further underlined by the detection of a 16S rRNA gene from a recently described urease-negative *Helicobacter* species (Trebesius *et al.*, 2000) in the stomach of nonimmunized mice (Table 1). This species was isolated earlier from the caecum of IL-10 deficient mice, where it induced colitis and typhlitis (Vaughan *et al.*, 2000). The inflammatory potential of microorganisms other than *H. pylori* was recently illustrated by *Acinetobacter lwoffii*-induced gastritis in a mouse model (Zavros *et al.*, 2002a). The finding that bacteria that are naturally located in the lower intestinal tract (Salzman *et al.*, 2002) colonize the stomach in *H. pylori*-infected animals provides strong evidence that *H. pylori* enables gut bacteria to adapt to selective gastric conditions. Many 16S rRNA gene sequences matched typical representatives of the lower intestinal flora of the mouse (Salzman *et al.*, 2002), such as members of the *Eubacterium rectale*/*Clostridium coccoides* group, *Lactococcus* and *Streptococcus* spp. or operationally defined taxonomic groups such as bacteria of the Cytophaga–Flavobacter–Bacteroides phylum. The route by which the gut bacteria reach the stomach may be linked to the coprophagous behavior of mice rather than to upstream migration from the lower intestinal tract. The reasons for the observed increase in diversity of the stomach flora due to *H. pylori* infection remain unclear. Reduced gastric acid secretion or pathological tissue damage induced by *H. pylori* infection would provide a straightforward explanation for the increased bacterial diversity, but both parameters were not significantly correlated with the effect of *H. pylori* infection on the microflora. The fact that any histological signs of pathology were absent is consistent with the pH measurements because several studies have

shown that increased gastric pH correlates with inflammation (Feldman *et al*., 1996; El Omar *et al*., 1997; Zavros *et al*., 2002b). Although the reasons for the *H. pylori*-induced increase in gastric microflora diversity remain unclear, *H. pylori* may improve the growth conditions for gut bacteria by production of ammonia and bicarbonate from urea, both of which could serve as substrates for other bacteria. Furthermore, stress generated by food deprivation reduces the level of lactobacilli in the murine stomach (Tannock & Savage, 1974) and *H. pylori* infection can be considered a form of stress. In addition, *H. pylori* expresses a bactericidal peptide, which was shown to be toxic for other bacteria *in vitro* (Putsep *et al*., 1999). Taken together, these factors could result in opportunities and novel ecological niches for nonresident bacteria in the gastric environment. The fact that the reduced *H. pylori* concentration in vaccinated mice no longer affected the gastric microflora points towards the possibility that a *H. pylori* vaccine may ameliorate gastric conditions and may prevent disease even if it does not eradicate infection, a concept reminiscent of antimalaria vaccines (Richie & Saul, 2002). Finally, the fact that many 16S rRNA gene sequences corresponded to species that have not yet been cultured underscores the molecular approach taken for microflora analysis. Because pathology may induce further alterations in the ecosystem, it might be rewarding to apply these techniques for the analysis of *H. pylori*-induced microflora changes in other mouse strains, which were shown to develop more severe signs of gastric immunopathology as well as differential localizations of inflammation (Sakagami *et al*., 1996).

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